Creatine Synthesis and Methionine Partitioning During TPN in Yucatan Miniature Piglets

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Abstract

Methionine is an indispensable amino acid in the diet of neonates and has a metabolic role in the synthesis of creatine, phosphatidylcholine (PC) as well as protein synthesis and other transmethylation reactions. Creatine synthesis consumes significant amounts of arginine (a conditionally essential amino acid) and methionine, while PC requires methionine for its synthesis. Total parenteral nutrition (TPN) is typically marginal in these amino acids, and devoid in creatine. Therefore, our objective was to quantify the partitioning of methyl groups among transmethylation reactions and the sparing effect of dietary creatine and GAA on protein synthesis. Adequate supply of dietary methionine resulted in significantly greater hepatic fractional synthesis rate (FSR) of PC. Furthermore, GAA supplementation increased FSR of creatine by approximately 175%, but only when adequate dietary methionine was supplied. Moreover, piglet weight gain was similar to that of piglets receiving adequate arginine, suggesting arginine was spared for growth. Overall, supplemental GAA could be considered as a novel TPN ingredient to increase the synthesis of creatine and spare arginine for growth, but only when adequate methionine is provided.

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List of Abbreviations

^{3}H	Tritium
5-MTHF	5-methyltetrahydrofoltate
Adeq	Adequate
ADP	Adenosine diphosphate
AGAT	Arginine:glycine amidinotransferase
ANOVA	Analysis of variance
ANSA	1-amino-2-naphthol-6-sulpohnic acid
Arg	Arginine
ASL	Argininosuccinate lyase
ASR	Absolute synthesis rate
ASS	Argininosuccinate synthase
ATP	Adenosine triphosphate
BHMT	Betaine-homocysteine methyltransferase
CBS	Cystathionine β-synthase
CGL	Cystathionine γ-lyase
CpG	Cytosine-phosphate-guanine
CRE	Creatine
DNA	Deoxyribonucleic acid
DNMT	DNA methyltransferase
DMG	Dimethylglycine
DPM	Disintegrations per minute
EDTA	Ethylenediaminetetraacetic acid

ELBW	Extremely low birth weight
FAO	Food and Agriculture Organization
FSR	Fractional synthesis rate
GAA	Guanidinoacetate
GAMT	Guanidinoacetate methyltransferase
HPLC	High-performance liquid chromatography
IG	Intragastric
IV	Intravenous
MeOH	Methanol
Met	Methionine
MS	Methionine synthase
N_2	Nitrogen
NEC	Necrotizing enterocolitis
NRC	National Research Council
P5C	Pyrroline-5-carboxylate
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PEMT	Phosphatidylethanolamine N-methyltransferase
PITC	Phenylisothiocyanate
PN	Parenteral nutrition
PNALD	Parenteral nutrition associated liver disease
PTFE	Polytetrafluoroethylene
SAH	S-adenosylhomocysteine

SAM	S-adenosylmethionine
Na^+	Sodium
SDS	Sodium dodecyl sulfate
SLC6A8	Sodium and chloride dependent transporter
SMA	Superior mesenteric artery
SRA	Specific radioactivity
TEA	Triethylamine
TFA	Trifluoroacetic acid
THF	Tetrahydrofolate
TLC	Thin layer chromatography
TPN	Total parenteral nutrition
UNU	United Nations University
WHO	World Health Organization

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1. Introduction

1.1 Total Parenteral Nutrition (TPN)

Total parenteral nutrition (TPN) was first introduced clinically in the early 1970's. Since TPN was first introduced, it has revolutionized how intestinal disease and dysfunction are treated. TPN is commonly used during times of intestinal distress, such as necrotizing enterocolitis (NEC), short bowel disease or feeding intolerance experienced by premature infants (Johnson, 2014). Due to the underdevelopment of the gut when an infant is born premature, it is very difficult to meet their nutritional needs as they have limited energy reserves (Brunton, Ball, & Pencharz, 2000). Feeding intolerances often develop and caloric intake is restricted, leading to growth retardation. TPN has allowed for increased survival and growth in pre-term infants over the last number of decades as an alternative method of nutrient delivery (Schutzman, Porat, Salvador, & Janeczko, 2008).

1.1.1 TPN Dietary Composition

TPN consists of all essential macro- and micro-nutrients. Carbohydrates are provided as a monosaccharide in the form of dextrose. Protein is commonly provided as crystalline amino acids with elevated essential to non-essential amino acid ratio, while cysteine is commonly added to parenteral nutrition (PN) solutions for neonates to prevent impairment of protein synthesis (Johnson, 2014a). It is estimated that protein retention during PN is approximately 70% (Schutzman et al., 2008). Therefore, in a clinical setting,

a range of approximately 3-4 g/kg/day of amino acids is recommended to promote growth in a premature infant (Johnson, 2014a). Fat is delivered in the diet as 20% lipid emulsions, commonly provided as a soybean oil emulsion in the form of Intralipid (Schutzman et al., 2008). Lipids are an important energy source for the neonate. It provides premature infants high energy for fat deposition during growth and spares energy from amino acids for protein synthesis (Johnson, 2014a). The recommended caloric intake for neonates receiving TPN is approximately 85-127 kcal/kg/day. This caloric intake is estimated to help the infant grow at approximately 15 g/kg/day (Johnson, 2014a). Understanding the energy requirements for the neonate is essential when administering TPN.

Micronutrients are provided in TPN as electrolytes, trace minerals and complex multiple vitamin solutions. These micronutrients are essential to the proper growth and development of the neonate (Johnson, 2014b). Research continues to refine PN solutions to alleviate complications associated with TPN, while increasing growth rates of infants.

1.1.2 TPN Drawbacks

Prolonged administration of TPN has been shown to lead to negative effects in both neonates and adult patients (Schutzman et al., 2008). One of the major drawbacks of TPN as an intervention method is gut atrophy. TPN by-passes first-pass splanchnic (ie. gut and liver) metabolism, and therefore does not utilize the gut for the digestion and absorption of essential nutrients. Niinikoski and colleagues demonstrated that only 8 hours after the introduction of TPN in three-week-old piglets, portal and superior mesenteric artery (SMA) blood flow decreased by more than 30% in comparison to

enterally fed pigs. These findings demonstrated the detrimental effects of TPN on the integrity of the gut, resulting in villous atrophy and decreased protein synthesis after 48 hours (Niinikoski et al., 2004). Gut atrophy from prolonged TPN can become problematic for the metabolism in the neonate. For example, the synthesis of the conditionally essential amino acid arginine occurs in the gut; and therefore, its synthesis becomes impaired with gut atrophy (Brunton et al., 2000).

Gut atrophy can lead to more serious conditions. NEC is a gastrointestinal infection resulting in mucosal inflammation and disruption in gut integrity. NEC is commonly seen in low birth weight infants and can result in intestinal perforation, surgery, and death (Dinesh et al., 2014). TPN affects the maturity of the gut and in piglets, introduction of formula feeding after TPN can lead to gut dysfunction and NEC. On the other hand, colostrum milk improved gut maturity and decreased the likelihood of NEC after administration of TPN (Bjornvad et al., 2008). Moreover, further research needs to be conducted to determine the optimal volume and rate of introduction of enteral feeds for premature infants transitioning from TPN. Gut atrophy and intestinal damage from disease such as NEC also down-regulate the synthesis of arginine (Brunton et al., 2000). As early as 1997, decreased arginine concentrations in pre-term infants were identified during the time of diagnosis of NEC (Zamora et al., 1997). Moreover, this observation led to research on the possible benefits of arginine supplementation for the prevention of all stages of NEC in premature infants (Amin et al., 2002).

Another problematic risk factor of TPN is prolonged implantation of central venous lines for the administration of TPN. The longer a patient is receiving TPN, the greater the likelihood of infection or vascular thrombosis (Schutzman et al., 2008).

