The effect of neonatal total parenteral nutrition on glucose metabolism in neonates and adult Yucatan miniature pigs

By

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Abstract:

Total parenteral nutrition (TPN) is used when oral nutrition is not possible, but may cause metabolic disturbances, increasing the risk of Type 2 diabetes mellitus (T2DM). We hypothesize that TPN feeding early in life can alter glucose metabolism in a way that persists into adulthood, leading to the development of biomarkers associated with T2D. Additionally, we hypothesized that supplementing TPN with betaine and creatine could potentially correct these changes, and that intrauterine growth-restriction (IUGR) could exacerbate TPN-induced changes. We assigned 32 female Yucatan miniature piglets to four groups: normal birth weight receiving TPN (TPN); sow-fed (SF); normal birthweight TPN supplemented with betaine and creatine (TPN-B+C); and IUGR piglets fed TPN (TPN-IUGR). After 2 weeks on TPN (or SF), glucose metabolism and insulin sensitivity was assessed. All pigs were then fed an oral diet for ~10 mo, and glucose metabolism tests were repeated. TPN feeding showed significantly more sensitive glucose metabolism, which were more pronounced immediately after TPN but remained significant 10 mo later. TPN also increased insulin sensitivity, which was corrected by adding betaine and creatine. IUGR did not exacerbate TPN effects. This study suggests that TPN in early life can permanently impact glucose metabolism into adulthood, but these changes do not align with T2DM.

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ADA- American Diabetes Association

AGAT- Arginine: glycine amidinotransferase

ATP- Adenosine triphosphate

AUC- Area under the curve

Bet- Betaine

BF- Breastfed

BHMT- Betaine-homocysteine methyltransferase

BG- Blood glucose

BW- Body weight

CBS- Cystathionine beta synthase

CGL- Cystathionine gamma-lyase

Cre- Creatine

DM- Diabetes mellitus

DNMT- DNA methyltransferase

DR- Diabetic retinopathy

EDTA- Ethylenediaminetetraacetic acid

ELISA- Enzyme-linked immunosorbent assay

ER- Endoplasmic reticulum

FPG- Fasting plasma glucose

FGR- Fetal growth restriction

G6P- Glucose 6-phosphate

GAA- Guanidinoacetate

GAMT- Guanidinoacetate *N*-methyltransferase

GO- Glucose oxidase

Hcys- Homocysteine

HbA1c- Glycated hemoglobin

HDL- High-density lipoprotein

HGP- Hepatic glucose production

HSC- Health Science Centre IFG- Impaired fasted glucose

IR- Insulin resistance

IST- Insulin sensitivity test

IV- Intravenous

IUGR- Intrauterine growth restriction

IVGTT- Intravenous glucose tolerance test

LDL- Low- density lipoprotein

MAT- Methionine adenosyltransferase

MS- Methionine synthase

MTHFR- Methylenetetrahydrofolate reductase

NW- Normal birth weight

NAFLD- Non-alcoholic fatty liver disease

OGTT- Oral glucose tolerance test

PC- Phosphatidylcholine

PEMT- Phosphatidylethanolamine *N*-methyltransferase

PN- Parenteral nutrition

RM- Remethylation

SAH- S-adenosylhomocysteine

SAHH- S-adenosylhomocysteine hydrolase

SAM- S-adenosylmethionine

SF- Sow-fed

T1DM- Type 1 diabetes mellitus

T2DM- Type 2 diabetes mellitus

THF- Tetrahydrofolate

TM- Transmethylation

TPN- Total parenteral nutrition

TS- Transsulfuration

VLDL- Very low-density lipoprotein

VLBW- Very low birth weight

WHO- World Health Organization

1. Introduction

1.1. The global burden of diabetes:

In 1964, 30 million people were estimated to have diabetes. In the next 40 years, the World Health Organization (WHO) reported that 171 million people worldwide had diabetes (Ogurtsova et al., 2017). According to the International Diabetes Federation, the global prevalence of diabetes was 246 million people in 2006, 285 million people in 2009, 366 million people in 2011, and 382 million people in 2013 (Cho et al., 2018). The number of T2DM cases is projected to increase from 415 million in 2015 to 642 million in 2040 (Petrie et al., 2018).

In Canada, the prevalence of the population with diabetes was 2.4 million in 2009 (6.8% of the adult population) (Cheng & Lau, 2013). Healthcare professionals and health policymakers are urged to improve their efforts to prevent diabetes. Diabetes mellitus was the seventh leading cause of death in Canada, responsible for 7,557 deaths in 2022, and is linked to a reduced lifespan of 5 to 15 years on average (David et al., 2024). Diabetes can cause many serious side effects, such as heart disease, stroke, blindness, kidney failure, and the amputation of limbs. All of these can significantly reduce the quality of life (Cheng & Lau, 2013).

1.1.1. Definition of diabetes:

Diabetes is an incurable metabolic condition with increased blood glucose levels; this is caused by either a deficiency in insulin secretion, a lack of insulin production, or a combination of both. Consequently, insulin deficiency increases glucose levels in the bloodstream (American Diabetes Association, 2013).

1.1.2. Insulin structure:

Insulin consists of an acidic (A) and a basic (B) chain, usually containing 21 and 30 amino acids, respectively. Disulfide bonds connect these two polypeptides. Many substances affect the "release" of insulin, including carbohydrates, sulfonylureas, amino acids, hormones, beta (β) adrenergic agents and possibly lipids (Grodsky,1971). Insulin is synthesized as a single-chain precursor, preproinsulin, with an N-terminal signal sequence and a connecting peptide linking the A and B chains of the insulin molecule. Nascent proinsulin is directed into the regulated secretory pathway, converted to insulin, and stored as microcrystals, as in Figure 1.1 (Dodson & Steiner, 1998; Wilcox, 2005).

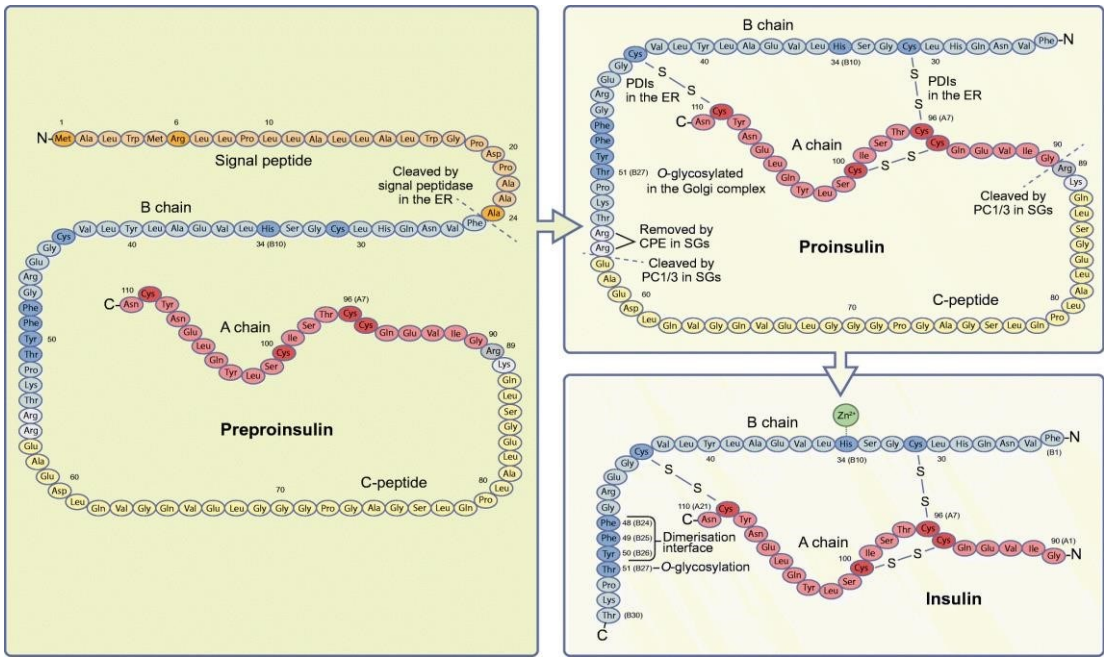

Figure 1.1: Insulin synthesis from preproinsulin (Vasiljević et al., 2020).

The insulin peptide undergoes a complex process of synthesis. Initially, insulin is created as a preproinsulin molecule consisting of 110 amino acids, encompassing a signal peptide, a B chain, a connecting C-peptide, and an A chain. The signal peptide guides preproinsulin to the endoplasmic reticulum (ER), cleaving it by signal peptidase and transforming it into proinsulin. A protease enzyme breaks down proinsulin with 86 amino acids in the Golgi apparatus. As a result, C-peptide is removed, and insulin is formed. Insulin is a protein hormone secreted by beta cells of the islets of the pancreas in response to high blood glucose (Vasiljević et al., 2020).

1.1.3. Insulin action:

Insulin acts by binding plasma membrane receptors on the cell surface of the target cells. Insulin receptors are glycoproteins containing two subunits, the alpha 2 subunit and the beta 2 subunit; the alpha unit is extracellular, to which insulin binds, whereas the beta subunit is cytoplasmic. After a dietary meal, insulin signals the cells of insulin-sensitive tissues, such as skeletal muscle, to increase their ability to use glucose. Insulin induces liver glycogenesis and inhibits glycogenolysis and gluconeogenesis in the liver (Aronoff et al., 2004).

1.1.4. Insulin resistance:

Insulin resistance refers to the impaired ability to respond to insulin action by muscle cells, adipose tissue, and the liver. As a result, this can result in high levels of glucose in the blood. Insulin resistance is characterized by insulinemia and hyperglycemia (Liu et al., 2020).

1.1.5. Classification of diabetes:

Diabetes can be classified into type 1 diabetes mellitus (T1DM) and type 2 diabetes mellitus (T2DM).

1.1.6. Type 1 diabetes:

T1DM is an autoimmune disease affecting children and teens (Bansal & Pinney, 2017), and it is estimated to affect between 5% and 10% of all diabetes cases worldwide. It is caused by the pancreas losing beta cells, leading to decreased insulin production and high blood glucose levels (Mobasseri et al., 2022).

1.1.7. Type 2 diabetes:

Type 2 diabetes is the most prevalent form of diabetes, accounting for between 90 and 95% of all individuals with diabetes. The primary risk factors for developing this condition are obesity, a family history and insulin resistance. As time progresses, the pancreatic beta cells fail to maintain insulin production, making insulin treatment necessary (Bansal & Pinney, 2017).

1.1.8. Complications of diabetes mellitus:

Cardiovascular disease:

People with T2DM are more likely to have short- and long-term complications. These can include macrovascular diseases such as high blood pressure, high cholesterol, heart attack, stroke, vascular disease, and microvascular diseases such as retinopathy, neuropathy, kidney disease, and cancer. Heart disease is a leading cause of death and morbidity in people with prediabetes and type 2 diabetes. This can be caused by oxidative stress, which significantly impacts the body's ability to repair itself. It can also cause LDL oxidation (Wu et al., 2014).

Diabetic neuropathy:

Diabetic neuropathy can lead to foot ulcers, amputations, non-healing skin ulcers and even sexual dysfunction. It can also cause skin infections like cellulitis, bone of the foot, and gangrene. (Wu et al., 2014).

Diabetic nephropathy:

Diabetic nephropathy is one of the most significant microvascular complications. The first symptom is the presence of microalbumin (a small protein) in the urine. Routine

urine tests cannot detect microalbumin, but specific tests can detect it. Early detection can prevent the development of kidney disease. However, this is often missed because routine urine testing is not sensitive enough to find microalbuminuria (Wu et al., 2014).

Diabetic retinopathy:

Diabetic retinopathy (DR) is a major DM complication (Ye et al., 2023). The retina requires a high amount of oxygen to convert light into electrical signals within the rods and cones. Prolonged hyperglycemia can lead to microvascular damage in the retinal blood vessels, causing edema and/or bleeding within the retina. Dysglycemia in the retina often occurs before diabetes, as almost 20% of newly diagnosed diabetic patients develop retinopathy (Wu et al., 2014).

Cancers:

T2DM is characterized by hyperinsulinemia, which has been shown to increase the risk of many types of cancer, including colon, liver, bladder, breast, and kidney. The risk of developing these types of cancer is linked to various risk factors, such as being overweight, obese, sedentary, smoking, and consuming too much saturated fat and refined carbohydrates (Wu et al., 2014).

1.2.1. Definition of variables:

According to the Canadian Diabetes Guidelines, diabetes mellitus is diagnosed when fasting plasma glucose (FPG) is ≥7 mmol/L or when the plasma glucose level is ≥11.1 mmol/L after a 2-hour oral glucose tolerance test (OGTT). Additionally, a diagnosis can be made if the glycated hemoglobin (A1C) level is ≥6.5% (Punthakee et al., 2018).

1.2.2. Biomarker of glucose metabolism:

Definition of biomarker:

Fasting plasma glucose (FPG):

FPG is widely used for screening T2DM and pre-diabetes in clinical practice because it is less time-consuming and inexpensive compared to the alternatives and is available worldwide (Kohansal et al., 2022).

Oral glucose tolerance test (OGTT):

According to the WHO, the oral glucose tolerance test (OGTT) is the gold standard and is used to diagnose diabetes and prediabetes. The OGTT measures the body's response to a liquid glucose dose. It measures the blood glucose response before and after 2 hours to 75 g of an oral glucose load (Philip, 2016).

Glycated hemoglobin (HbA1c):

HbA1c is an important indicator of long-term glycemic control with the ability to reflect average plasma glucose over the previous eight to twelve weeks (Sherwani et al., 2016). It can be performed anytime and does not require special preparation such as fasting. These properties have made it the preferred test for assessing glycemic control in people with diabetes. More recently, there has been substantial interest in using it as a diagnostic test for diabetes and as a screening test for persons at high risk of diabetes. Results defined as non-diabetes usually fall within the 4.0%-5.6% HbA1c range, whereas prediabetes usually has HbA1c levels of 5.7%–6.4%, while those with 6.4% or higher HbA1c levels have diabetes (Sherwani et al., 2016).

1.2.3. Glucose metabolism:

Glucose is a primary energy source for all living things (Guo et al., 2017). Glucose is the major macronutrient and essential homeostatic factor in the regulation of energy metabolism. In healthy people, a normal fasting plasma glucose concentration ranges from 4.4 to 6.1 mmol/L, as shown by Gromova et al. (2021). The liver plays an important role in glucose homeostasis by storing large amounts of glucose through glycogenesis and releasing glucose through glycogenolysis and gluconeogenesis (Ribeiro & Antunes, 2018). The pancreas is another important site for blood glucose control. A small amount of insulin is released from the pancreas as soon as a person begins to eat. When a large amount of glucose enters the blood, the pancreas releases insulin to promote the synthesis and storage of glycogen in the liver as well as the uptake of glucose by muscle and fat cells. Insulin also reduces hepatic gluconeogenesis (Wardlaw et al., 2004). Other effects of insulin include stimulating fat synthesis, promoting triglyceride storage in adipocytes, promoting protein synthesis in the liver and muscle, and increasing cell proliferation. Insulin activity is carefully regulated in response to circulating glucose concentrations**.** Other hormones counteract the effects of insulin when a person does not consume carbohydrates for several hours; blood glucose levels are maintained by the hormone glucagon, which is also secreted by the pancreas. This hormone causes the breakdown of glycogen in the liver, leading to the release of glucose into the blood. Glucagon also enhances gluconeogenesis. Glucagon helps restore blood glucose to normal levels. Blood glucose levels are also affected by epinephrine, norepinephrine, cortisol, and other hormones (Figure 1.2) (Wardlaw et al.,

2004). However, any insulin action or response defect can lead to hyperglycemia (Vauhkonen et al., 1998).