Although from a clinical standpoint, the likelihood of infection is reduced with proper dressing techniques at the site of administration (Costello et al., 2008). The benefits associated with the administration of TPN during times of gastrointestinal distress outweigh the risks, since the survival rate of premature infants continues to increase (Schutzman et al., 2008).

1.1.3 Parenteral Nutrition-Associated Liver Disease (PNALD) & Cholestasis

The most common complication associated with TPN administration in neonates is the development of parenteral nutrition-associated liver disease (PNALD), that commonly presents as liver cholestasis (Kelly, 1998). TPN-induced liver cholestasis is characterized by bile duct regeneration, portal inflammation and fibrosis (Guglielmi et al., 2008). Over the past number of years, much research has been conducted to prevent the onset and reduce the severity of PNALD, but it continues to be a consequence with prolonged TPN administration. Schutzman and colleagues reported that 8-50% of extremely low birth weight (ELBW) infants receiving TPN for only 2 weeks demonstrated signs of biochemical cholestasis. Moreover, the incidence spiked to approximately 90% of ELBW infants receiving TPN for more than 90 days (Schutzman et al., 2008).

Many risk factors are associated with the development of TPN-induced liver cholestasis. These risk factors include liver dysfunction, sepsis, infection and nutritional deficiencies (Vlaardingerbroek et al., 2014). At this point in time, there is no definitive clinical method to prevent or alleviate liver cholestasis from prolonged TPN other than termination of administration. Recent research on prevention and reversal of PNALD has

focused on the lipid component of PN. Soybean oil has been the main source of lipids in commercial emulsions used in TPN for decades, but recent research suggests that this could be the cause of TPN-induced cholestasis. Soybean oil is high in phytosterols which have been shown to damage the biliary tract and disrupt bile flow (Clayton et al., 1998). Recently, Vlaardingerbroek and colleagues demonstrated that a 15% fish oil lipid emulsion protected against PNALD in pre-term piglets in comparison to TPN containing soybean oil lipid emulsion (Vlaardingerbroek et al., 2014). However, unlike Clayton et al., they found that phytosterols were not associated with the development of PNALD. These results provide evidence that lipid emulsions in TPN require further research to possibly prevent or alleviate TPN-induced cholestasis in pre-term infants.

1.2 Creatine Biosynthesis

Creatine biosynthesis is a major synthesis pathway that occurs in mammals and is a major consumer of labile methyl groups (Brosnan et al., 2009). Creatine synthesis primarily occurs in the liver and kidney (Wyss & Kaddurah-Daouk, 2000). In the kidney, arginine transfers an amidino group to glycine to form guanidinoacetate (GAA) and ornithine via the enzyme arginine:glycine amidinotransferase (AGAT). GAA is then transported to the liver and methylated by S-adenosyl methionine (SAM) via the enzyme guanidinoacetate methyltransferase (GAMT). One labile methyl group is transferred to GAA resulting in creatine and S-adenosyl homocysteine (SAH) (Brosnan & Brosnan, 2016). Creatine can then be transported to other tissues with high energy demand, such as brain and skeletal muscle. Creatine itself can be phosphorylated via creatine kinase to phosphocreatine which is used as an energy buffer to convert adenosine triphosphate

(ATP) to adenosine diphosphate (ADP) in a reversible reaction (Wyss & Kaddurah-Daouk, 2000). This rapid conversion and regeneration of ATP has made creatine supplementation a popular ergogenic aid in the fitness world.

The two enzymes involved in creatine biosynthesis, AGAT and GAMT, in some cases do not function properly due to a result of an inborn error of metabolism. As a result, an inborn error in the function of AGAT and GAMT may result in creatine deficiency in the brain and muscle. An inborn error in GAMT can lead to mental retardation at an early age (Sykut-Cegielska et al., 2004). However, it should be noted that large doses of creatine supplementation in infants suffering from AGAT or GAMT deficiency will normalize creatine levels if started at birth (Braissant et al., 2011; Schulze & Battini, 2007). Moreover, the success of this therapy has been demonstrated in AGAT knockout mice by Nabuurs and colleagues. Mice fed a creatine-free chow diet demonstrated disturbed muscle energy metabolism that could be rescued by supplementation with dietary creatine (Nabuurs et al., 2013). The clinical severity of these inborn errors of AGAT or GAMT in infants and the need for early intervention to prevent lifelong damage raises the question of whether infants should be screened at birth for defects in creatine synthesis.

1.2.1 Amino Acids & Metabolites Involved in Creatine Synthesis

1.2.1.1 Glycine

Glycine is a non-essential amino acid in the neonate and the adult, except in avian species where it is considered essential (Baker, 2009). Glycine can be synthesized via an inter-organ pathway primarily involving the liver and kidney (Wang et al., 2013). It is

synthesized from serine, threonine, choline, or hydroxyproline. Although it is non-essential, glycine plays many important roles such as the synthesis of protein and glutathione (GSH), conjugation of bile acids in mammals and in our case most importantly, de novo synthesis of creatine (Vlaardingerbroek et al., 2011). As described above, glycine's role in creatine synthesis occurs in the kidney. Glycine accepts an amidino group from arginine via the enzyme AGAT. It has been estimated that glycine accounts for approximately 11.3% of total protein in the piglet (Wu et al., 1999). Moreover, Brosnan et al. estimated that the glycine used for creatine synthesis only amounts to an equivalent of approximately 2.7% of net glycine incorporated into protein (Brosnan et al., 2009).

Aside from creatine synthesis, glycine plays a major role in protein synthesis and growth in the neonatal piglet. It has been estimated that protein synthesis accounts for approximately 80% of whole body glycine needs by animals, including piglets (Wu et al., 2004). Moreover, because of these high demands for protein synthesis and growth, there is the question of whether de novo synthesis of glycine can fully meet the demands of the growing neonate. Recently, strong evidence has been published to suggest that glycine synthesis is inadequate to meet the energy and growth demands of the growing neonatal piglet, and therefore glycine should be considered a dietary essential amino acid (Rezaei et al., 2013).

Glycine also has a major role in the synthesis of GSH, a major intracellular antioxidant, especially in early development when GSH pools are expanding (Vlaardingerbroek et al., 2011). Under certain circumstances in the neonate, in particular

during critical illness, it may be necessary to increase dietary glycine to maintain adequate synthesis of GSH, to prevent further illness and complication.

1.2.1.2 Arginine

Arginine is a conditionally essential amino acid depending on the stage of development (Dinesh et al., 2014). Arginine plays a major role in the synthesis of creatine by transferring an amidino group to glycine to make GAA via AGAT, as described above. Along with creatine, arginine plays a role as a precursor for the synthesis of other important amino acids and molecules such as proline, ornithine, polyamines and nitric oxide (NO). Furthermore, arginine plays a role in the secretion of important hormones including growth hormone, insulin, prolactin and glucagon (Vlaardingerbroek et al., 2011).

a. Arginine de novo Synthesis in Adults

Arginine synthesis differs between the growing neonate and the adult. In adults and most other mammals, arginine is considered a non-essential amino acid and is synthesized primarily through the intestinal-renal axis (Brosnan & Brosnan, 2004). In the small intestine, dietary arginine, glutamine, glutamate and proline are converted to citrulline through a sequential set of reactions and released into the portal blood. Circulating citrulline is primarily taken up by the kidney where two major enzymes, argininosuccinate synthetase (ASS) and argininosuccinate lyase (ASL), convert citrulline to arginine for release back into circulation (Brosnan & Brosnan, 2004).

The importance of the intestine in the synthesis of citrulline and arginine is further

emphasized by the localization of three major enzymes responsible for the conversion of glutamine, glutamate and proline to citrulline. These are pyrroline-5-carboxylate (P5C) synthase, proline oxidase, and *N*-acetylglutamate (NAG) synthase. The enterocyte is the only mammalian cell type to express all three of these enzymes (Flynn & Wu, 1996). It should be noted that the rate of arginine synthesis in humans depends on the delivery of citrulline to the kidney and not on the dietary availability of arginine (Castillo et al., 1994). Humans with functional kidneys demonstrate high rates of arginine synthesis from both endogenous and exogenous citrulline (Brosnan & Brosnan, 2004). Furthermore, it has been demonstrated that normal functioning kidneys synthesize approximately 1.75 g/day of arginine, while patients with chronic renal insufficiency still produce approximately 0.7 g/day of arginine (Tizianello et al., 1980). In context, synthesis of 2 g arginine/day is compared to approximately 4-5 g/day consumed in a typical Western diet (Brosnan & Brosnan, 2004). These findings demonstrate the importance of the intestinal-renal axis on the synthesis of arginine in humans and mammals.

b. Arginine de novo Synthesis in Neonates

In the neonate, arginine is considered a conditionally essential amino acid important for growth and many metabolic processes (Dinesh et al., 2014). Unlike the adult, neonatal arginine synthesis is localized and primarily occurs in the enterocytes of the gut (Vlaardingerbroek et al., 2011). Although the neonate is capable of synthesizing arginine, this is considered inadequate to meet the demands of the growing neonate; therefore, arginine is considered a conditionally essential amino acid (Dinesh et al., 2014).

Unlike the adult, arginine is not synthesized in the intestinal-renal axis. But, like the adult, arginine is the precursor for the synthesis of NO (Vlaardingerbroek et al., 2011). Playing an important role in immune and vasodilator activities, NO is an important molecule for the development of the neonate. In 2005, Fu and colleagues found that animal models of small intestine ischemia/reperfusion demonstrated increased intestinal blood flow due to increased NO when supplemented with arginine (Fu et al., 2005). These findings demonstrate the important homeostatic role between maintaining gut integrity and arginine synthesis in the growing neonate.

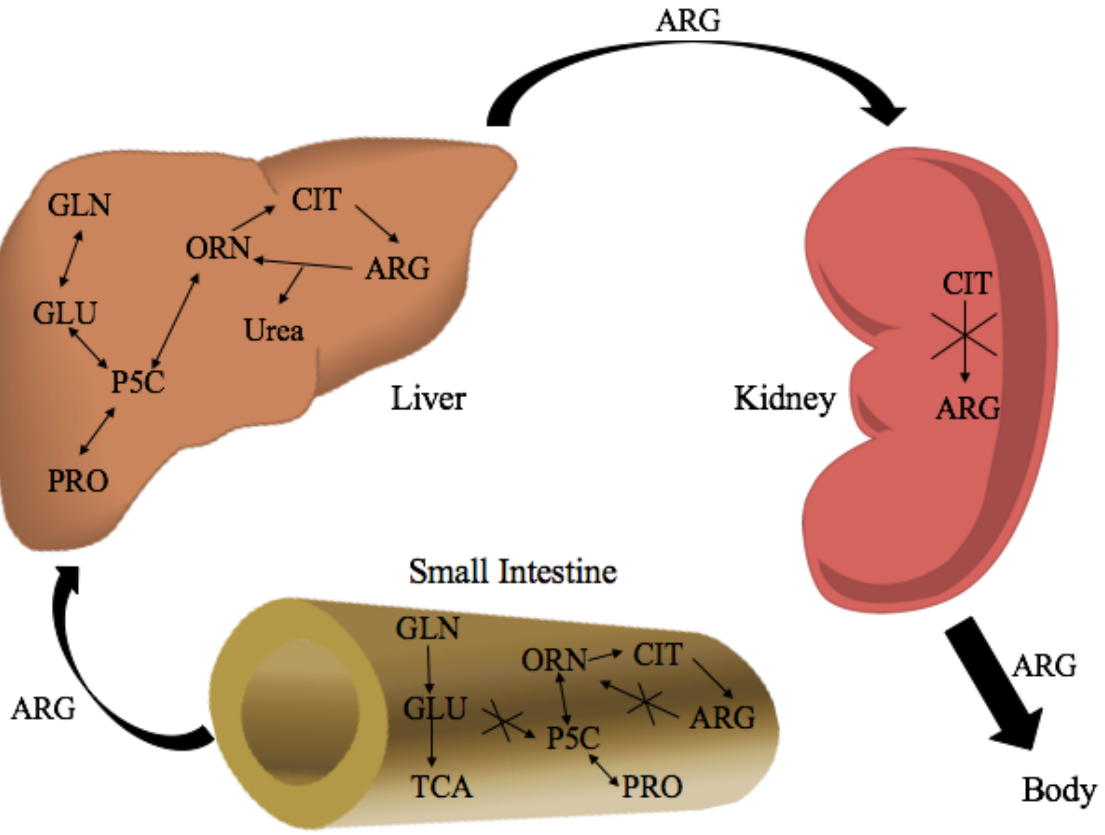

Figure 1.1: Arginine Synthesis in the Neonatal Gut (Adapted from: Vlaardingerbroek et al., 2011).

ARG, arginine; CIT, citrulline; GLN, glutamine; GLU, glutamate; ORN, ornithine; PRO, proline; P5C, 1-pyrrolline-5-carboxylic acid; TCA, tricarboxylic acid.

c. Arginine Requirement in Neonates

Because arginine is a conditionally essential amino acid in the growing neonate, the neonatal arginine requirement is dependent on the stage of development. During early development, energy demands are high due to high protein turnover (Dinesh et al., 2014). For example, as piglets get older, the dietary arginine requirement decreases. Piglets at approximately 4 kg body weight require 2.4% of their crude protein intake as arginine while 100 kg pigs only require 1.4% (Ball et al., 2007). The National Research Council (NRC) estimates the arginine requirement of growing piglets at 0.40 g/kg body weight/day (National Research Council, 1998). However, findings suggest that a minimum arginine intake of 0.20 g/kg/day of arginine is required to avoid hyperammonemia in piglets (Wilkinson et al., 2004). Another estimate using a factorial approach concluded that one-week-old piglets have an arginine requirement of approximately 1.1 g/kg/day to meet whole body needs, compared to the arginine intake from sow's milk which is estimated at 0.42 g/kg/day (Wu et al., 2004). Therefore, the neonatal piglet relies substantially on de novo synthesis to meet the arginine requirement. Although the NRC requirement has been established for growing piglets, recent findings have suggested that arginine supplementation increases the rate of growth in neonates. In 2004, 7- to 21-day-old piglets supplemented with 0.2% and 0.4% arginine in milk replacer demonstrated improved weight gains of 28% and 66%, respectively, in comparison to the control group fed arginine at the concentration in sow milk (Kim & Wu, 2004).

d. Arginine Requirement & de novo Synthesis during TPN

Prolonged usage of TPN has a significant impact on gut integrity and growth in the neonate. Gut atrophy has been shown to result in lower synthesis of arginine in neonatal piglets (Bertolo et al., 2003). Therefore, the arginine requirement for neonates during TPN is higher than that for neonates receiving breast milk or enteral diets. Arginine concentrations in PN solutions range from as high as 12.3 g/100 g amino acids, to as low as 4.7 g/100 g amino acids (Vlaardingerbroek et al., 2011). Moreover, in commercially available PN solutions, the arginine concentration is more than any other conditionally essential and non-essential amino acid. Studies on determining the optimum arginine concentration in PN solutions rely on the balance between maximizing growth and decreasing the likelihood of PNALD with high amino acid intake (Johnson, 2014a). The effect of prolonged administration of TPN to neonates on arginine status has been investigated by studying the role of dietary proline in arginine synthesis.