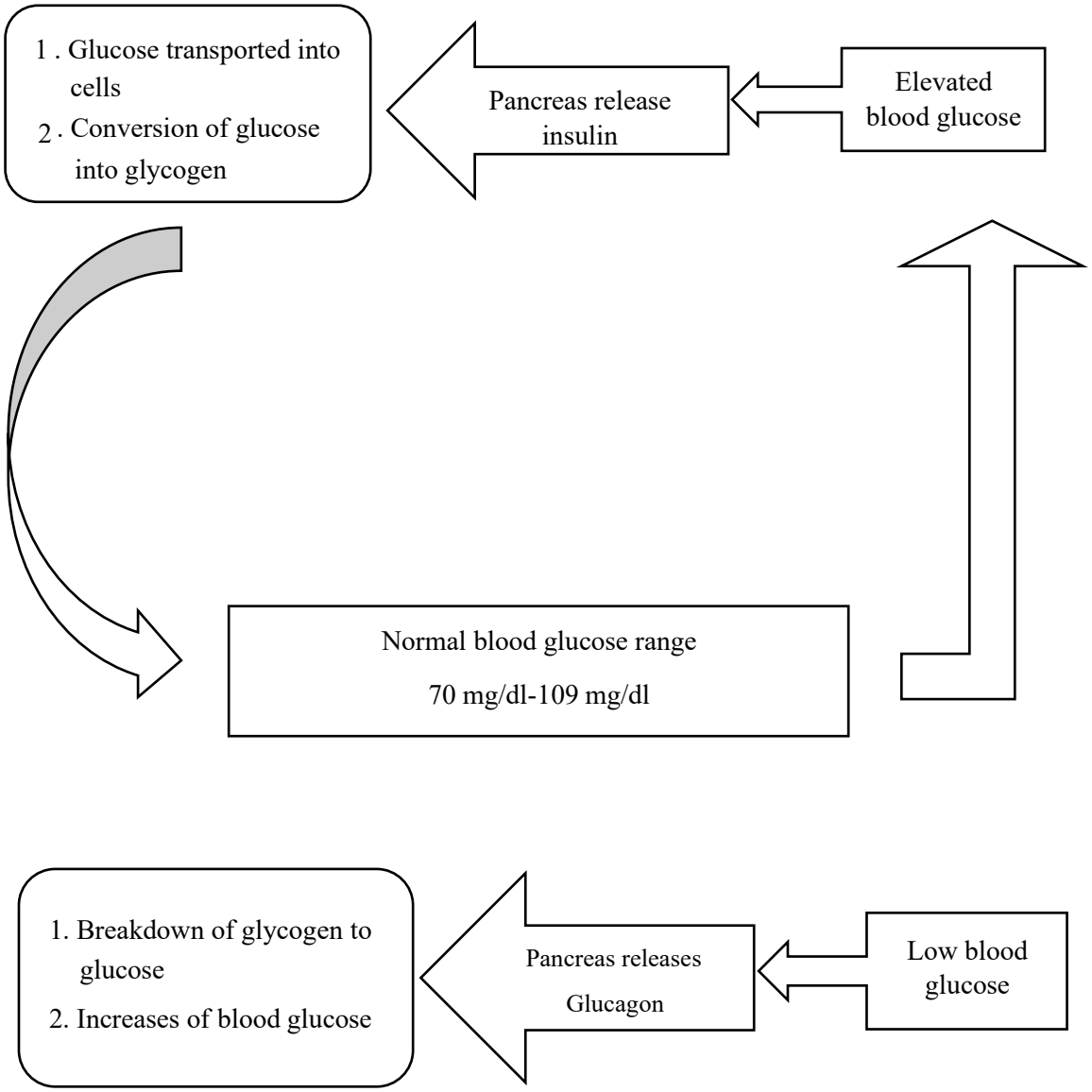

Figure 1.2: Regulation of blood glucose (Wardlaw et al., 2004).

1.3. Total Parenteral Nutrition (TPN):

Total parenteral nutrition (TPN) is frequently used to prolong the lives of neonates who cannot tolerate oral feeding. When intravenous (IV) nutrition is the only way to address the patient's nutritional needs, parenteral IV nutrition is used (Calkins et al., 2014; Elhassan & Kaiser, 2011; Sudha et al., 2006.). A parenteral diet consists of dextrose, amino acids, lipids, electrolytes, vitamins, and minerals (Alchaer et al., 2020) (Chaudhari & Kadam, 2006). The term "total parenteral nutrition" (TPN) has been used since it was first established in 1960 (Duran, 2005). In 1966, Dudrick implanted a catheter for the first time in a beagle puppy before using it on infants with intestinal disorders (Chaudhari & Kadam, 2006). Despite the reality that TPN has had many benefits since it was first introduced, TPN also has many side effects, including hyperglycemia (Stoll et al., 2010) and liver impairment (Nowak, 2020).

Hyperglycemia represents one of the most prevalent complications in patients who receive elevated glucose doses as part of their nutritional support (Llop et al., 2012). High blood glucose levels during TPN also increase the risk of complications such as cardiac issues, acute renal failure, and respiratory problems, increasing mortality risk (Llop et al., 2012). Moreover, observational studies have reported a 33% fatality rate among individuals receiving TPN who experience elevated blood glucose levels and elevated susceptibility to infections and heart complications (Pasquel et al., 2010). Numerous factors can contribute to elevated blood glucose levels in individuals receiving TPN. Patients requiring TPN often experience critical illness and severe malnutrition, and they are commonly administered TPN solutions with a high glucose content. Elevated glucose levels can result in elevated levels of counter-regulatory hormones and cytokines (Alchaer et al.,

2020). According to Alchaer et al., 2020, hyperglycemia during parenteral support is caused by decreased skeletal muscle glucose uptake and increased liver glucose production. Although the exact cause of neonatal hyperglycemia is unknown, several factors, including insulin resistance, poor pancreatic-cell secretion, an immature glucose transport system, and a small mass of insulin-dependent tissues, are thought to play a role in its development. The most frequent cause of hyperglycemia is excessive parenteral glucose and fat infusion. Stress-reactive hormones also interfere with the actions of insulin and glucagon in preterm neonates, causing hyperglycemia by increasing endogenous hepatic glucose synthesis and decreasing peripheral glucose consumption (Stoll et al., 2012).

1.4. Low birthweight:

Low birthweight (LBW) is when a human newborn is born with a body weight of less than 2500 g. It is estimated that 16% of newborns worldwide suffer from this condition, which can lead to neurodevelopmental disorders, cardiovascular and metabolic issues, and hypertension (Domell, 2017). In 2010, a study revealed that over 15 million infants worldwide were born prematurely each year (Glass et al., 2015), with 40-95% underweight when discharged from the hospital (Robinson et al., 2016). About 3-7% of all infants are affected by intrauterine growth restriction (IUGR), which is a form of LBW (Romo et al., 2009). A study also mentioned that around 15-20% of infants in the world are born yearly with low birthweight (under 2500 g) (Moreira et al., 2018). These can also be classified as very low birth weight (below 1500 g) or extremely low birth weight (less than 1000 g). These infants are unique because they have high nutritional needs (Cutland et al., 2017).

IUGR or fetal growth restriction (FGR) could be due to maternal influences such as insufficient nutrition, chronic maternal conditions, multiple pregnancies, genetic elements, placenta issues (vascular damage to the placenta), infections within the uterus or unspecified factors (maternal smoking, alcohol consumption or severe malnutrition) (Ergaz et al., 2005). Although there is still much to learn about IUGR, it is commonly defined as a condition where the fetus shows poor growth *in utero* (Devaskar & Chu, 2016).This is frequently brought on by placental insufficiency, in which the fetus receives inadequate amounts of oxygen and nutrients, which lowers blood oxygen levels, insulin and plasma glucose levels and increases the amounts of counter-regulatory hormones, which can lead to changes in metabolic pathways to support hepatic glucose production (HGP). IUGR increases the risk of perinatal complications, including stillbirth, and predisposes the infant to the development of type 2 diabetes and cardiovascular disease in childhood and adult age. IUGR is generally associated with fetal genetic abnormalities (syndromes, chromosomal abnormalities), maternal factors (vascular disease, persistent hypoxia, and toxins), and placental aetiologies. It is thought that 40% of birth weight is due to genetic factors, and the remaining 60% is due to fetal environmental exposures (Devaskar & Chu, 2016). Infants with IUGR are at high risk of immediate postnatal complications. Furthermore, infants experiencing catch-up growth in the postnatal period can result in long-term adverse outcomes, including neurodevelopmental impairment, a heightened risk of cardiovascular disease, and the development of metabolic syndrome, which can persist throughout their lifetime. (Kesavan & Devaskar, 2019). Therefore, IUGR-term infants were more likely to develop chronic diseases later in life (Robinson et al., 2016). Low birth weight has been linked to increased obesity, insulin resistance, and T2D, all of which are

part of the metabolic syndrome (Jornayvaz et al., 2016). Numerous studies have demonstrated that prenatal nutrition leading to low birth weight is linked to developing chronic diseases (such as diabetes, hypertension, and obesity in adulthood) (McMillen & Robinson, 2005). However, the exact mechanism of programming of T2DMis not fully understood (Berends et al., 2013); it is possible that epigenetic alterations in DNA methylation during early life could program metabolic mechanisms in adulthood, possibly due to the lack of methyl nutrients responsible for methylation of DNA.

1.4.1. Obesity:

Obesity is a chronic disease with a multifactorial etiology, including genetics, environment, metabolism, lifestyle, and behavioural components. Obesity is excessive body fat accumulation resulting from impaired energy balance. Obesity is associated with health risks and non-communicable chronic diseases, including T2DM (Rippe et al., 1998). Obesity is also associated with non-alcoholic fatty liver disease (NAFLD), characterized by an accumulation of fat in the liver. This fat buildup can occur with or without inflammation and fibrosis, a condition termed steatohepatitis. It has now been recognized that NAFLD is linked to significant metabolic and cardiovascular issues, including the development of T2D, metabolic syndrome, and coronary heart disease. Additionally, individuals with NAFLD experience dyslipidemias, including high triglycerides and low HDL cholesterol, and are at a greater risk of developing T2DM (Fabbrini et al., 2010).

Research has indicated that the early intake of certain nutrients can impact DNA methylation, including one-carbon metabolism components such as methionine, betaine, or choline, which contribute to methyl groups for methylation reactions (Randunu & Bertolo, 2020). The deficiency of these nutrients can disrupt one-carbon metabolism and S-

Adenosyl methionine synthesis (SAM), the methyl donor for DNA and histone proteins. Consequently, disturbances in DNA methylation may contribute to the development of chronic conditions such as cardiovascular disease, obesity, and the onset of T2DM (Nilsson & Ling, 2017).

1.5. Methionine cycle metabolism:

Methionine, an essential amino acid crucial for cellular growth and development, is one of the four sulfur-containing amino acids, alongside cysteine, homocysteine, and taurine. It is present in both food sources and the gastrointestinal microbes of humans. Methionine plays a vital role in protein synthesis in prokaryotes and eukaryotes, also acting as an endogenous antioxidant on protein surfaces (Parkhitko et al., 2019). Its significance extends to being a key component for protein synthesis and the formation of enzymes involved in transmethylation processes (Robinson, 2016). Furthermore, methionine serves as a methyl donor in over 50 common transmethylation reactions (McBreairty & Bertolo, 2016). The breakdown of methionine metabolism can be categorized into three distinct phases: transmethylation (TM, involving the conversion of methionine to homocysteine), transsulfuration (TS, conversion of homocysteine to cysteine), and remethylation (RM, where homocysteine is converted back to methionine) (Figure 1.3). S-adenosylmethionine (SAM), a critical component of methionine metabolism, was first discovered by Cantoni in 1953 (Brosnan & Brosnan, 2006). Diets lacking in methionine or choline result in reduced SAM levels and an increased accumulation of lipids in the liver (Deminice, 2016). SAM is converted to S-adenosylhomocysteine (SAH), which can further be transformed into homocysteine and adenosine (Zaho et al., 2018). In addition to homocysteine removal

through transsulfuration and cysteine synthesis, remethylation is another important pathway for homocysteine elimination. Betaine, choline, and folate are precursors for the remethylation pathways and have been shown to reduce hyperhomocysteinemia (Robinson et al., 2016), which has been associated with reduced development of cardiovascular disease (Kumar et al., 2017). Remethylation of homocysteine occurs by transferring a methyl group from a methyl donor and reforming methionine either by methionine synthase (MS) or betaine homocysteine methyltransferase (BHMT). (Krish et al., 2010). Otherwise, HCys is irreversibly converted into cysteine through the transsulfuration pathway (Figure 1.3) (Blom & Smulders, 2010).

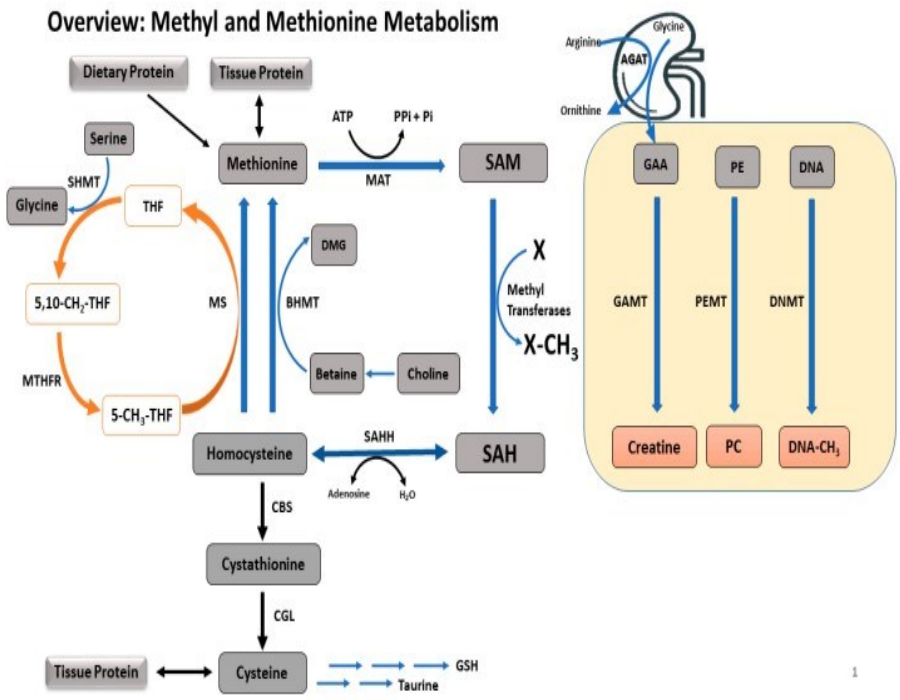

Figure 1.3: Methionine cycle metabolism (Randunu & Bertolo, 2020).