In 1999, Brunton et al. administered a complete TPN diet for 1 week to piglets, and then administered a PN solution that was complete except devoid in arginine. Within hours, this diet led to arginine deficiency as demonstrated by severe hyperammonemia (Brunton et al., 1999). When proline (a precursor of arginine) was also removed from the diet, these effects were exacerbated. However, the same study was performed using enteral diets and arginine deficiency was partially ameliorated as proline was converted to arginine by the intact gut. This study outlines the important role the gut plays in the de novo synthesis of arginine from proline in neonates and demonstrated the effect of TPN-induced gut atrophy on the rate of arginine synthesis. Using an isotope tracer study, the conversion of proline to arginine was estimated at 17% when proline was introduced into

the small intestinal lumen, but when first pass metabolism was by-passed, the conversion rate was negligible (Bertolo et al., 2003). Prolonged TPN administration in the neonate leads to a significant impact on first-pass metabolism and de novo synthesis of arginine. Therefore, the length of exposure to PN and concentration of arginine within PN solutions should be considered when neonates require TPN.

1.2.1.3 Guanidinoacetate (GAA)

Guanidinoacetate (GAA) is synthesized in the kidney from arginine and glycine via the enzyme AGAT, as described above (Brosnan & Brosnan, 2016). GAA is primarily taken up by the liver and readily converted to creatine via GAMT. Creatine is used by the body for high energy demand processes and has been extensively studied as an ergogenic aid for athletes to increase performance in high-intensity sports (Wyss & Kaddurah-Daouk, 2000). Less is known about GAA's effect as a supplement to promote growth and creatine synthesis. A recent human study in 2014 demonstrated a dose response to GAA supplementation on GAA and creatine concentrations in plasma (Ostojic et al., 2014). That study demonstrated GAA's possible effects including upregulating creatine synthesis to provide excess for creatine stores for energy metabolism. Those findings were supported by research from our group, which found that GAA supplementation to young pigs was better than dietary creatine at increasing creatine concentrations in hepatic and muscle tissues (McBreairty et al., 2015). However, as our group noted, the higher conversion of GAA to creatine leads to an increased consumption of available methyl groups. Therefore, dietary GAA supplementation may place a burden on available methyl groups and affect other important transmethylation reactions, including PC and

protein synthesis.

An abnormality in GAMT activity may lead to GAA toxicity and possible mental retardation in neonates. Therefore, these findings suggest that GAA supplementation may have possible toxic side effects. To asses this in humans, Ostojic et al. demonstrated that a 6-week supplementation of oral GAA in adults did not lead to any significant negative physiological effects (Ostojic et al., 2013). However, hepatocytes isolated from chow-fed rats exposed to various concentrations of GAA in vitro demonstrated higher caspase-3 activity, which is an indicator of apoptosis and cell death (Kharbanda et al., 2014). Further research is needed to determine whether GAA supplementation has toxic side effects at specific concentrations, particularly in growing animals and humans.

1.2.1.4 Methionine

Methionine is the only essential sulfur-containing amino acid and is required in the diet in both adults and growing neonates. Methionine also plays a major role in many transmethylation reactions (Finkelstein et al., 1988). In the cell, methionine is adenylated to SAM via the enzyme S-adenosylmethionine synthetase, also known as L-methionine S-adenosyltransferase (MAT). SAM acts as a universal methyl donor for over 100 transmethylation reactions (Stead et al., 2006). Methionine is also the major source of cysteine and taurine. Cysteine is the limiting amino acid for the synthesis of glutathione, an important antioxidant for the cell, while taurine is also important for normal cell function (Vlaardingerbroek et al., 2011). Methionine's role in many major biological processes results in the synthesis of homocysteine. Moreover, folate also plays a role in methionine metabolism via the remethylation of homocysteine with 5-

methyltetrahydrofolate (5-MTHF) via the enzyme methionine synthase (MS) (Bertolo & McBreairty, 2013). Methionine's involvement in many transmethylation reactions and biological processes demonstrates its importance biochemically, so it is not surprising that it is a principal metabolite of investigations into cardiovascular diseases (Durand et al., 2001).

a. Methionine Requirement in Neonates

Methionine plays an important role in transmethylation reactions and protein synthesis during early development when growth is at a high rate. Therefore, it is important to ensure the neonate receives the optimum amount of methionine in their diet without excessively exceeding the requirement, due to risk factors associated with high levels of methionine intake (Garlick, 2006). The WHO/FAO/UNU consultation has estimated the methionine intake based on breast milk to be the optimal nutrition for infants $\leq$ 6 months of age, which is 28 mg/kg/day (WHO/FAO/UNU, 2007). Interestingly, Huang et al. have more recently used the indicator amino acid oxidation technique to determine the methionine requirement in newborn infants, and reported it to be ~30% higher than that provided in human milk, at 38 mg/kg/day, even though cysteine was provided in excess (Huang et al., 2012). As the infant grows and matures, less methionine is required for transmethylation reactions and protein synthesis. As demonstrated by Humayun and colleagues, the methionine requirement is lowered to approximately 6.55 mg/kg/day in school-aged children, in the presence of cysteine (Humayun et al., 2006).

It has been estimated that the equivalent of 35% of dietary methionine intake is

consumed for de novo creatine synthesis in neonatal piglets (Brosnan et al., 2009). Methionine's role in many major transmethylation reactions, including creatine biosynthesis, only further emphasizes the importance of determining the optimal methionine requirement in the neonate

b. Transmethylation Reactions

As mentioned above, a substantial proportion of methionine in the neonate is used for transmethylation reactions. These reactions include, but are not limited to, creatine biosynthesis, DNA methylation, phosphatidylcholine (PC) synthesis, protein methylation, polyamine and carnitine synthesis, betaine and choline synthesis, and sarcosine synthesis (Bertolo & McBreairty, 2013). It has been estimated that creatine synthesis consumes approximately 70% of labile methyl groups in adults (Brosnan et al., 2009), although PC synthesis is also a major consumer of methyl groups. The distribution of dietary methionine and labile methyl groups between protein synthesis and transmethylation pathways in the neonate needs further investigation as variations in dietary methionine can limit available methionine (Bertolo & McBreairty, 2013).

i. Phosphatidylcholine Synthesis

Phosphatidylcholine is the major phospholipid component of all lipoproteins and is required for lipoprotein assembly and secretion. PC is synthesized via two major pathways: the CDP-choline pathway and the phosphatidylethanolamine N-methyltransferase (PEMT) pathway (Cole et al., 2012). The ubiquitous CDP-choline pathway, more commonly known as the Kennedy pathway, contributes the majority of PC

to the pool and synthesizes PC via three enzymatic steps, converting choline to PC (Kennedy & Weiss, 1956). The PEMT pathway, which is localized to the liver, contributes approximately 30% of total PC and is SAM-dependent (Reo et al., 2002; Sundler & Akesson, 1975) Phosphatidylethanolamine (PE) is converted to PC in a three step reaction via PEMT requiring three labile methyl groups (Cole et al., 2012).

Although PEMT activity only provides 20-40% of total PC, both the PEMT and the Kennedy pathway work synergistically. It has been shown that when PEMT is inhibited, PC synthesis is three-fold higher via the Kennedy pathway. Moreover, mice receiving a diet deficient in choline have an up-regulation of PEMT activity and the PC and choline are supplied through the PEMT pathway (Vance et al., 1997; Walkey et al., 1998) These findings demonstrate the coordination between pathways to ensure adequate PC and choline are supplied. The percentage of labile methyl groups consumed for PC synthesis is still disputed. Previously, results suggested that PEMT activity only consumes approximately 15% of total SAM (Mudd & Poole, 1975). Whereas, recent findings showed that the deletion of PEMT in mice causes a 50% decrease in plasma homocysteine (Noga & Vance, 2003). These findings suggest that PEMT consumes more than 15% of SAM due to the large decrease in plasma homocysteine. More research is needed to quantify the burden of PEMT on methyl groups.