Methionine is transformed into S-adenosylmethionine (SAM), a key methyl donor in more than 50 reactions, such as DNA methylation, creatine production, and phosphatidylcholine synthesis (PC). These reactions result in a methylated product and S-adenosylhomocysteine (SAH), which is further converted to homocysteine. Homocysteine can then be converted to cysteine through the transsulfuration pathway or remethylated back into methionine via two remethylation pathways. (Randunu & Bertolo, 2020)

1.6. Remethylation reactions:

Methionine is converted to SAM, which is converted into SAH via transmethylation. When SAH undergoes hydrolysis, it converts into homocysteine. As discussed, homocysteine can follow two different paths: it can be either converted back into methionine through remethylation, or it can irreversibly transsulfurated to produce cysteine. Remethylation, leading to methionine, can occur through two mechanisms: one by accepting a methyl group from betaine, facilitated by BHMT in the liver or kidney, and the other by using 5methyltetrahydrofolate with the assistance of methionine synthase in various tissues (Le et al., 2014; McBreairty et al., 2016). Methyl donors, such as betaine (synthesized from choline) and folate, have effectively alleviated hyperhomocysteinemia.

1.7. Transmethylation reactions:

In the transmethylation pathway, S-adenosylmethionine (SAM) is synthesized from ATP and methionine by SAM synthetase (SAM-S), also known as methionine adenosyltransferase (MAT). The process of the methyl cycle pathway is to metabolize SAM into S-adenosylhomocysteine (SAH) through the transfer of a methyl group to methylated metabolites. SAH is subsequently converted into homocysteine. These methylated metabolites have a variety of critical functions in the body, and the major transmethylated products include creatine, phosphatidylcholine, and methylated DNA (Bertolo & McBrearity, 2013).

1.7.1. Creatine:

Creatine plays a crucial role in neonatal muscle function and neurological development. In infants, particularly during periods of rapid growth, there is a significant increase in the storage of creatine to meet the demands of growing tissues. Neonates receive

their creatine from breast milk (or infant formula) or via *de novo* synthesis. However, up to 77 percent of a neonate's daily creatine needs must be produced endogenously, meaning they need many precursors amino acids, including arginine and methionine, to synthesize creatine (Dinesh, 2021). Over 90% of the creatine and phosphocreatine in the body are primarily found in muscle tissue (Brosnan & Brosnan, 2007). Around one-third of this creatine exists in its basic form, with two-thirds as phosphocreatine. Phosphocreatine is an energy buffer in tissues with fluctuating energy demands, such as muscle (Post et al., 2019). Approximately 1.6%-1.7% of the entire creatine pool is spontaneously converted into creatinine and excreted by the kidney daily (Da Silva et al., 2014; Post et al., 2019). Consequently, *de novo* creatine synthesis is required to maintain a steady pool in the body (Post et al., 2019). Creatine is synthesized in two reactions from three amino acids: arginine, glycine, and methionine. The kidneys are highly active in the formation of guanidinoacetate (GAA) with the arginine: glycine amidinotransferase (AGAT) enzyme (Brosnan et al., 2011), while the liver is highly active in the synthesis of creatine from GAA via the guanidinoacetate Nmethyltransferase (GAMT) enzyme. AGAT transfers an amidino group from arginine to glycine to form GAA and ornithine. In the liver, SAM from methionine is donated to a methyl group to guanidinoacetate to form creatine using GAMT (Figure 1.4). Creatine can then be transported to other tissues that require high energy, such as the brain and skeletal muscle. However, the brain also has lower quantities of both enzymes and may synthesize creatine for itself (Brosnan et al., 2011). The availability and demand for methionine during early development can be affected by variations in the dietary intake of various methionine-related nutrients. For instance, a higher requirement for creatine synthesis (such as a low creatine diet) may divert methionine intake to creatine synthesis

and away from protein and PC synthesis. In contrast, an increase in the intake of remethylation nutrients (such as betaine, choline or folate) may lead to an increase in protein synthesis by making methionine more available. Because the same hepatic SAM pool is used for PC synthesis via PEMT and creatine synthesis via GAMT, creatine supplementation will reduce creatine synthesis via GAMT and may spare SAM availability for PC production via PEMT.

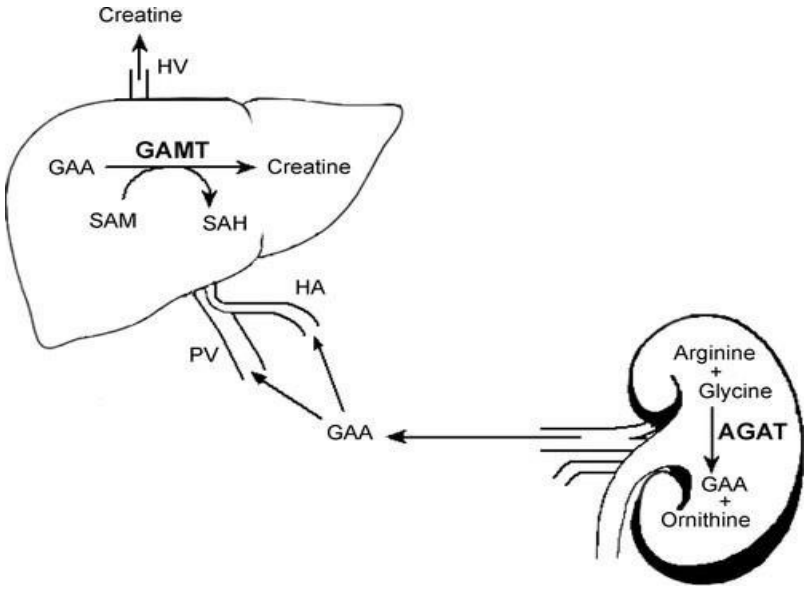

Figure 1.4: Creatine biosynthetic pathway (De Silva et al., 2009). AGAT transfers an amidino group from arginine to glycine, producing GAA and ornithine then GAMT catalyzes the methylation of GAA using SAM as a methyl donor. This reaction forms creatine and SAH. HA, hepatic artery; PV, portal vein; HV, hepatic vein; SAM, S-adenosylmethionine; SAH, S-adenosylhomocysteine; GAMT, guanidinoacetate N-methyltransferase; AGAT, L-arginine: glycine amidinotransferase; GAA, guanidino acetic acid. (De Silva et al., 2009).

1.7.2. Phosphatidylcholine (PC):

PC plays a vital role in preventing fatty liver diseases. PC is synthesized via the phosphatidylethanolamine methyltransferase (PEMT) pathway by donating three methyl groups to PE to produce PC. PEMT is responsible for 30% of hepatic PC synthesis. PC can also be synthesized through the CDP-choline enzyme pathway, i.e., the Kennedy pathway, which represents 70% of hepatic PC synthesis (McBreairty & Bertolo, 2016).

The production of PC involves a process where PEMT utilizes 3 methyl groups from SAM as a methyl donor to convert phosphatidylethanolamine (PE) into PC. Any alterations in this PC synthesis pathway can impact methionine metabolism and the availability of methyl groups (McBreairty et al., 2013). In fatty liver disease, liver dysfunction is characterized by the deposition of triglycerides and other lipids in the liver (Heeren & Scheja, 2021) due to the reduction of phosphatidylcholine (PC) synthesis. PC is required to synthesize very low-density lipoprotein (VLDL) for secretion from hepatocytes (Deminice et al., 2011). The VLDL particles carry hepatic fat from the liver to the tissues. NAFLD development is not yet fully understood, but insulin resistance appears to be a critical contributing factor, with obesity as the most common cause of the resistance (Kitade et al., 2017), storage of body fat progression of obesity and type 2 diabetes (Dewidar et al., 2020). So, by increasing synthesis of PC, lipids could be prevented from accumulating in the liver (Lee et al., 2014). Supplementation with betaine (or its precursor choline) is an important methyl donor for the remethylation of homocysteine to methionine, so may protect the liver from fat accumulation and lipid peroxidation (Deminice, 2011).

1.7.3. Epigenetics:

Epigenetics is defined as heritable changes in gene function but not a change in the nucleotide sequence. Epigenetic regulation includes DNA methylation, histone modifications, and non-coding RNA. However, DNA methylation appears to be most specific to epigenetic programming related to nutrition and adult diseases (Wei et al., 2016). DNA methylation refers to transferring a methyl group onto the C5 position of cytosine to form 5-methylcytosine. Methylation of the DNA is catalyzed by DNA methyltransferases (DNMTs) that transfer a methyl group from SAM to methylate the cytosine residues (Keil & Lean, 2016; Moore & Fan, 2013).

Research has shown that altered DNA methylation in tissues including in pancreas, liver, skeletal muscle, and adipose tissue likely plays a key role in glucose regulation, in type 2 diabetic patients. Some risk factors of type 2 diabetes, such as above-normal body weight, obesity, physical inactivity, a family history, and an unhealthy diet, have been shown to affect DNA methylation of genes related to insulin resistance (Jin & Liu, 2018). As a result, identifying DNA methylation alterations could improve the understanding of the pathogenicity of type 2 diabetes and improve the development of novel treatments (Nilsson & Ling 2017). Moreover, the susceptibility to develop T2DM in adulthood could be due to altered methylation of DNA, which can permanently program metabolism if altered early in life. Early nutrition could change methyl availability and DNA methylation patterns, which can change gene expressions in various metabolic pathways. However, supplementation of dietary methyl groups, which comes from methionine, as well as betaine, choline, or folate, can also regulate gene expression

(Randunu & Bertolo, 2020). Because neonates synthesize 75% of their creatine requirement (Dinesh et al., 2021), creatine synthesis consumes a significant proportion of dietary methionine; by supplementing creatine, we can further spare methyl groups in the body, for DNA methylation as well as other methyl-dependent pathways. Evidence shows that an imbalance of dietary methionine or methyl nutrients, prenatally or postnatally, plays a role in disease programming via altered DNA methylation (Randunu & Bertolo, 2020). It has been well established in both human and animal models that IUGR can "program" an increased risk of hypertension, cardiovascular disease, diabetes, and obesity in later life, but it is unclear if IUGR is related to an imbalance of methyl nutrients (Evans 2006).

Although DNA methylation is quantitatively a small consumer of methyl groups, compared to creatine and PC synthesis in piglets (McBreairty et al., 2013), the potential for diet to cause epigenetic changes during early development has enormous implications for infant nutrition guidelines (McBreairty & Bertolo, 2016). Therefore, we expected that feeding betaine and creatine could increase the availability of methyl groups for DNA methylation during TPN and prevent the programming of T2DM.

1.8. Choline:

The dietary intake of choline varies among individuals, leading to insufficient consumption for many people. Choline intake plays a role in regulating transmethylation through the action of betaine homocysteine methyltransferase (BHMT). Choline, along with its byproduct betaine, regulates the levels of S-adenosylhomocysteine and S-adenosylmethionine, which are crucial for epigenetic modifications. Choline availability and other methyl-group donors in the diet can influence DNA and histone methylation,

which are integral to various epigenetic processes. For instance, feeding pregnant agouti mice high-choline, high-methyl diets can hypermethylate genes which change their offspring's coat colour and body weight (Zeisel, 2017). Moreover, choline can be used in the CDP-choline pathway to make PC, thereby reducing the need for SAM to synthesize PC via PEMT, further sparing methyl groups from being used in that pathway.

1.9. Betaine:

Betaine, or trimethyl glycine, is found at high concentrations in several foodstuffs, including wheat bran, wheat germ, spinach, beets, and microorganisms (Zaho et al., 2018). Betaine helps to maintain intracellular osmotic pressure. Betaine also controls the surface tension of water and protects cells. However, a key function of betaine is remethylating methionine by donating its methyl group to homocysteine, converting it to methionine in the liver and kidney via betaine-homocysteine methyltransferase (BHMT) (Arumugam et al., 2021). Studies have shown that when betaine was administered to animals with fatty liver disease, it led to elevated levels of SAM, and reduced fatty liver (Deminice et al., 2015). Similarly, piglets fed diets rich in betaine exhibited increased levels of methionine and SAM (Robinson et al., 2018). Furthermore, the introduction of betaine into the diets of humans resulted in higher methionine concentrations (Cai et al., 2014). Betaine consumption reduced fat deposition in animals (Wang et al., 2010) and liver disease, which is associated with insulin resistance (Kathirvel et al., 2010). Therefore, supplementation of betaine (a methyl donor) could protect the liver from an accumulation of fat (Deminice et al., 2011), which can prevent insulin resistance and T2D. Consequently, available SAM is critical in regulating transmethylation reactions within the methionine cycle.

1.10. Rationale:

TPN feeding is an abnormal delivery method for nutrients that can permanently alter glucose metabolism when fed early in life. Moreover, IUGR infants are at a higher risk for developing glucose intolerance and insulin resistance than infants with a normal birth weight. TPN feeding is also associated with the potential programming of T2DM later in life. Furthermore, the combination of TPN and IUGR could worsen all these conditions for the individual.

Strategies to mitigate these harmful outcomes from TPN use and IUGR are important for reducing morbidities and mortalities in this population. One potential approach is the supplementation of betaine and creatine, which could increase methionine availability for enhanced DNA methylation and higher PC synthesis. Higher PC synthesis could prevent fatty liver disease, which is a common occurrence during TPN feeding. Preventing fatty liver disease could lower the risk of diabetes.

1.10.1. Hypotheses:

I hypothesized that early TPN will disrupt glucose metabolism in adult Yucatan pigs as measured by changes in biomarkers of T2DM. However, supplementation of betaine and creatine to TPN will prevent these reduce the severity of these changes in glucose metabolism. I also hypothesized that TPN-fed Yucatan piglets who are also IUGR would develop obesity and develop worse biomarkers of T2DM than pigs who were not IUGR.

1.10.2. Objectives:

The objectives are to determine if: early TPN can lead to biomarkers of T2DM in adulthood; if supplementation of betaine and creatine to TPN will prevent biomarkers of

T2DM; and if IUGR combined with TPN will exacerbate the programming of biomarkers of T2DM (Figure 2.1).

2. Methods

The Animal Care Committee at the Memorial University of Newfoundland approved all the procedures according to Canadian Council on Animal Care guidelines. Thirty-two female Yucatan miniature piglets were assigned into four groups: normal birthweight fed control TPN (TPN); normal birthweight fed TPN with supplemental betaine and creatine (TPN-B+C); intrauterine growth-restricted fed control TPN (TPN IUGR); and sow fed (SF), as listed in Figure 1. The IUGR piglets were those born with an initial body weight of ~65% of the largest littermate. After birth, all piglets were allowed to suckle colostrum and milk for ~7 days, then removed from the sow for surgical catheterization (described below). SF piglets were put back with the sow and allowed to suckle for two weeks. The remaining piglet groups were fed their respective TPN for two weeks until day 14. Glucose metabolism was assessed in all groups after 14 days, using the intravenous glucose tolerance test (IVGTT) (day 15) and insulin sensitivity test (IST) (day 16). The piglets were fed a sow milk replacer until 28 days old, followed by a grow-out phase. During the grow-out phase, the pigs were fed a standard commercial grain-based grower feed until the end of the study. At 9 months of age, pigs underwent a second surgery to implant the same catheters. The glucose metabolism tests were repeated after recovery at ~10 months of age (Figure 2.1.1). Body weight and blood collection were recorded every two weeks throughout the grow-out phase.

TPN groups Study Design:

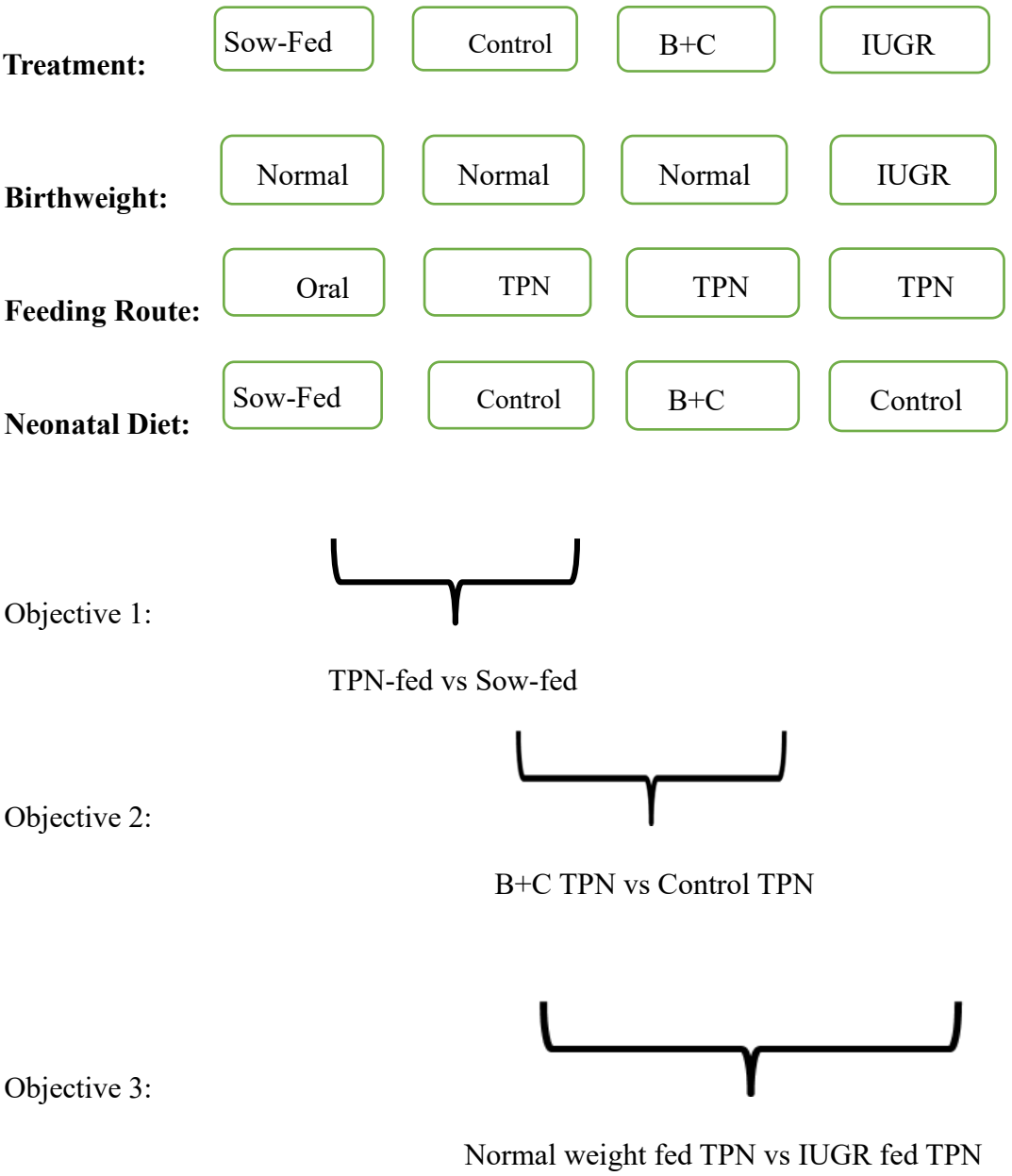

Figure 2.1: TPN groups study design, including statistical contrasts (i.e., objectives). Each column represents the four treatment groups.

Abbreviations: TPN- Total Parenteral Nutrition; IUGR- Intrauterine Growth-Restricted; B+C- Betaine and Creatine supplemented TPN.

TPN Programming Study Design:

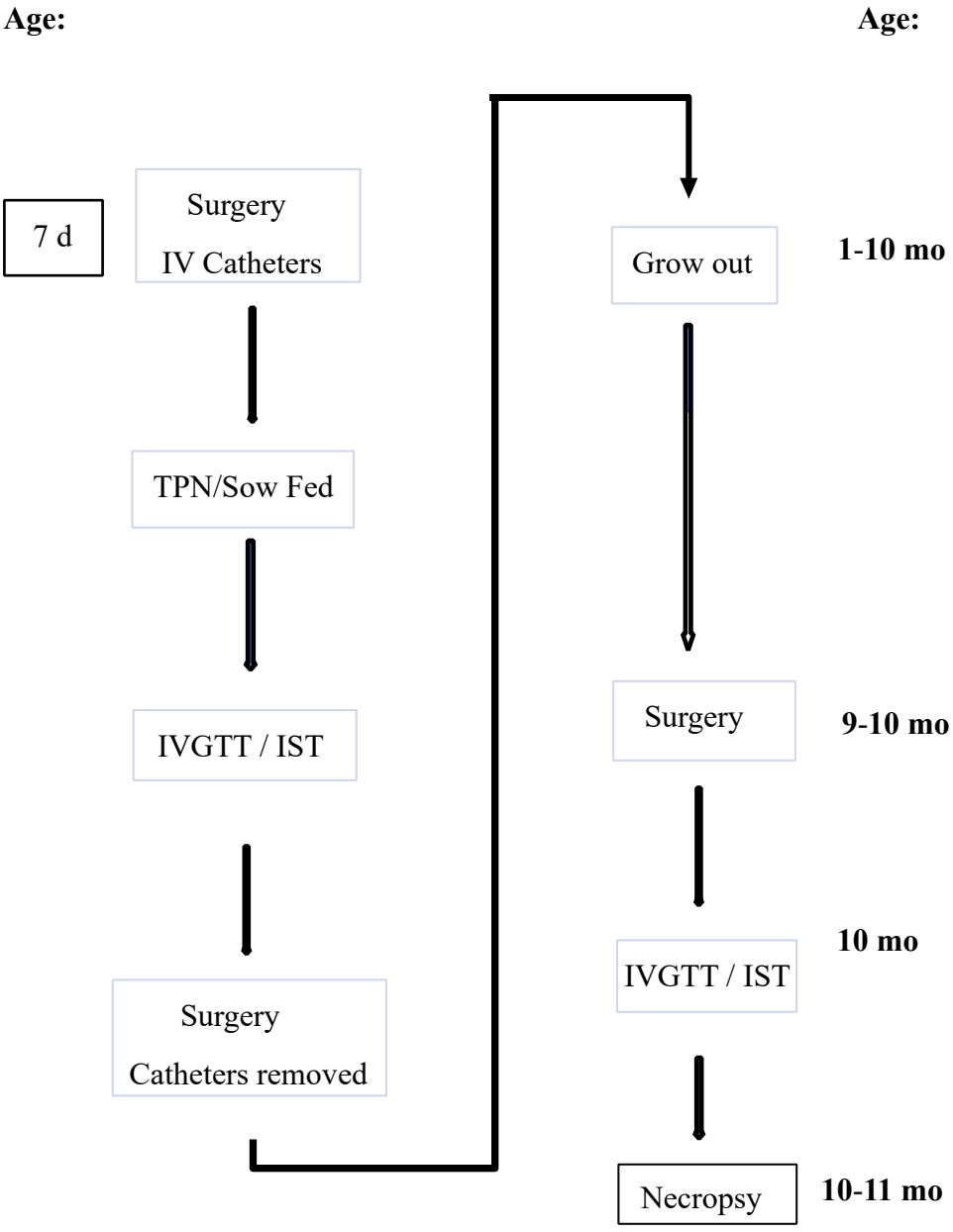

Figure 2.1.1. TPN Programming Study Design. Piglets were started on TPN at 7 d old and fed TPN or Sow-fed for 14 days. IVGTT/IST tests performed after TPN and then catheters removed. Piglets then fed for 10 mo on grower feed. IVGTT/IST tests repeated and then animals euthanized. TPN-Total Parenteral Nutrition; IV-Intravenous; IVGTT-Intravenous glucose tolerance test; IST-Insulin sensitivity test.

2.1.1. Piglet surgery:

At birth, all piglets were permitted to feed on the sow for 7 days, after which the piglets were separated from the sow and transported from the vivarium to the main campus for surgical catheterization. A pre-anesthesia injection of 0.5 mg/kg acepromazine was administered, followed by a ketamine injection of 20 mg/kg. The piglets were then administered gas anesthesia, which consisted of 1-2% isoflurane delivered with oxygen at 1.5 L/min. Subsequently, two intravenous catheters were implanted; one was placed in the femoral vein for blood sampling and the other in the jugular vein for TPN infusion. To prevent the formation of blood clots, heparinized saline solution was flushed into the catheters daily. Piglets allocated to a sow fed group were returned to the sow at the vivarium and housed following the standard protocol. To protect the catheters, piglets were wrapped with Vetrap; no catheters were lost during the suckling period. Pigs were allowed monitored for 2-3 hours after surgery to ensure the sow accepted the return of the piglet, and suckling was successful. The piglets that were allocated to one of three TPN-fed groups remained in the animal care facility on the main campus and were housed separately in round metabolic cages that were 0.5 m in height and 1 m in diameter. The cages allowed for visual and auditory contact with other pigs. After surgery (d 0), the piglets were placed into jackets that facilitated the delivery of the test TPN diets (described below) through a tether-swivel system. The size-adjustable jackets had an anchor button that attached to a tether, and the tether connected to a swivel, allowing free movement within the cages. The room temperature was adjusted to 26-28°C, and heat lamps were used for additional warmth. In addition, various toys were used for environmental enrichment (e.g., squeaky toys, chew toys, metal chains, plastic cups, balls, etc.). The TPN diets were infused by hospital-grade

pressure-sensitive peristaltic pumps, starting immediately after surgery at 50% of the target infusion rate (12 mL/kg/h) on the day of surgery (d0). The next day (d1), the rate was increased to 75%, and the full nutrient requirements were met with a 100% infusion rate. The piglets continued their TPN diet for two weeks.

2.1.2. Diet description and preparation:

The control TPN (TPN) and the TPN-IUGR group were provided with the same TPN diet formulation, whereas the TPN-B+C diet had supplemental betaine and creatine (Table 2.1, 2.2 and 2.3). The TPN solutions were prepared in the laboratory following the methods described by Dodge et al. (2012) under sterile conditions. Just before infusion, each diet bag received the addition of 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 SMOFlipid (Fresenius-Kabi). All essential vitamins and minerals were included in amounts exceeding 100% of the recommended requirements for neonatal piglets (National Research Council 2012).

Table 2.1. Diet composition of solid grower diet

Energy %	**%**
Carbohydrate	67
Fat	12
Protein	21

Nutrients g/kg	
Whole wheat shorts	400.5
Canola	49.0
Meat meal	19.0
Limestone	13.0
Corn gluten meal	40.0
Ground barley	297.0
Oats	175.0
Vitamin mix	0.8
Mineral mix	1.0

Table 2.2. TPN diet

Amino Acid Profile	Control Diet (g/L)	B+C Diet (g/L)
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	3.00	3.00
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
Valine	2.89	2.89
Threonine	2.23	2.23
Betaine hydrochloride	0	1.29
Creatine monohydrate	0	0.57

Table 2.3. Chemical Profile of TPN:

Chemical Profile	Control Diet (g/L)	B+C Diet (g/L)
Dextrose ($C_6H_{12}O_6$)	90.3	90.3
Potassium phosphate KH_2PO_4 trihydrate	1.57	1.57
KH_2PO_4 monobasic	1.085	1.085
Potassium acetate (CH_3CO_2K)	1.47	1.47
Sodium chloride (NaCl)	2.17	2.17
Magnesium sulfate ($MgSO_4$)	0.78	0.78
$ZnSO_4$ heptahydrate	0.089	0.089
Calcium gluconate ($C_{12}H_{22}CaO_{14}$)	6.41	6.41

2.1.3. Intravenous glucose tolerance test (IVGTT):

An intravenous glucose tolerance test (IVGTT) is used to assess the ability of the pig to remove glucose from the blood. IVGTT is less stressful in pigs than an oral glucose tolerance test, which requires orogastric intubation for a complete delivery of glucose. The stress to restrain the pig during orogastric intubation affects blood glucose concentrations dramatically, so the IVGTT was employed to deliver the glucose dose by indwelling catheters. In piglets, IVGTT was performed after two weeks of TPN or sow feeding, and again at 10 months of age, 5 days after surgery to re-implant the catheters. To measure fasting glucose concentration, three blood samples were collected at -10, -5, and 0 min before a glucose bolus (50% solution in saline; 0.5 g/kg BW) was injected over one minute

to deliver 0.5 g glucose/kg (McKnight et al., 2012). After the intravenous glucose infusion, blood samples were collected every 5 minutes until glucose concentrations returned to baseline concentrations. Blood glucose was immediately measured using a glucometer (Ascensia blood glucose meter, Toronto, Ont., Canada) and the remaining blood samples were transferred to EDTA tubes and centrifuged at 3000 g for 5 min to isolate plasma. Plasma was stored at -80 degrees C for further analysis. The following parameters were calculated from IVGTT time course data: time to peak for glucose and insulin, time for glucose and insulin concentration to return to baseline, the slope of glucose removal from the blood (glucose clearance), the peak glucose concentration, and area under the curve for glucose (gAUC) and insulin (iAUC).

2.1.4. Insulin sensitivity test (IST):

The insulin sensitivity tests were administered the day after IVGTT after 18 hours of fasting. Three samples were taken at -10, -5, and 0 min to measure fasting blood glucose concentration. At -5 min, a dose of somatostatin (4 μg/kg) was injected to suppress endogenous insulin secretion. At time 0, a bolus of 50% glucose solution (0.5 g/kg) was administered intravenously, blood samples were collected, and glucose was measured every three minutes until the glucose concentration was stable. Subsequently, a bolus of insulin (0.05 U/kg BWT) was infused, and blood samples were collected every 5 minutes until glucose reached fasting levels. Glucose clearance was calculated by plotting the natural log of plasma glucose concentration against time and calculating the slope after administering insulin.

2.1.5. Glucose assay:

Plasma glucose concentrations were measured using a Sigma-Aldrich glucose oxidase (GO) assay (Sigma-Aldrich, Saint Louis, Missouri, USA). The kit contained glucose oxidase, peroxidase and *o*-dianisidine reagents and glucose concentrations were measured using a spectrophotometric plate reader at 450 nm.

2.1.6. Standard preparation:

A series of dilutions were prepared from a starting concentration of 100 mg/dL (equivalent to 5.6 mmol/L) glucose to establish a standard curve, as listed in Table 2.4.