ii. DNA Methylation

DNA methylation also consumes labile methyl groups. DNA methyltransferase (DNMT) transfers a methyl group to cytosine residues in cytosine-phosphate-guanine (CpG) dinucleotides of DNA and converts SAM to SAH. Although DNA consumes

significantly smaller amounts of labile methyl groups than creatine or PC (McBreairty et al., 2013), it can still have a significant effect on epigenetic changes, – the heritable modification of gene expression without the alteration of a genetic sequence, usually in early development (Waterland, 2006). Dietary availability of methyl groups can also affect DNA methylation. For example, animals fed a methyl-deficient diet resulted in hypomethylated DNA (Wainfan et al., 1989). Moreover, methionine supplementation during early development can lead to increased methylation at certain genes and cause permanent changes in DNA expression (Waterland & Jirtle, 2003)

Although dietary methyl supplementation can change DNA methylation, it is still unclear whether high methionine supplementation induces DNA hyper- or hypo-methylation (Waterland, 2006). It is possible that methionine's interaction with the transsulfuration pathway via homocysteine is a major factor directing global DNA methylation. Higher levels of homocysteine are linked to higher intracellular levels of SAH, and therefore a lower SAM/SAH ratio. The intracellular increase in concentrations of SAH have been linked to tissue specific changes in DNA methylation. For example, higher plasma SAH concentrations were associated with lower global DNA methylation in human leukocytes (Yi et al., 2000). On the other hand, there have been other studies disputing the relationship between tissue specific elevation in SAH concentrations and decreases in global DNA methylation (Heil et al., 2007).

Nutritional supplementation of methionine or methyl groups appears to have a significant effect on global DNA methylation, with tissue specificity, but the consequences of supplementing high methionine and its effects on DNA methylation warrant further investigation.

c. Remethylation & Transsulfuration

Unlike many other amino acids, methionine can be recycled through remethylation of homocysteine. As previously discussed, methionine is transmethylated via MAT to generate the universal methyl donor SAM, which subsequently generates SAH after donating its methyl group (Stead et al., 2006). SAH is then hydrolyzed to homocysteine and adenosine by SAH hydrolase. Homocysteine can then be remethylated to methionine via one of two major pathways via methionine synthase (MS) or betaine:homocysteine methyltransferase (BHMT). MS is a vitamin B_{12}-dependent enzyme which uses 5-methyl-tetrahydrofolate (5-MTHF) as a methyl donor for homocysteine's conversion to methionine and reproduces tetrahydrofolate (THF). BHMT uses betaine, a product of choline oxidation, as a methyl donor to generate methionine and dimethylglycine (DMG) (Finkelstein, 1998).

Lower activities of both MS and BHMT have been linked with increased concentrations of circulating plasma homocysteine (Schalinske & Smazal, 2012). Although methionine is readily trans- and remethylated, methionine can still undergo complete catabolism. This occurs in an irreversible reaction where homocysteine is converted to cysteine via the transsulfuration pathway. Homocysteine conversion occurs in a two-part reaction via two B_6-dependent enzymes: cystathionine-β-synthase (CBS) and cystathionine-γ-lyase (CGL) (Schalinske & Smazal, 2012). There is evidence to suggest that the upregulation of CBS activity is an important factor when determining and/or controlling homocysteine levels (Wang et al., 2004), and in turn may lower the

risk of cardiovascular and linked metabolic diseases, which are usually associated with high plasma homocysteine levels (Schalinske & Smazal, 2012).

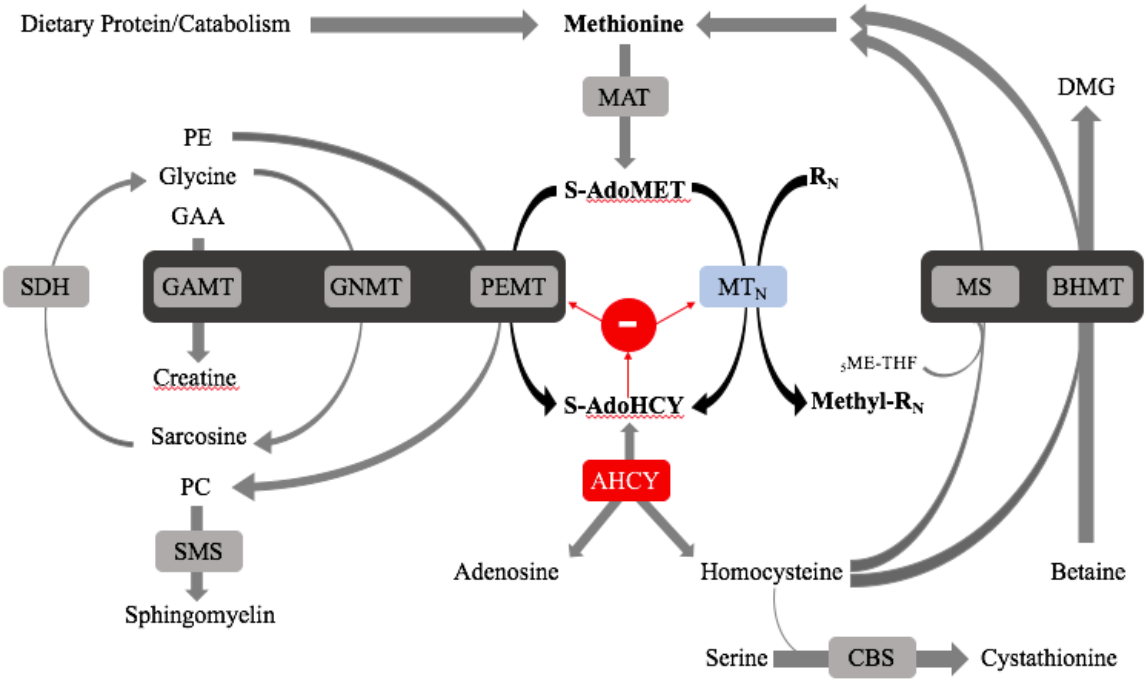

Figure 1.2: Methionine Cycle and Transmethylation Partitioning (Adapted from: Strauss et al., 2015).

5Me-THF, 5-methyltetrahydrofolate; AHCY, AdoHcy hydrolase; BHMT, betaine:homocysteine methyltransferase; CBS, cystathionine β-synthase; DMG, dimethylglycine; GAA, guanidinoacetate; GAMT, guanidinoacetate methyltransferase; GNMT, glycine N-methyltransferase; MAT, methionine adenosyltransferase; MS, methionine synthase; MT_N, more than 100 other methyltransferase enzymes; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PEMT, phosphatidylethanolamine N-methyltransferase; R_N, diverse cellular substrates; ; S-AdoHcy, S-adenosylhomocysteine; S-AdoMet, S-adenosylmethionine; SDH, sarcosine dehydrogenase; SMS, sphingomyelin synthase; THF, tetrahydrofolate

d. Methionine Requirement in TPN

Amino acid levels within parenteral solutions have been investigated in recent years to determine the optimum concentrations required to increase growth while lowering TPN-associated risks in neonates (Brunton et al., 2000). Dietary methionine levels have been investigated with and without cysteine supplementation (Shoveller et al., 2003a; Shoveller et al., 2003b). Cysteine's ability to spare methionine means that both amino acids need to be considered when determining requirements. Oral feeding studies in piglets demonstrated that cysteine can spare methionine by up to 70% (Baker et al., 1969). Studies in TPN also demonstrated this sparing effect. When cysteine is absent from parenteral diets, the methionine requirement during TPN was estimated to be 0.29 g/kg/day, which is approximately 30% less than the requirement for enterally fed piglets (Shoveller et al., 2003a). When cysteine was added to the parenteral diet, the methionine requirement decreased to 0.18 g/kg/day, which is approximately 70% of the enteral requirement in the presence of excess cysteine (Shoveller et al., 2003b), similar to the sparing effect of cysteine in oral feeding.

Methionine levels in parenteral nutrition solutions are typically investigated in the absence of cysteine due to its poor stability in solution because it oxidizes easily to cystine, which is insoluble (Vlaardingerbroek et al., 2011). A drawback of providing the complete sulfur amino acid requirement (i.e., methionine plus cysteine) in the form of methionine is that this approach leads to higher levels of homocysteine in the neonate (Shoveller et al., 2004). Moreover, splanchnic tissues play a major role in methionine metabolism. The difference between enteral and parenteral methionine requirements is attributed to the 30% utilization of sulfur amino acids by the gut (Shoveller et al., 2003a).