Table 2.4. Standard preparation:

	Concentration (mmol/L)	ddH_2O (µL)	Stock Glucose (µL) (100 mg/dL)
F	0.2	964	36

	Concentration (mmol/L)	ddH_2O (µL)	Vol. from the next highest concentration
E	0.1	250	250 (of 0.2 mmol/L)
D	0.05	250	250 (of 0.1 mmol/L)
C	0.025	250	250 (of 0.5 mmol/L)
B	0.0125	250	250 (of 0.025 mmol/L)
A	0.000	500	0

2.1.7. Procedure:

One capsule of Glucose Oxidase/Peroxidase reagent was dissolved in 39.2 mL of deionized water in an amber vial. Then, 5.0 mg of *o*-Dianisidine dihydrochloride powder was resuspended in 1.0 mL of deionized water in a separate amber vial. Following that, 0.8 mL of the resuspended *o*-Dianisidine dihydrochloride was added to the vial containing the Glucose Oxidase/Peroxidase reagent, which was stored in a cooler at 4°C. On experiment day, the plasma samples for IVGTT and IST were removed from a -80°C freezer and allowed to thaw on ice for approximately one hour. Once fully thawed, these samples were vortexed to ensure proper mixing. To create 100x diluted plasma samples, we took 10.0 µL of each sample and transferred it into labelled 1.5 mL Eppendorf tubes; then 990 µL of deionized water was added to each labelled Eppendorf tube, followed by thorough vortexing to achieve a complete mixture. In the next step, we prepared a 96-well microplate (Grenier bio-one, Frickenhausen, Germany) for the assay. Then, 60.0 µL of the diluted plasma sample was loaded into wells A1-H10 on the microplate. Additionally, we introduced 60.0 µL of each glucose standard, labelled as A-F, into wells A11-H12 on the same plate. We used a multi-micropipette to add 120.0 µL of the Glucose Oxidase/Peroxidase/o-Dianisidine reagent into each well. The plate was then securely covered and incubated for 25 minutes at a temperature of 37°C. After incubation, samples were read at 450 nm using a plate reader to measure the absorbance of each diluted sample. Finally, the absorbance readings from the IVGTT and IST plasma samples were compared to the glucose standard curve to calculate the plasma glucose concentration.

2.1.8. Insulin concentration using Enzyme-Linked Immunosorbent Assay (ELISA):

The Human Insulin ELISA (Enzyme-Linked Immunosorbent Assay) kit from Abcam was used to determine the amount of insulin in plasma. This test uses a 96-well plate covered with a human insulin-specific antibody. Standards and samples are pipetted into the wells, and the immobilized antibody binds the insulin in the sample to the wells. After washing the wells, a biotinylated anti-human insulin antibody is then added. After removing the unbound biotinylated antibody, the HRP-conjugated Streptavidin is pipetted into the wells. After the final washing, a TMB substrate solution is added to the wells, and colour develops in direct relation to the amount of bound insulin. At 450 nm, the colour intensity is measured and is converted by the stop solution from blue to yellow. To calculate an unknown insulin concentration, a standard curve was created. This test was validated to detect pig insulin in plasma in previous experiments.

2.2. Grow out phase:

After weaning, at 1 month old, pigs were returned to the vivarium, housed in groups of size-matched piglets for the grow-out phase, and fed the as a group, according to Vivarium feeding schedule. Feed intake was calculated biweekly by separating pigs into individual cages to avoid competition and feeding *ad libitum*. Feed intake was calculated by subtracting the remaining feed after 6 hours from the starting feed amount (g/kg body weight/d)**.** At these intake tests, body weight and blood samples were also taken. Blood samples were collected via a brachiocephalic vein puncture with the pig restrained on its back in a V-trough. For the pigs between 1-3 months old, 10 mL of blood was collected, and for the pigs between 3-8 months, 20 mL of blood was collected. Plasma was immediately separated and stored at -80 until further analysis.

2.2.1. Mature pigs surgery:

At ~9 months old, pigs were fasted overnight and placed under anesthesia to implant a blood pressure telemeter into the femoral artery, as well as two catheters in the femoral vein to collect blood. Ketamine, lorazepam and xylazine were used as pre-anesthetics and pigs were maintained on isoflurane delivered with oxygen. Tygon catheters (i.d. 1.0 mm, o.d. 1.8 mm) were inserted in the femoral vein and advanced to the inferior vena cava. The other ends were tunnelled under the skin and exteriorized between the shoulder blades on the back. After the surgery, the pigs were given 0.005 mg/kg of buprenorphine for pain and antibiotics (10 mL of Borgal).

2.2.2. Post-surgery:

All animals were closely monitored for any evidence of fever, dehydration, or sepsis, as well as any behavioural alterations, as well as urine and feces output.

2.2.3. Diabetes tests:

After 5 days of recovery in individual pens, the IVGTT and IST tests were conducted on subsequent days. Other *in vivo* tests (e.g. blood pressure measurement, salt challenge, fat tolerance test) were also conducted by another student for their thesis.

2.2.4. Necropsy:

Five days after all of the in vivo tests were completed (approximately 1 mo post-surgery), pigs were anesthetized with sodium pentobarbital (30 mg/kg body weight) and mechanically ventilated with oxygen. Various organs were removed and sampled for later analysis. Pigs were killed by exsanguination during organ removal.

2.2.5. Growth rate calculation:

The growth rate was determined by taking the difference between two body weights and dividing by number of days between those weights. Fractional growth rate (FGR) was calculated by dividing body weight gain for a given period of time by the initial body weight of that period.

2.2.6. Calculations and Statistical Analysis:

All data were analyzed statistically using Graph Pad Prism 10 software. A one-way analysis of variance (ANOVA) and Dunnett's multiple comparisons test were used to compare each diet group to the reference group (TPN control). A difference was considered significant if the P value was < 0.05. The mean and standard deviation for data are presented. The AUC (Area Under the Curve) for the IVGTT was calculated from the baseline glucose concentrations, which was calculated before glucose was infused for the IVGTT to the baseline at the end of the experiment. GraphPad Prism 10.0 (GraphPad Software Inc.) was used for this calculation. The peak of glucose concentration, the time to reach this peak, and the time to return to the baseline glucose concentration during the IVGTT were calculated utilizing GraphPad Prism 10.0 (GraphPad Software Inc.). The slope to glucose baseline was also measured via linear regression from the peak glucose concentration to the baseline for both IVGTT and IST. This was performed after the IST data were transformed to natural logarithm glucose concentration using GraphPad Prism 10.0.

3. Results

Yucatan pigs typically reach sexual maturity between the ages of four and seven months (McKnight et al., 2012). To put the growth data in context, we categorized growth into four distinct phases: neonatal (0-15 days), pre-sexual maturity (45 days- 4 months), peri-sexual maturity (4-7 months), and post-sexual maturity (7-9 months). The TPN-IUGR piglets exhibited a lower body weight than the TPN-control group ($p=0.0045$) at birth. Furthermore, even at 21 days of age, the TPN-IUGR piglets had a lower body weight than the control TPN group ($p=0.0121$). However, by the time they reached 45 d old, the piglets showed no significant differences in body weight ($p>0.05$).

In this study, we found that pigs at 9 months of age in the TPN-IUGR group did not differ in body weight compared to the TPN-control group ($p=0.0593$); the TPN-B+C and Sow groups also were not different than the TPN-control group. The growth rate in IUGR pigs was faster from 4-6 months old than in the TPN-control group ($p=0.044$). Relative daily feed intake (g/kg body weight/d) was significantly lower in IUGR pigs compared to controls at ages 4-7 and 7-9 months old ($p=0.0001$ and $p=0.0104$, respectively); Sow-fed pigs were also different from control TPN from 4-7 months old ($p=0.0238$), as in Table 3.1.

3.1 Growth parameters:

Table 3.1: Growth parameters during phases of growth in control, betaine and creatine (B+C), intrauterine growth restricted (IUGR), and sow-fed (SOW) pigs.

Development period	Age	Control	B+C	IUGR	SOW
Bodyweight (kg):					
Birth	0 d	0.99+0.17	1.02+0.12	0.72+0.11**	1.00+0.13
TPN start	7 d	1.74±0.15	1.74±0.23	1.27±0.18**	1.70±0.44.
Neonate	15 d	2.44±0.22	2.50±0.24	1.79±0.29*	2.54±0.38
TPN end	21 d	3.02±0.19	3.10±0.25	2.40±0.21*	3.51±0.79
Pre-sexual maturity	45 d	4.23±1.33	4.31±1.08	3.87±1.00	5.27±1.11
Peri-sexual maturity	4 mo	14.46±3.66	15.35±3.32	17.25±2.98	17.70±2.79
Peri-sexual maturity	7 mo	31.04+5.61	29.9+7.17	36.70+4.97	34.00+4.88
Post-sexual maturity	~8-9 mo	40.25±6.44	40.20±5.84	46.16±6.23	41.91±6.23
Necropsy	~10 mo	43.36±6.55	43.51±6.77	48.07±3.68	45.657±9
Daily growth rate (g/d)					
Neonate	0-15 d	97.2±14.3	99.0±18.1	71.6±19.5	103.3±24.3
TPN period	7-21 d	98.6±5.8	105.0±6.8	85.0 ±7.8	139.1±44.0**
Pre-sexual maturity	0-45 d	112.0±37.7	113.9±34.3	78.6±19.9	158.8±37.2*
Pre-sexual maturity	45 d-4 mo	136.4±37.7	147.1±31.0	193.3±41.0**	165.7±31.1
Peri-sexual maturity	4-6 mo	150.1±46.2	153.1±52.2	223.4±53.2*	176.8±70.9
Peri-sexual maturity	4-7 mo	156.6± 40.0	162.5±65.3	217.3±22.4	181.1±57.4
Post-sexual maturity	7~9 mo	195.04±39.0	170.4±64.2	181.4±87.1	157.7±38.1
Study period	7 d-9 mo	142.4±23.7	142.4±21.4	168.6±18.2	147.9±23.4

Fractional growth rate (g/kg body weight/d)					
Neonate	0-15 d	49.6±6.1	53.2±8.3	58.7±7.9	74.0±32.9*
Pre-sexual sexual	0-45 d	12.4±2.6	34.08±17.4*	13.72±2.3	52.4±23.0****
Pre-sexual maturity	45 d-4 mo	30.9±8.2	27.93±4.4	46.77±18.0*	27.5±6.
Peri-sexual maturity	4-7 mo	9.10±2.7	7.52±2.6	11.9±3.4	9.2±4.3
Post-sexual maturity	7-9 mo	5.79±2.7	8.87±2.5	3.6±1.3	8.3±1.1
Average daily feed intake g/d					
Pre-sexual maturity	45 d-4 mo	564±269	556±238	508±225	645±264
Peri-sexual maturity	4-7 mo	1339±252	1231±225	1246±233	1353±229
Post-sexual maturity	7-9 mo	1739±190	1671±98	1522±59	1737±212
Fractional daily feed intake (g/kg body weight/d)					
Pre-sexual maturity	45 d-4 mo	58.5±8.5	60.8±7.0	46.3±10.5*	51.31±6.0
Peri-sexual maturity	4-7 mo	58.3±3.5	59.2±2	45.71±7****	51.9±4.3*
Post-sexual maturity	7-9 mo	45.9±4.9	45.4±6.5	35.13±6.4*	43.0±3.9

Data are expressed as mean ±SD. One-way ANOVA and Dunnett's multiple comparisons were used to compare with the control TPN group. Significant differences were identified if $p < 0.05$. *P<0.05, **P<0.001, ***P<0.0001.

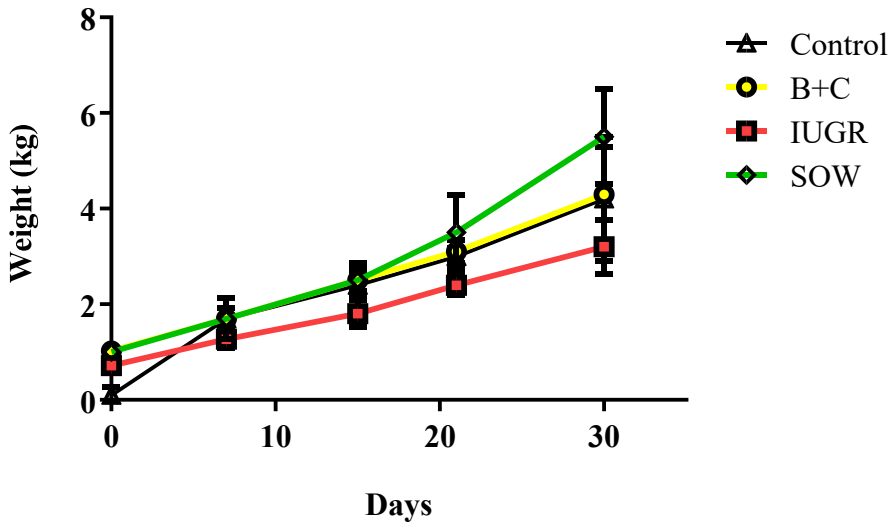

Figure 3.1: Piglet body weight from birth to 1 month old. Body weights were measured weekly from birth to 1 month old. Symbols represent mean ±SD with n=8 for TPN-control, TPN-B+C, and the Sow-fed.

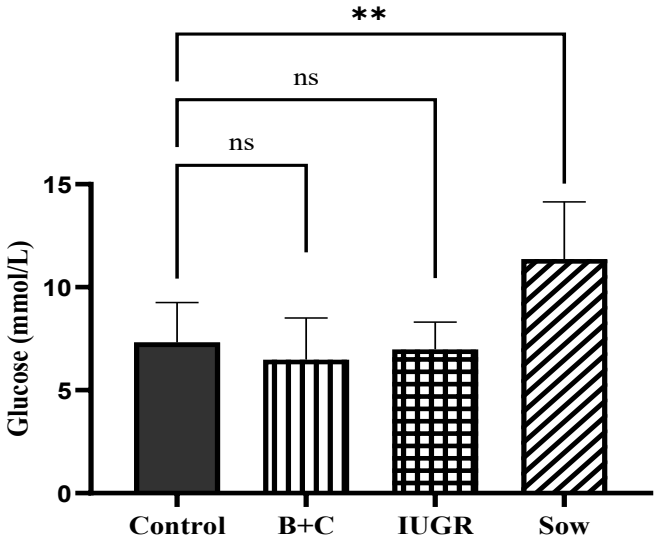

Figure 3.2: Fasting plasma glucose concentrations. Fasting plasma glucose was measured before the piglet IVGTT test (~ 3 weeks old) on all groups of piglets (n= 8 for TPN-control, TPN- B+C, TPN- IUGR; n = 7 for Sow-fed. Bars represent means ± SD. One-way ANOVA followed by Dunnett's multiple comparisons ($p < 0.05$) was used to test for diet differences. Significant differences were observed in the Sow-fed group ($p=0.0017$), but no differences were observed between the control TPN and B+C ($p=0.8$) or IUGR ($p=0.9$) groups.