Overall, when considering sulfur amino acid requirements in parenteral solutions for neonates, a balance between methionine and cysteine should be emphasized. When methionine is in excess, higher levels of homocysteine is a drawback and is associated with TPN cholestasis (Courtney-Martin et al., 2010).

e. Methionine Toxicity

Methionine toxicity has been studied due to its link to possible side effects of high levels of plasma homocysteine and sulfur toxicity. The possible side effect of high levels of homocysteine is usually linked to an increased risk of cardiovascular diseases (Garlick, 2006). Whether it is an increase in dietary methionine intake that causes this increased cardiovascular disease risk has yet to be determined. In 2001, Hanratty and colleagues investigated the difference in forearm blood flow response to high oral intakes of methionine versus homocysteine. Both treatments resulted in higher concentrations of plasma homocysteine and resulted in the same forearm blood flow response (Hanratty et al., 2001). This result suggests that it is homocysteine that is responsible for endothelial vascular dysfunction. Moreover, it has been demonstrated that significant changes in plasma homocysteine concentrations are only seen when methionine intake is five times the requirement (Ward et al., 2001). Methionine, being the only sulfur-containing essential amino acid, has also been studied to determine if there are possible toxic side effects of increased levels of plasma methionine, particularly in infants. Mudd and colleagues investigated 10 infants who suffered from hypermethioninemia or hyperhomocysteinemia (Mudd et al., 2003). Despite plasma methionine levels rising to a high of 6830 μmol/L with normal plasma levels ranging from 10-40 μmol/L, there were

no apparent long term clinical side effects of hypermethioninemia associated with these infants. So it appears that high dietary methionine becomes toxic via its role in increasing plasma homocysteine levels.

1.3 Creatine Additives

In addition to its extensive use as a sports supplement and nutraceutical (Wyss & Kaddurah-Daouk, 2000), creatine has recently gained interest as a supplement in patients suffering from neurodegenerative diseases (Gualano et al., 2012) and in the agriculture industry to promote growth (Maddock et al., 2002; Young et al., 2005). Moreover, creatine has shown promising effects as a treatment for fatty liver and as a PN additive due to its ability to spare methionine and possibly arginine.

1.3.1 Creatine as an Ergogenic Aid

Creatine as a sports supplement for athletes has been extensively studied over the last number of decades. Despite creatine being synthesized within the body and supplied through the diet, typically, through red meat and fish, it is also the most widely used sports supplement to increase strength and growth (Gualano et al., 2012). Creatine's rapid conversion to phosphocreatine via creatine kinase explains its effectiveness as a rapid source of ATP regeneration during times of high intensity training or exercise. Therefore, creatine supplementation may allow for increased phosphocreatine stores in muscle and help increase energy and performance, by preventing the depletion of phosphocreatine (Wyss & Kaddurah-Daouk, 2000).

A recent study demonstrated that combining creatine monohydrate as a

supplement with bicarbonate beverages in trained men increased their peak and mean power in repeated sprint performance (Barber et al., 2013). Moreover, creatine supplementation has been shown to have positive effects in the elderly population in the prevention or delay of sarcopenia, which is a loss of muscle tissue as a part of aging. Recent findings have shown that creatine supplementation paired with a resistance training program significantly decreased the likelihood of sarcopenic changes seen in older adults (Devries & Phillips, 2014). The positive effects seen with creatine supplementation are still being investigated in a variety of populations. Creatine supplementation also has positive effects in patients suffering from neurodegenerative diseases such as Alzheimer's, cancer and rheumatoid arthritis (Gualano et al., 2012). Further research is needed in these areas to develop a successful therapy.

1.3.2 Creatine Usage in Neonates and TPN

Creatine synthesis is a major consumer of labile methyl groups, consuming approximately 70% in the neonate (Brosnan et al., 2009). Depending on the protein source, creatine concentrations in infant formula have a broad range from 334 μmol/L in cow's milk formula to approximately 10 μmol/L in soy-based infant formula. Human breast milk provides approximately 70 μmol/L of creatine (Edison et al., 2013). Variations in concentration between infant formula and physiological levels in human milk accounts for some of the variability in findings on the ratio of creatine synthesized de novo versus dietary intake. In 2009, Brosnan et al. found that only 25% of the accrued creatine in neonatal piglets was supplied by the sow's milk (Brosnan et al., 2009).

Similarly, infants receiving cow-milk based infant formula consumed approximately 35% of their creatine via diet (Edison et al., 2013). These findings demonstrate the importance of creatine biosynthesis during the neonatal phase which includes very high rates of transmethylation and protein turnover.

The large percent of labile methyl groups consumed for creatine synthesis during the neonatal period places a burden on available methionine for other transmethylation reactions. During times of intestinal distress or illness, infants are placed on TPN, which is devoid of creatine (Dinesh et al., 2017). This raises the question of whether a larger demand is placed on labile methyl groups for de novo synthesis.

1.4 Protein Synthesis in Neonatal Piglets

Methionine and arginine are essential amino acids in the neonatal piglet for protein synthesis and other non-protein pathways (Brosnan et al., 2009). Because of the very rapid protein turnover rates in piglets, supplying adequate amounts of arginine and methionine is essential for adequate growth. Moreover, these amino acids are required for the rapidly expanding pools of non-protein products. Approximately 70% of creatine is synthesized de novo in piglets (Brosnan et al., 2009), so receiving adequate amounts of dietary methionine must also accommodate creatine synthesis. Furthermore, increasing the demand for one transmethylation product can potentially limit methionine availability for protein synthesis and/or other transmethylation products (McBreairty & Bertolo, 2016).

On the other hand, arginine is equally important for optimizing the rate of protein synthesis in the neonatal piglet given it is essential in neonates. Arginine's essentiality is

even more pronounced during TPN feeding due to decreased endogenous synthesis by the atrophied gut (Bertolo et al., 2000). Because arginine is also required for GAA and creatine synthesis, which is very high in neonates, providing adequate dietary arginine must also consider the creatine content of the diet, especially during TPN feeding.

1.5 Creatine Transport & Excretion

The bioavailability of dietary creatine has been estimated at approximately 80% in adults (MacNeil et al., 2005), whereas in the neonatal animal it has been shown that the bioavailability of dietary creatine is likely even higher (Brosnan et al., 2009).

Creatine is primarily synthesized in the kidney and liver where AGAT and GAMT activity are highly expressed, respectively, as described above. A majority of the body's creatine is synthesized in the liver and exported via Na^+ -dependent transporters. These Na^+ -dependent transporter are members of the "neurotransmitter" transmitter family, similar to GABA and taurine transporters (Wyss & Kaddurah-Daouk, 2000). Expression of creatine transporters is highest in kidney, heart and skeletal muscle, while lower in brain, small and large intestine. Creatine is transported into the skeletal muscle by a sodium and chloride dependent transporter (SLC6A8) (Brosnan & Brosnan, 2007). Major consumers of creatine include high energy demand tissues such as skeletal and cardiac muscle, brain, spermatozoa and retina (Wyss & Kaddurah-Daouk, 2000). However, more than 90% of the body's creatine and phosphocreatine is present in the muscle (Brosnan & Brosnan, 2007).

Creatine and phosphocreatine are spontaneously converted to creatinine in a non-enzymatic chemical reaction, which is excreted in the urine Approximately, 1.5-2% of

creatine is excreted as creatinine per day (Brosnan & Brosnan, 2016). Therefore, this loss must be replaced by either the diet or de novo synthesis.

1.6 Neonatal Piglets as a Model for Pre-Term Infants

The neonatal piglet has been a research model for the pre-term infant for many years because of the piglet's similar digestive anatomy and physiology (Miller & Ullrey, 1987). Moreover, the piglet's large size (compared to rodents) and accelerated growth are very beneficial to research metabolic effects of diet and allow for differences to be detected in a shorter period of time (Miller & Ullrey, 1987). Research involving neonatal amino acid metabolism is well established in the piglet. The neonatal piglet has very similar amino acid requirements to that of an infant (Wykes et al., 1993) and piglet amino acid requirement estimates have been shown to be transferrable to the human infant after adjusting for the accelerated growth rate of the piglet (Chapman et al., 2009). This study involves methionine and arginine metabolism in the neonate, which requires knowledge of the methionine and arginine requirements in the piglet. Shoveller and colleagues have established the methionine requirement in both parenterally and enterally fed piglets with and without excess cysteine (Shoveller et al., 2003a; Shoveller et al., 2003b). Moreover, Bertolo and colleagues have estimated the adequate arginine requirement for muscle protein synthesis in the orally (Bertolo et al., 2003) and parenterally fed piglet (Brunton et al., 2003). Therefore, piglets are a well-established model for clinically relevant research applicable to the human neonate.

1.7 Rationale, Hypothesis & Objectives

Creatine synthesis requires three amino acids, arginine, methionine and glycine (Brosnan et al., 2009). Creatine synthesis consumes large amounts of methionine (via labile methyl groups), while also consuming arginine. Approximately 70% of creatine accretion is synthesized *de novo*, which consumes the equivalent of 35% of dietary methionine and 20% of dietary arginine in the suckling piglet. Methionine and arginine are considered essential and conditionally essential, respectively, in the neonate (Dinesh et al., 2014; J. Finkelstein et al., 1988). Moreover, during times of gut atrophy as in TPN, arginine synthesis is even lower which requires more dietary arginine for protein synthesis and growth (Brunton et al., 1999). It has been proposed that creatine supplementation may in fact spare arginine and glycine for growth (Hegsted et al., 1941), but only when arginine is limited (Wietlake et al., 1954). During times of limited methionine intake our group has demonstrated that creatine synthesis is more sensitive to methionine and labile methyl group availability than other transmethylation pathways (McBreairty et al., 2013). Commercial TPN solutions are devoid of creatine and so arginine and methionine requirements must consider synthesis of 100% of creatine needs. On the other hand, supplementation of TPN with creatine during marginal supply of arginine and methionine may spare both amino acids making them more available for growth and protein synthesis.

GAA supplementation is effective at increasing creatine stores (Almquist et al., 1941; McBreairty & Bertolo, 2016), although the methylation of GAA to creatine is not feedback regulated, so GAA supplementation in excess may increase the burden on methyl groups and methionine (Bertolo & McBreairty, 2013). On the other hand,

supplementation with GAA may spare arginine for growth and protein synthesis, as seen in broiler chicks fed an arginine deficient diet (Dilger et al., 2013). Therefore, during TPN, supplementation with GAA may be effective at increasing creatine muscle stores while also sparing arginine for protein synthesis, but only if extra methyl groups and methionine are provided.

We hypothesized that during TPN feeding, dietary creatine and GAA supplementation will spare methionine and arginine for protein synthesis and transmethylation reactions in Yucatan miniature piglets.

Objectives:

01. To quantify the partitioning of methyl groups among transmethylation reactions, including creatine and PC synthesis and DNA methylation.

02. To quantify the sparing effect of creatine and GAA on methionine and/or arginine availability for growth, nitrogen retention and protein synthesis.

03. To determine if supplemental methionine is specifically required to facilitate GAA methylation to creatine with supplemental GAA.

2. Materials and Methods

2.1 Animals and Surgical Procedures

Our hypothesis was tested in the Yucatan miniature piglet model. All animal procedures were reviewed and approved by the Institutional Animal Care Committee at Memorial University of Newfoundland and conformed to the guidelines of the Canadian Council on Animal Care. Thirty-one, 6 – 10 day old piglets were obtained from a breeding colony at Memorial University of Newfoundland (St. John's, NL, Canada). A sample size of six per group was calculated with significance level of 0.05 and power of 0.80 to detect a 10% difference using previous variance estimates from data for transmethylation partitioning in piglets; however, our Base + GAA treatment group had a sample size of seven, as one of our first piglets in this group showed signs of sickness. Our experiment used a randomized complete block (by litter) design obtaining five piglets from each litter to maintain blocking. However, six piglets were obtained for the last block to replace the sick piglet in the first block; therefore, two piglets were placed in the Base + GAA treatment group (n=7). However, all data were included in analyses for piglets in Base + GAA (n=7), unless otherwise stated.

On the morning of surgery (Day 0) piglets were transported from the breeding colony by Animal Care Services to the surgical room located in the Biotechnology building at Memorial University of Newfoundland (St. John's, NL, Canada). Upon arrival, piglets were weighed and received an intramuscular injection of Ketamine hydrochloride (Bimeda Canada, Cambridge, ON, Canada) and Acepromazine (Vetoquinol. QC, Canada) mixture at 22 mg/kg of body weight and 0.5 mg/kg of body weight, respectively. Afterwards, anesthesia was maintained by 1.5% Isoflurane (Abbott

Laboratories Inc., Canada) mixed with oxygen (1.5 L/minute). A rectal thermometer was inserted for monitoring of piglet's body temperature. Heart rate and oxygen saturation were monitored using a pulse oximeter attached to the piglet's ear. Piglet cleaning before surgery consisted of a three-step process: first, piglets were scrubbed and washed with Prepodyne solution, second, piglets were washed with 70% Isopropanol (Fisher Scientific, Ottawa, ON, Canada) and lastly, piglets received a final wash of Prepodyne solution prior to surgery. All surgical tools, gowns and drapes used in the surgical procedures were sterilized using an autoclave sterilizer and 45-minute sterilizing dry cycle.

Prior to surgery, piglets received a dose of Buprenorphine analgesic (Temgesic, Reckitt Benckiser Healthcare, UK) at 0.03 mg/kg of body weight. This analgesic was repeated 12 h post-surgery and anytime piglets demonstrated signs of pain at 0.015 mg/kg of body weight. For surgery, each piglet was implanted with two venous catheters: a jugular catheter for administration of diet for 7 d of TPN and a femoral catheter for blood sampling. A small paramedian incision was made and a silastic catheter was inserted into the external jugular vein and advanced to the cranial vena cava. A second incision was made and a silastic catheter was implanted into the left femoral vein and advanced to the caudal vena cava. Incisions were closed and piglets received an IV injection of 0.5 mL of antibiotic Borgal (Trimethoprim 40 mg/mL and sulfadoxine 200 mg/mL; Intervet Canada Ltd, Canada) diluted in 10 mL of saline to prevent the development of infection. Heart rate, oxygen levels, body temperature and respiration rate were all monitored and recorded in fifteen-minute intervals throughout the surgical procedure.

Post-surgery, piglets were transported to a piglet housing area and placed in individual cages using a jacket and tether system. This system allowed the piglets to free roam while ensuring the safety of the implanted catheters. The piglet housing area was programmed on a 12-hour light and dark cycle.