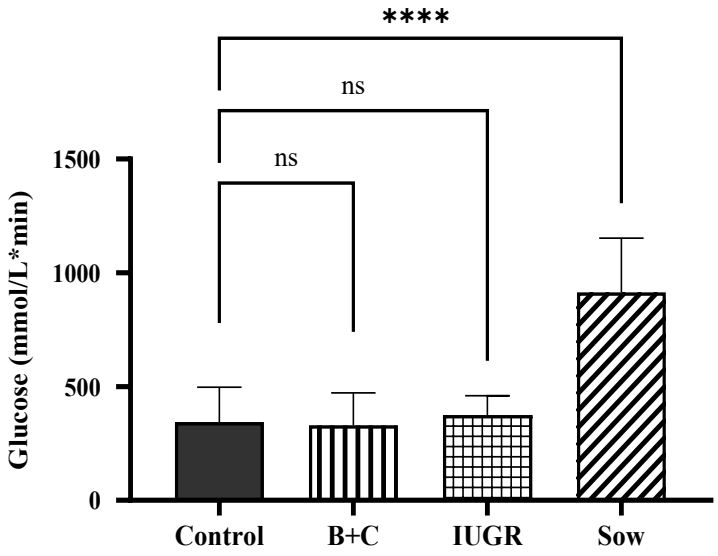

Figure 3.3: AUC from IVGTT conducted immediately after treatments. Bars represent means ± SD for the area under the curve for the TPN- control, TPN-B+C, TPN- IUGR and Sow-fed groups (n=8 for Control and B+C, n=9 for IUGR, n=7 for Sow). One-way ANOVA followed by Dunnett's multiple comparisons ($p < 0.05$) was used to test for diet differences. Control TPN had a significantly lower area under the curve (p=0.0001) compared with the Sow-fed piglets. B+C and the IUGR groups showed no significant differences (p=0.9 and p=0.9) compared to TPN- control.

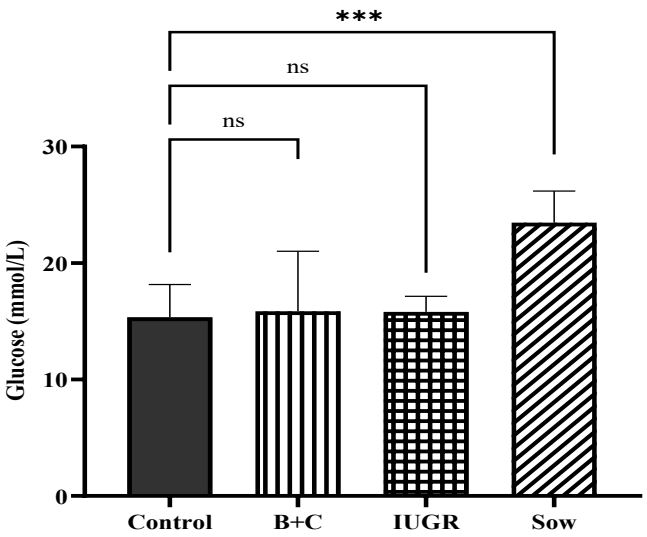

Figure 3.4: Effect of diet on peak glucose concentration from IVGTT conducted immediately after treatments. Peak glucose concentration for intravenous glucose tolerance test (IVGTT). Bars represent mean ± SD with n=8 for TPN-control and TPN-B+C, n=9 for TPN-IUGR, n=7 for the Sowfed group. One-way ANOVA followed by Dunnett's multiple comparisons ($p< 0.05$) was used to test for diet differences. No significant effect was observed in the TPN- B+C and the TPN-IUGR groups compared to the TPN- control ($p=0.9$ and $p=0.9$, respectively). However, there was lower peak glucose in the TPN- control groups compared to the Sowfed ($p=0.0001$).

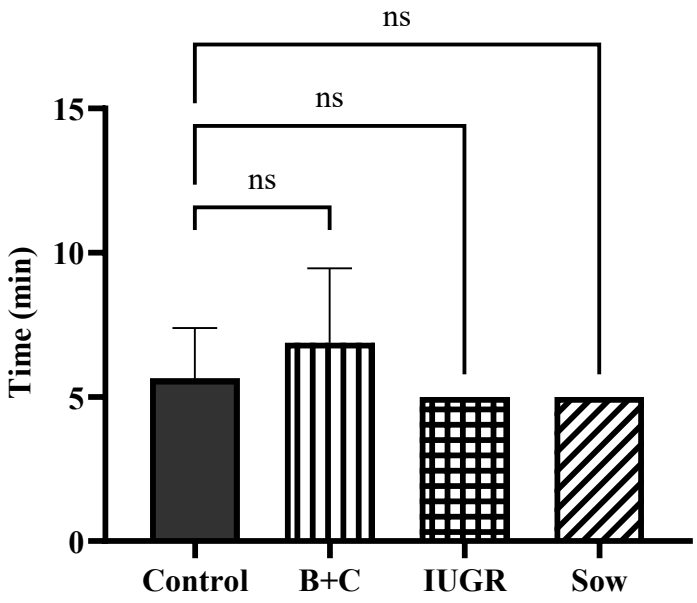

Figure 3.5: Effect of diet on time to peak glucose concentration for IVGTT conducted immediately after treatments. Data represent the time to peak glucose concentration (TTP). Bars represent mean ± SD with n=8 for TPN-control and TPN- B+C, n=9 for TPN-IUGR, and n=7 for the Sow-fed. One-way ANOVA followed by Dunnett's multiple comparisons ($p<0.05$) was used to test for diet differences. There was no statistically significant difference from TPN-control for the TPN- B+C ($p=0.3$), TPN-IUGR ($p=0.8$) or the Sow-fed groups ($p=0.9$). In both the IUGR and Sow-fed groups, glucose levels peaked 5 minutes after the glucose injection in all pigs.

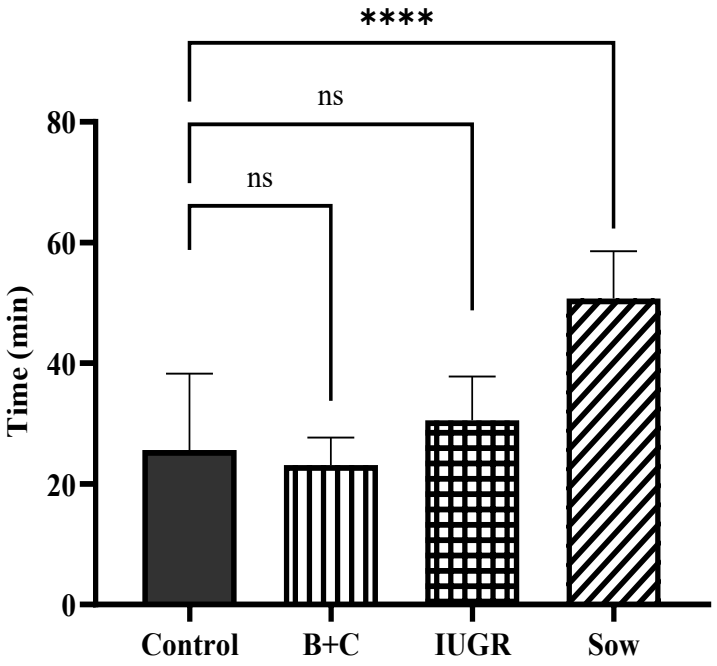

Figure 3.6: Piglets time to baseline (TTBL) conducted immediately after treatments. Data show the time to return to baseline glucose concentrations (TTBL). Bars represent mean ±SD with n=8 for TPN-control and TPNB+C, n=9 for TPN-IUGR, n=7 for the Sow-fed. One-way ANOVA followed by Dunnett's multiple comparisons compared to the TPN-control showed no statistically significant differences between the TPN-control and TPN-B+C or TPN-IUGR groups (p=0.08 and p=0.5, respectively). However, the TPN- control group had lower TTBL than the Sow-fed group (p=0.0001).

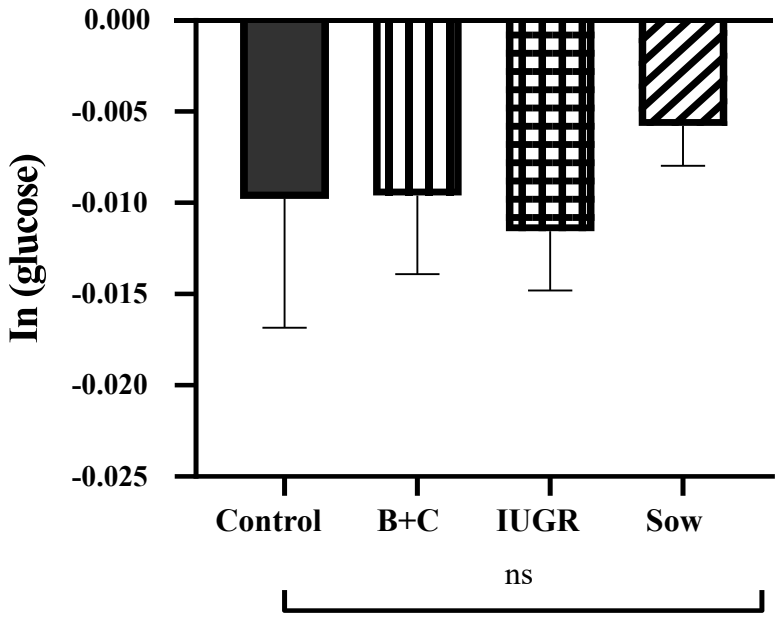

Figure 3.7: Slope of glucose clearance from the IVGTT conducted immediately after treatments. Bars represent mean ± SD with n=8 for TPN-control and TPN- B+C, n=9 for TPN-IUGR, n=7 for Sow-fed. One-way ANOVA followed by Dunnett's multiple comparisons ($p < 0.05$) was used to test for diet differences. No significant effects were observed for TPN-B+C, TPN-IUGR or Sow-fed groups, compared to the TPN- control ($p=0.9$, $p=0.7$, $p=0.2$, respectively).

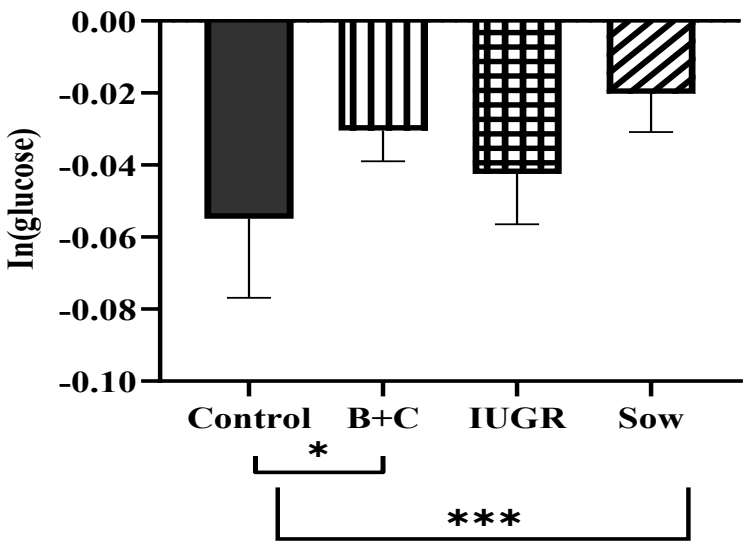

Figure 3.8: Slope of glucose clearance from insulin sensitivity test (IST) conducted immediately after treatments. Bars represent mean ± SD with n=8 for TPN-control and TPN-B+C, n=9 for TPN-IUGR, n=7 for Sow-fed. One-way ANOVA followed by Dunnett's multiple comparisons ($p< 0.05$) was used to test for diet differences. Compared to the TPN-control group, TPN-B+C and Sow-fed animals had higher slopes ($p=0.018$ and $p=0.0006$, respectively) than control. TPN-IUGR group did not differ significantly from the TPN-control group ($p=0.2$).

3.3. Insulin concentrations:

Table 3.2: Insulin concentrations from IVGTT immediately after pigs were fed experimental TPN diets (Piglets) and 9 months later (Adult pigs).

	Control	B+C	IUGR	SOW
Piglets (µIU/ml)				
Peak insulin	207.4±84.2	184.7±75.9	128.9±88.3	184.8±115.5
Corrected peak insulin	115.8±34.9	132.1±49.0	106.0.3±55.3	119.8±58.0
TTP	13.8±3.8	13.1±3.9	16.0±6.0	10.0±7.0
TTBL	37.0±10.4	48.8±13.6	45.7±9.7	42.0±12.1
AUC	1899±1039	2269±940	1601±695	1969±1190
Adult pigs (µIU/ml)				
Peak insulin	215.9±125.3	200.5±96.2	192.2±79.0	164.2±52.3
Corrected Peak insulin	130.2±67.8	90.0±41.2	135.8±59.9	97.2±45.1
TTP	9.3±4.9	13.7±4.4	10.0±3.4	10.7±5.7
TTBL	41.5±9.3	38.13±8	41.67±10.3	35.71±9.3
AUC	3748±1885	4512±1379	3990±1352	4987±1895
Corrected insulin AUC	2406±1162	1597±867	2229±470	1473±388

Data are expressed as mean ±SD. One-way ANOVA and Dunnett's multiple comparisons were used to compare treatment groups to the control TPN group.

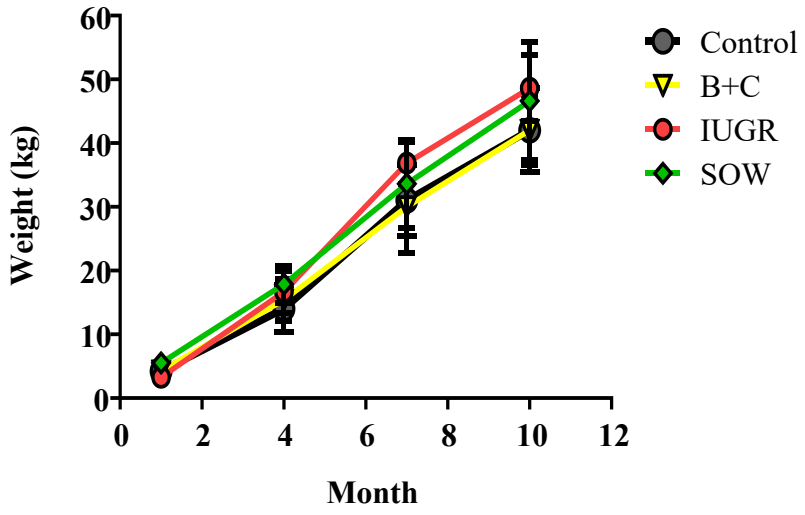

Figure 3.9: Body weight from 1 to 10 months old. Symbols represent mean ± SD with n=8 for TPN-Control, TPN-B+C and for TPN- IUGR, n=7 for Sow-fed.

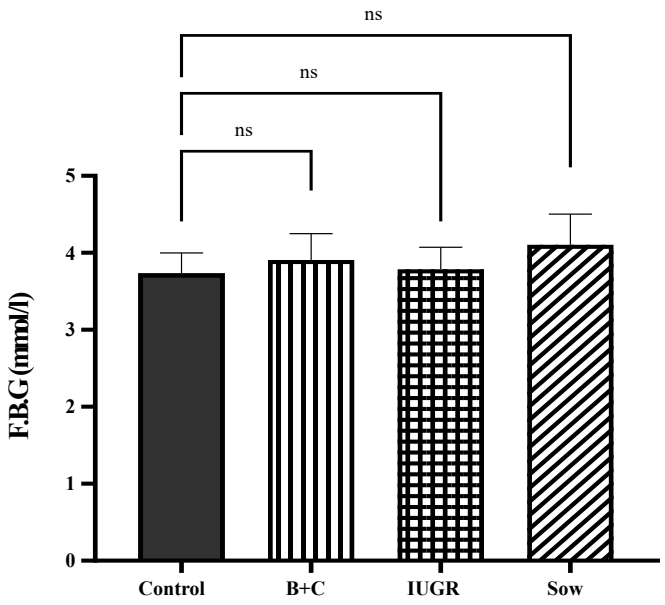

Figure 3.10: Fasting blood glucose concentrations from IVGTT conducted 9 months after treatments. Bars represent means ± standard deviation with n= 8 for TPN-control, TPN-B+C, TPNIUGR; n = 7 for Sow. One-way ANOVA followed by Dunnett's multiple comparisons ($p < 0.05$) were used to test for diet differences compared to control. No differences among groups were observed when compared with the control TPN.

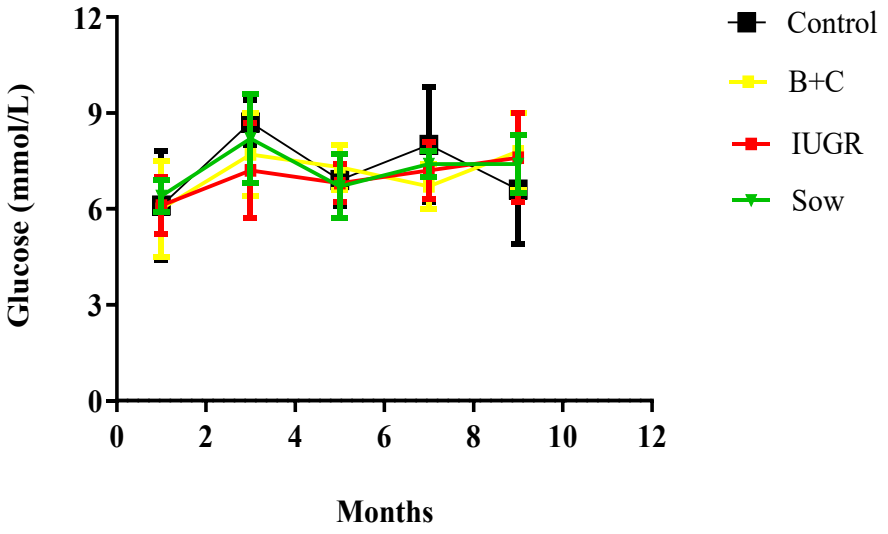

Figure 3:11: Monthly fasted plasma glucose concentration. After overnight fasting, monthly plasma glucose concentrations were measured for all pigs (TPN-control, n=8, TPN-B+C, n=7, Sow-fed, n=7, and TPN-IUGR, n=7) and were not statistically different among the treatment groups. Each symbol from the graph represents the mean ±SD plasma glucose in each treatment group.

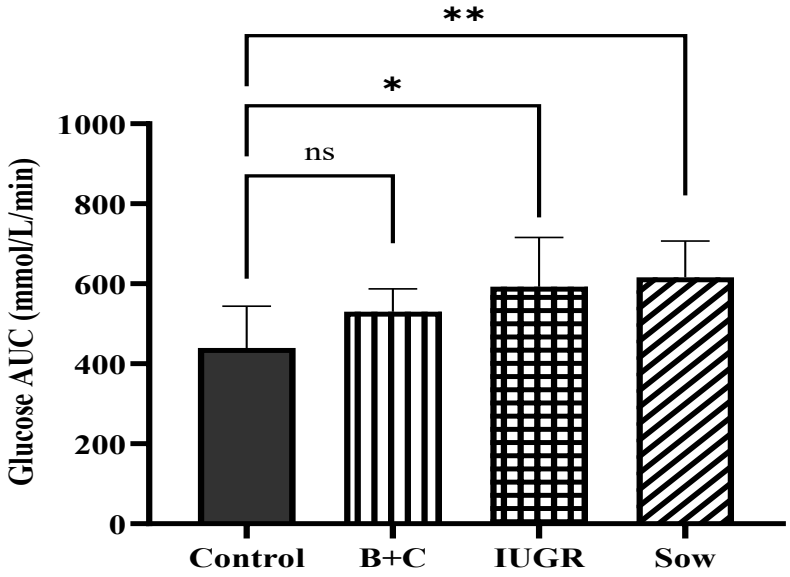

Figure 3.12: Area under glucose curve (AUC) from IVGTT conducted 9 months after treatments. Bars represent mean ± SD with n=8 for TPN-control and TPN-B+C, n=7 for TPN-IUGR and Sow-fed. One-way ANOVA followed by Dunnett's multiple comparisons ($p< 0.05$) was used to test for diet differences. Control TPN demonstrated a significantly lower glucose area under the curve (AUC) when compared with the Sow treatment and IUGR group ($p=0.0042$, $p=0.0134$, respectively). On the other hand, betaine and creatine supplementation showed no significant difference from Control ($p=0.2$).

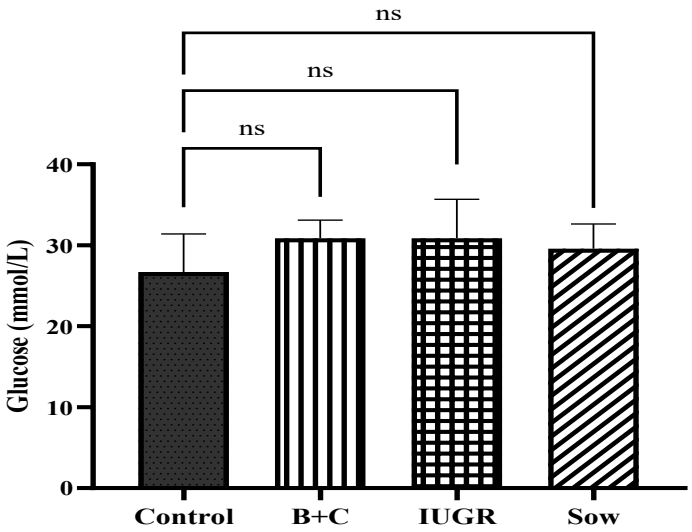

Figure 3.13: Peak glucose concentration from IVGTT conducted 9 months after treatments. Bars represent mean ± SD with n=8 for TPN-control, TPN-B+C and TPN-IUGR, n=7 for Sow-fed. One-way ANOVA followed by Dunnett's multiple comparisons ($p<0.05$) was used to test for diet differences vs Control. There tended to be a significant effect in the TPN- B+C and Sow-fed groups compared to the TPN- control ($p=0.1$ and $p=0.1$, respectively). However, there were no significant effects in the TPN-IUGR group compared to the TPN- control group ($p=0.3$).

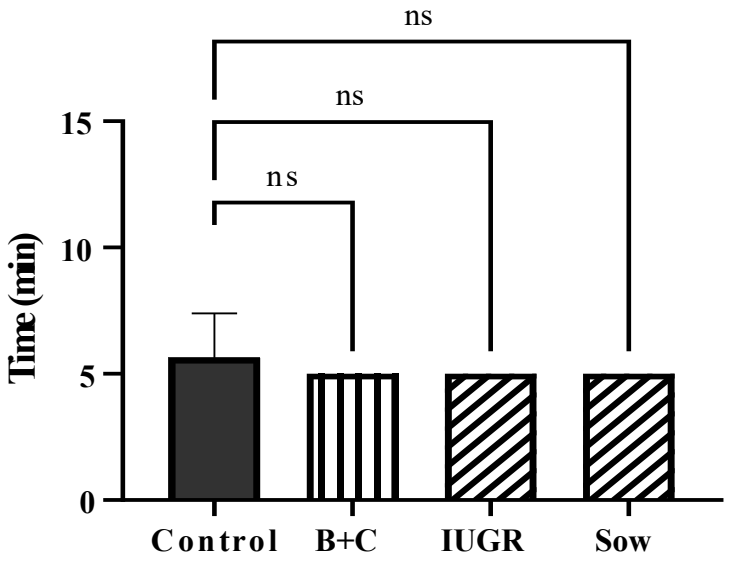

Figure 3.14: Time to peak glucose concentration (TTP) from the IVGTT conducted 9 months after treatments. Bars represent mean ± SD with n=8 for TPN-control, TPN-B+C and TPN-IUGR, and n=7 for Sow-fed. One-way ANOVA followed by Dunnett's multiple comparisons ($p< 0.05$) was used to test for diet differences vs Control. There was no statistically significant difference from TPN control for the TPN- B+C, TPN-IUGR or Sow-fed groups ($p=0.4$ and $p=0.4$, $p=0.4$ respectively). In B+C, IUGR and Sow-fed groups, glucose levels peaked 5 minutes after glucose injection in all pigs.

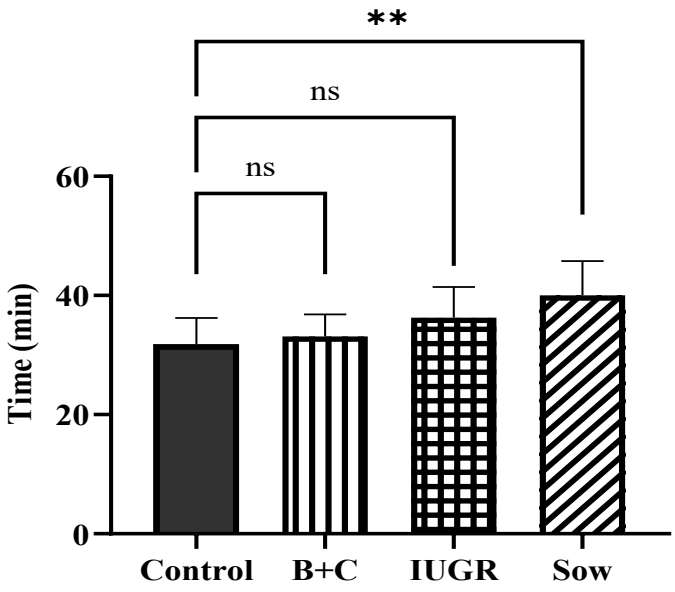

Figure 3.15: Time to baseline glucose concentration (TTBL) from the IVGTT conducted 9 months after treatments. Bars represent mean ± SD with n=8 for TPN-control, TPN-B+C and TPN-IUGR, n=7 for Sow-fed. One-way ANOVA followed by Dunnett's multiple comparisons ($p< 0.05$) was used to test for diet differences vs Control. There was no statistically significant difference between the TPN-B+C and TPN-IUGR groups ($p=0.9$ and $p=0.2$, respectively) compared to Control. However, there was significantly higher TTBL ($p=0.0073$) in the sow-fed group compared to control.

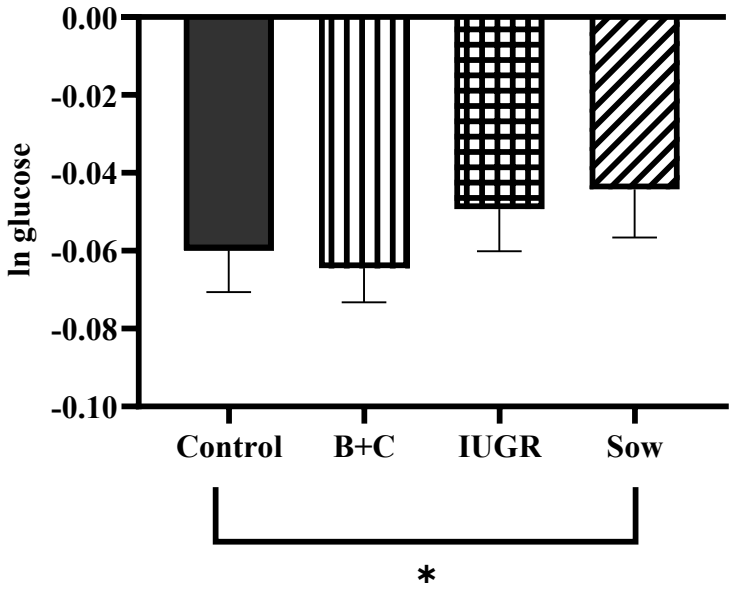

Figure 3.16: Slope of glucose clearance from IVGTT conducted 9 months after treatments. Bars represent mean ± SD with n=8 for TPN-control, TPN B+C and TPN-IUGR, n=7 for Sow-fed. One-way ANOVA followed by Dunnett's multiple comparisons ($p< 0.05$) was used to test for diet differences vs Control. No significant effect was observed in the TPN-B+C and TPN-IUGR groups compared to the TPN control ($p=0.7$ and $p=0.2$, respectively). However, there was a faster glucose clearance rate in the Sow-fed group than in the TPN-control group ($p=0.033$).

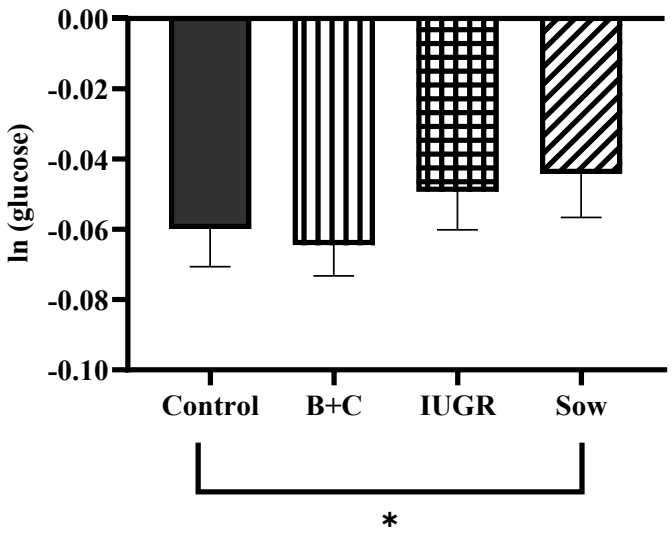

Figure 3.17: Slope of glucose clearance to insulin infusion from the IST conducted 9 months after treatments. Bars represent mean ± SD with n=8 for TPN-control, TPN-B+C and TPN-IUGR, n=6 for Sow-fed. One-way ANOVA followed by Dunnett's multiple comparisons ($p < 0.05$) was used to test for diet differences vs Control. No significant effects were observed in the TPN-B+C, Sow-fed, or TPN-IUGR groups compared to the TPN-control ($p=0.9$, $p=0.9$, $p=0.9$, respectively).

5. Discussion

The Yucatan miniature piglet is an excellent model for studying parenteral effects on nutrient metabolism because of human nutritional, physiological, and metabolic similarities. Moreover, pigs are a good model for diabetes and many other chronic diseases, such as cardiovascular disease and obesity (McKnight et al., 2012). In this study, female pigs were chosen because previous research indicated that they have higher visceral fat levels and worse glucose tolerance than males (McKnight et al., 2012). Therefore, female Yucatan miniature pigs were selected for this research.

The uptake of glucose by tissues is regulated by insulin sensitivity. Faster clearance of glucose from the bloodstream is indicative of high insulin sensitivity. The IUGR piglets were born small and were defined as approximately 65% of the normal birth weight of the largest littermate (McKnight et al., 2012). We hypothesized that IUGR pigs will increase body size quickly to catch up, which could lead to obesity and subsequently lead to biomarkers of T2D. We also hypothesized that early TPN will alter glucose metabolism in Yucatan pigs in later life and induce biomarkers of T2DM. At the end of 2 weeks of TPN feeding, at approximately 21 days of age, the IUGR piglets had significantly lower mean body weight than the TPN control (Table 3.1). Our findings suggest that the piglets with IUGR did not exhibit catch-up growth during the TPN feeding phase. This lack of growth acceleration might be attributed to the controlled TPN feeding rate relative to body weight. The TPN was infused at a rate to maintain a normal growth rate and was not increased to allow early catch-up growth.

Our study demonstrated that piglets fed by the sow exhibited a significantly higher growth rate compared to those in the TPN control group ($p=0.0034$). Table 3.1. TPN

infusion rate is determined to match the average growth rate of sow-fed piglets. However, in our study, this enhanced growth rate may be linked to the increased volume of milk suckled after the removal of the other piglets from the sow; it is well known that a smaller number of piglets will grow faster on a sow, due to lower competition for the best teats, leading to a notable increase in their growth rate.

TPN-fed pigs with IUGR showed an elevated fractional growth rate in the 45d-4 month period compared to TPN-fed pigs with normal birth weight, as listed in Table 3.1, demonstrating compensatory growth due to IUGR. Additionally, between 4 and 6 months of age, IUGR pigs exhibited a higher growth rate (Table 3.1), resulting in no significant difference in body weight among groups by 6 months, confirming that IUGR pigs achieved complete catch-up growth before reaching sexual maturity (i.e. ~7 months old). This finding is similar to the results from a previous study conducted in our laboratory by Myrie et al. (2017). Moreover, at 4 months old, there were no differences in body weight between the IUGR, B+C, or sow-fed groups compared to the control TPN group ($p>0.05$); these data suggest that the IUGR piglets caught up even earlier post-weaning, before reaching sexual maturity. In humans, children born with IUGR do not exhibit compensatory growth until they reach the age of six; consequently, these children continue to be shorter in stature (Batista et al., 2012). Moreover, McMillen and Robinson (2005) reported that neonatal animals that have experienced malnutrition or illness may exhibit compensatory growth; this phenomenon is not exclusive to low-birthweight infants. It was estimated that 30% of babies born within the normal range of birth weights may experience catch-up growth (Kays & Hindmarsh, 2006). Regardless, IUGR piglets in our study caught up in body weight, but whether the body composition was different is unclear. In our previous studies,

this catch-up growth was due to higher body fat deposition, but we did not confirm this in our current study.

In the current study in Yucatan pigs, fasting blood glucose was measured immediately after two weeks of TPN feeding. Blood samples were taken after a 6-hour overnight fast, and plasma was separated for fasting glucose tests and other biochemical analyses. In a study of 71 male and female Yucatan pigs, Rispati et al (1993) found that normal fasting plasma glucose levels ranged from 2.4 to 7.4 mmol/L, and in parenterally fed Yorkshire White domestic piglets, blood glucose ranged from ~4 to 8 mmol/L. In our study, no significant differences were observed between the TPN groups supplemented with betaine and creatine or the IUGR group when compared to the TPN control group; moreover, these values were within the range mentioned above. Similarly, a study conducted by McKnight et al. (2012) in our laboratory found no significant differences in IUGR or normal-weight Yucatan adult pigs with fasting blood glucose of ~5.3 mmol/L. However, our study observed an unexpected outcome in sow piglets; they exhibited an elevated fasting blood glucose concentration compared to the TPN groups (Figure 3.2). Specifically, the average fasted plasma glucose concentration in the sow-fed group was 10.8±3.3 ($p = 0.0017$), while it was 7.2±2.2 in the TPN control group. It is possible that our short 6-hour fast needed to be extended to 8-12 hours to ensure a more accurate measurement of fasting plasma glucose concentration in the suckled pigs. Alternatively, delayed gastric emptying and the potential disruption of incretin hormones caused by TPN could explain these changes.

In addition, plasma glucose was also measured monthly (after overnight fasting) to assess glycemia or signs of type 2 diabetes as the pigs grew to adulthood. Plasma samples at the 1st, 3rd, 5th, 7th and 9th month of age were not statistically different among the treatment

groups (Figure 3.11). The current study also tested the piglets for glucose tolerance (IVGTT) after 14 days of TPN feeding or suckling and then again in adulthood. A glucose bolus was administered intravenously during this test, and blood samples were collected to assess glucose tolerance. TPN feeding led to a faster glucose clearance rate, which persisted into adulthood, as indicated by lower fasting glucose, the lower glucose area under the curve (AUC), shorter time to peak and shorter time from peak to the baseline from IVGTT, compared to sow-fed controls. Indeed, the peak plasma glucose concentration in the sow-fed piglets reached 23 mmol/L and was significantly higher than that in the TPN control group. Our findings contradicted our expectations; oral feeding showed a higher plasma glucose peak and glucose TTBL than in the TPN groups ($p=0.0001$). The faster glucose clearance after TPN treatments could possibly be related to a high insulin level or low level of counterregulatory hormones in piglets.

Unexpected outcomes were also notable. The sow-fed piglets exhibited a ~2.5x larger glucose area under the curve from the IVGTT than the TPN control group and ~50% higher fasting plasma glucose levels in comparison to the TPN control group. It is possible that the sow-fed piglets developed hyperglycemia due to stress from handling, unlike the TPN-fed pigs that were already confined in cages for two weeks. Stoll et al. (2012) mentioned that stress hormones could induce hepatic glucose production and reduce insulin action in premature infants, leading to hyperglycemia. Alternatively, it is possible that the sow-fed piglets responded normally, whereas the TPN-fed groups had altered metabolism. The areas under the curve (AUC) in the TPN treatment groups were much lower than the sow-fed piglets, indicating that piglets that received TPN feeding cleared exogenous glucose more rapidly. However, the result of a higher glucose AUC from the IVGTT in

sow-fed pigs persisted into adulthood, which suggests that those AUC data are normal and the high AUC as piglets was not due to handling stress, which was unique to sow-fed pigs among piglet groups. The adult pigs were all handled similarly and were adapted to handling.

The current study showed that glucose concentration AUC in Yucatan miniature piglets ranged from 330 to 913 mmol/L $^{-1}$·min^{-1} (Figure 3.3), whereas McKnight et al. (2012) reported 150-900 mmol/L^{-1}·min^{-1}. In the current study, during IVGTT in piglets, the slope of glucose from peak to baseline showed no differences between the Sow-fed or B+C groups, compared with the TPN control. However, IUGR piglets showed a faster glucose clearance rate than the TPN group, but those effects did not seem to persist into adulthood. So, it is possible that IUGR pigs are more insulin-sensitive early in life.

An insulin sensitivity test (IST) measures the cells' sensitivity to endogenous insulin. In IST, an insulin bolus is administered, and the glucose clearance in response to that insulin is assessed. The rate at which glucose is removed from the plasma reflects the glucose uptake by peripheral tissues. In this study, somatostatin, which has a short half-life, was administered intravenously to suppress endogenous insulin secretion, so the test is a response to a fixed amount of insulin dose. Following each blood sample, a consistent maintenance dose of insulin was given. Then, glucose infusion was administered, inducing hyperglycemia, and insulin bolus was administered. After the bolus, the plasma glucose clearance rate was assessed through serial blood sampling. In piglets, after IST was performed, sow-fed and B+C treatment had a lower negative slope of glucose clearance ($p<0.05$) compared to TPN control (Figure 3.8). This similar glucose clearance in the sow-fed and B+C groups could suggest that adding B+C to TPN could be a novel treatment that

might prevent the TPN-induced hypersensitivity to insulin observed in the TPN-control and IUGR groups. Insulin levels were also measured during the IVGTT in piglets and adult pigs. However, no notable differences were observed between the TPN control and the different treatment groups, suggesting neither piglets nor adult pigs exhibited insulin resistance or type 2 diabetes development**.**

Our hypothesis suggests that TPN feeding may have long-term effects on metabolism, potentially leading to glucose intolerance and an increased risk of type 2 diabetes in later life, especially in pigs with IUGR. Since TPN impairs glucose tolerance in premature infants or those with low body weight (LBW) in later life, we expected that TPN would alter glucose metabolism and induce glucose intolerance or insulin resistance in adult pigs, and the IUGR pigs could have an even higher risk of metabolic disease in later life. We also hypothesized that supplementation of betaine and creatine may prevent these effects. Therefore, at ~9 months of age, all treatment groups (TPN Control, B+C, IUGR and Sow fed) were tested for glucose intolerance and insulin sensitivity tests (IST).

Our study observed that pigs exposed to TPN did not exhibit signs of T2DM biomarkers in adulthood. This unexpected outcome made us consider possible explanations for the absence of anticipated metabolic effects. One possible explanation is that the study duration may have been insufficient for developing T2DM biomarkers. Metabolic changes, particularly those related to insulin resistance, often require an extended period to become evident. At 9 months of age, our pigs are equivalent to a young human adult. Although T2DM occurs at these young ages, it is far more prevalent in the middle age or the elderly. Extending the study duration could provide a more comprehensive understanding of the long-term effects of TPN on metabolic health. Another important observation was the

decreased fractional daily feed intake in pigs with IUGR compared to the TPN control group, as reflected by (g/kg body weight) from 45 d old to 4 months old (p=0.0492), 4-7 months old (p=0.0001), and 7-9 months old (p=0.0203) (Table 3.1). This lower feed intake is likely due to the obesity developed in the IUGR group. Their absolute feed intakes were not different, but the IUGR pigs were heavier. So, they most likely ate the same amount of food per metabolic live weight (i.e. lean weight). Additionally, the measurement of pigs' body weight at the end of the study did not reveal a significant difference between the IUGR group and the TPN control group. Interestingly, our study revealed similar results to previous research by McKnight et al. (2012), which found that IUGR pigs did not develop glucose intolerance, insulin resistance or T2DM. Similarly, Poore and Fowden (2004) observed no differences in body weight between feed-restricted domestic pigs and low-birth pigs at 12 months of age. However, their study reported an association between small birth weight and glucose metabolism in domestic pigs. One notable factor arising from this comparison is the difference in study duration, with Poore and Fowden (2004) conducting their study over a 12-month period, while our study had a shorter duration. Studies have mentioned that growth and adiposity are associated with increased sensitivity to insulin and insulin-like growth factor-1 (IGF-1). When animals exhibit enhanced responsiveness to these hormones, there is a subsequent augmentation in the absorption of nutrients into tissues, leading to the surplus being stored as fat in adipocytes, as elucidated by DeBlasio (2007).

Our study revealed a higher AUC in the IUGR adult pigs (Figure 3.12) (p=0.013), which agreed with our expectations. As a result, higher AUC could eventually, with time, lead to insulin resistance or biomarkers of T2DM in IUGR pigs.

As adult pigs, IUGR and Sow-fed pigs exhibited a larger AUC than the TPN control group (Figure 3.12). Sow-fed pigs displayed elevated AUC values in both early life and adulthood. Moreover, the time required to return the plasma glucose concentration to baseline showed a greater significant effect in the Sow-fed group, approximately 40 minutes, compared to the TPN control ($p=0.007$) (Figure 3.15). Together, these data demonstrate that the effects of TPN feeding on glycemia persisted into adulthood. Although TPN did not induce insulin resistance or biomarkers of T2DM, TPN feeding did lead to more efficient removal of glucose from the blood. These pigs were highly sensitive to insulin, as indicated by time to baseline (TTBL). Furthermore, from the peak to baseline, the glucose clearance rate in Sow-fed adult pigs demonstrated a faster removal of glucose from circulation than the TPN control group ($p=0.033$) (Figure 3.16). This supports the anticipated and confirmed hypothesis that Sow-fed (breastfeeding) is more advantageous and efficient than TPN feeding. Compared to the TPN control, IUGR, B+C, and sow-fed pigs also did not show any significant difference in the sensitivity of cells to endogenous insulin (IST) ($p>0.05$).

6-Conclusion:

Total parenteral nutrition (TPN) fulfills the nutritional needs of many ill or IUGR newborns who cannot receive nutrition orally due to gastrointestinal issues. Nevertheless, prolonged TPN use is linked to chronic conditions such as gut atrophy, liver disorders, sepsis, hepatic fibrosis, and fatty liver (Hui et al., 2006). IUGR, followed by rapid postnatal growth, is associated with an increased risk of developing obesity and T2DM (Ling & Ronn, 2019). The programming of this risk is associated with epigenetics, more specifically, DNA methylation. It is thought that methyl groups might be limiting in IUGR and TPN feeding, affecting epigenetics and risk for glucose intolerance and insulin resistance in later life. Adding methionine to TPN is one strategy to increase methyl groups, but it may lead to hyperhomocysteinemia. Therefore, an alternative strategy of adding betaine to TPN feeding in neonates could reduce homocysteine levels, while also increasing the availability of methyl groups, which would improve DNA methylation and possibly prevent glucose intolerance and insulin resistance in later life. Moreover, because methionine is needed to synthesize creatine, adding creatine to TPN can spare methionine, which may increase methyl groups for DNA methylation reactions (Randunu & Bertolo, 2020). TPN itself can induce hyperglycemia, which leads to many complications, including higher mortality rates. Hyperglycemia is associated with high blood glucose levels and reduced glucose utilization by tissues (Stoll et al., 2012). Whether this hyperglycemia persists into adulthood is unknown.

The rationale of the study:

Infants born with intrauterine growth restriction are more likely to develop glucose intolerance and insulin resistance. TPN feeding is also associated programming of glucose intolerance and insulin resistance, so the combination of TPN and low birth weight could be worse. However, supplementation of betaine and creatine could increase methionine availability for DNA methylation. TPN also directly alters glycemia in pigs, but whether these changes persist to adulthood is unknown. This study aimed to investigate neonatal TPN effects on glucose metabolism in early life and whether these changes persist into adulthood in Yucatan miniature pigs. So, we hypothesized that TPN would alter glucose metabolism in neonatal piglets and induce biomarkers of T2DM in adulthood. However, supplementation of betaine and creatine will prevent these changes by providing sufficient methyl groups for methylation reactions and DNA synthesis, thus ensuring normal epigenetics. Moreover, betaine is a methyl donor that can remethylate homocysteine to methionine, which would lower homocysteine concentration. At the same time, creatine could reduce the burden of creatine synthesis by supplying the methyl groups and sparing methionine for protein synthesis and growth. And because IUGR can independently program risk for T2DM, our final hypothesis was to determine if IUGR exacerbates the TPN-induced programming of T2DM biomarkers.

We found that TPN feeding altered glucose metabolism, unexpectedly enhancing glucose clearance, as evidenced by lower fasting glucose levels, reduced glucose area under the curve (AUC) from the IVGTT, decreased peak glucose concentration, and shorter time from peak to baseline (TTBL). These effects on glucose clearance were most pronounced immediately after stopping TPN but remained significant ~9 months later. While TPN

initially increased insulin sensitivity, this effect was not sustained after ~9 months. However, supplementing TPN with betaine and creatine improved insulin sensitivity, with no significant changes observed in glucose clearance parameters. Notably, the combination of low birth weight and TPN did not exacerbate TPN's effects.

In summary, TPN can induce lasting changes in metabolism, potentially elevating the risk of chronic conditions like insulin resistance and type 2 diabetes mellitus. These alterations in metabolism likely occur through epigenetic mechanisms influenced by imbalances in methyl nutrients such as betaine and creatine. Consequently, we conclude that TPN during early life can alter glucose metabolism into adulthood, though these changes are inconsistent with type 2 diabetes biomarkers. Further research is warranted to comprehensively understand TPN's effects on glucose metabolism and their health implications.

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