2.2 Experimental Design and Diet

All diets, trace minerals and vitamins used in our study were based on commercially available diets for infants (Vaminolact; Fresenius Kabi, Germany) with slight modifications made for neonatal piglets (Wykes et al., 1993). Complete elemental diets (Table 2.1) were prepared in the laboratory using free crystalline L-amino acids (Ajinomoto, Japan; Evonik Industries AG, Hanau-Wolfgang, Germany; Sigma-Aldrich, Oakville, Canada). Amino acids were individually weighed and placed in a large beaker to be thoroughly mixed. Two 4 L beakers with 1.5 L of pyrogen free water were placed on heaters with stirrer bars and heated to 50 - 70^{o}C. Amino acids were then added equally to each beaker and dissolved under nitrogen gas. After amino acids were dissolved, D-glucose (Sigma-Aldrich, Oakville, Canada) was weighed and placed equally into each beaker and dissolved. Minerals (Sigma-Aldrich, Oakville, Canada) were weighed and dissolved in individual beakers and then added to the amino acid mixtures. A large carboy for mixing of diet was placed on a scale to determine final weight of the diet, and amino acid mixtures were combined. Diet was then filtered through an AcroPak^{TM} 200 Supor^{R} Membrane filter (Pall Corporation, Switzerland) using a mechanical pump (EMD Millipore, Darmstadt, Germany) and placed into empty sterile bags (Baxter Corporation, Mississauga, ON, Canada) using a 20G 1-inch needle (BD – Canada, Mississauga, ON,

Canada) in a laminar flow hood. Each bag was individually weighed to ensure 750 mL (795 g) of diet was added and stored at 4^{o}C in a dark environment until ready to be used.

Piglets received TPN for 7 d via the implanted jugular catheter by a pressure-sensitive TPN pump (Baxter FloGard 6301, InfuSystem Inc., USA). Diet was pumped at a target rate of 13.5 mL/kg of body weight. Post-surgery (day 0), piglets received 50% of target rate. On day 1, the rate was increased to 75% for 12 h, then increased to 100% of target rate on the evening of Day 1 until end of study.

2.2.1 Dietary Treatment Groups

All piglets received the same complete elemental diet (Adaptation Diet), from Day 0 – Day 2 to ensure recovery from surgery. To test our hypotheses, on the morning of Day 3 piglets were randomized to one of five experimental dietary treatments (Table 2.1).

Table 2.1: Amino Acid Profiles of Complete Elemental and Treatment Diets

	Adaptation Diet	Base	Base + GAA	Base + Creatine	Adequate Arg & Met	Adequate Met + GAA
	g/L	g/L	g/L	g/L	g/L	g/L
Alanine	5.89	9.51	8.72	8.73	0.00	7.85
Arginine	3.65	2.02	2.02	2.02	6.62	2.02
Aspartate	3.32	3.47	3.47	3.47	2.31	3.47
Cysteine	0.76	0.83	0.83	0.83	0.83	0.83
Glutamate	5.72	6.00	6.00	6.00	6.00	6.00
Glycine	1.47	1.31	1.31	1.31	1.31	1.31
Histidine	1.69	1.76	1.76	1.76	1.76	1.76
Isoleucine	2.51	2.64	2.64	2.64	2.64	2.64
Leucine	5.67	5.94	5.94	5.94	5.94	5.94
Lysine-HCL	5.58	4.73	4.73	4.73	4.73	4.73
Methionine	1.04	0.368	0.368	0.368	1.84	1.84
Phenylalanine	3.00	2.24	2.24	2.24	2.24	2.24
Proline	4.52	4.73	4.73	4.73	4.73	4.73
Serine	3.11	3.19	3.19	3.19	3.19	3.19
Taurine	0.27	0.28	0.28	0.28	0.28	0.28
Tryptophan	1.14	1.21	1.21	1.21	1.21	1.21
Tyrosine	0.44	0.43	0.43	0.43	0.43	0.43
Threonine	2.23	3.03	3.03	3.03	3.03	3.03
Valine	2.89	3.03	3.03	3.03	3.03	3.03
GT*	0.00	1.42	1.42	1.42	1.42	1.42
Creatine (CMH**)	0.00	0.00	0.00	0.43	0.00	0.00
GAA	0.00	0.00	0.34	0.00	0.00	0.34

Dietary rate of amino acid infusion for piglets was set at 272 mL/kg/d. Diets were isonitrogenous by adjusting the concentrations of amino acids, alanine and aspartate.

*Glycine-Tyrosine dipeptide

**Creatine Monohydrate

Base Diet (Low Arginine & Low Methionine): Feeding a low arginine and low methionine diet as TPN allowed for increases in protein synthesis to be detectable (i.e.: by sparing either amino acid). Therefore, the Base Diet treatment served as a negative control.

Base + GAA (Low Arginine & Low Methionine plus GAA): Supplementing GAA in low arginine and low methionine in TPN tested whether GAA could spare arginine from creatine synthesis to be utilized for other uses.

Base + Creatine (Low Arginine & Low Methionine plus Creatine): Supplementing creatine in low arginine and low methionine TPN tested whether creatine could spare both arginine and/or methionine from creatine synthesis to be utilized in other transmethylation reactions or be used for protein synthesis.

Adequate Arg & Met (Adequate Arginine & Adequate Methionine): Feeding adequate amounts of arginine and methionine in TPN served as a positive control and tested whether creatine synthesis is maximized when precursor amino acids are adequately fed. In order to accommodate all products of these amino acids, approximately 150% of estimated piglet requirements were fed.

Adequate Met + GAA (Low Arginine & Adequate Methionine plus GAA): Supplementing GAA in low arginine and adequate methionine in TPN tested whether GAA can spare arginine for other uses, while ensuring there is enough methionine available for methylation of GAA for creatine synthesis.

2.2.2 Trace Mineral Inclusion

Trace mineral mixture (Appendix II) was prepared in the laboratory and provided at least 120% of NRC requirements for piglets (National Research Council, 1998). Individual trace minerals were weighted and placed into a 1 L beaker with 800 mL of pyrogen free water. Minerals were stirred and dissolved, then pyrogen free water was added to give a total volume of 1 L. Trace mineral mixture was then filtered using a 25 mm polytetrafluoroethylene (PTFE) 0.45 μm syringe filter (Canadian Life Science, Peterborough, ON, Canada) with a 60 cc syringe and a 20G ½ inch needle into an empty sterile bag. The mixture was then stored at 4^{o}C until needed. Prior to the administration of diet, 3 mL of trace mineral mixture was added using a 5 cc syringe and 20G ½ inch needle to the 750 mL amino acid mixture. After this, 1 mL of iron dextran (Ventoquinol Canada Inc., Canada) solution was added to the 750 mL amino acid mixture, which provides 2 mg of iron per kg of body weight.

2.2.3 Vitamin and Lipid Inclusion

Vitamin/K12 mixture (Appendix III) supplied by Baxter Pediatric Multivitamin for Infusion (Multi-12/K1 Pediatric multivitamins, Baxter Corporation, Mississauga, ON, Canada) provided at least 120% of NRC requirements for piglets in 3 mL (Wykes et al., 1993). Prior to the administration of diet, 1 mL of Vial 2 was mixed with 4 mL of Vial 1. After this mixture was prepared, 3 mL of the vitamin mixture was added to the 750 mL amino acid mixture. IV dietary lipid (Appendix IV) was supplied by Baxter Intralipid 20% (20% Intralipid, Baxter Corporation, Mississauga, ON, Canada); total energy was

provided at 1.1 MJ metabolizable energy/kg/d and lipid and carbohydrate each provided 50% of the non-protein energy (Wykes et al., 1993). Intralipid was added to the IV diet bags immediately prior to the administration of diet; 145 mL of the 20% Intralipid was added to 750 mL of diet using a 60 cc syringe and 18G 1 inch needle.

2.3 Isotope Infusion and Necropsy

On the morning of day 6, a stable isotope infusion study (Appendix V) was performed by another graduate student in our laboratory (A. Al-Jaroudi); the data from the stable isotope work are part of the M.Sc. thesis of A. Al-Jaroudi. On day 7, piglets received a 6-hour primed-continuous isotopic infusion of L-[methyl-^{3}H] methionine (American Radiolabeled Chemicals Inc, St. Louis, MO, USA). Prior to the infusion, baseline blood samples were taken from the femoral catheter. At time 0, a priming dose of 30 μCi/kg of L-[methyl-^{3}H] methionine was administered into the jugular catheter via a Y-connector attached to the TPN catheter. Immediately after the priming dose, a 6-hour continuous infusion of L-[methyl-^{3}H] methionine at a rate of 30 μCi/kg/h using a syringe pump was initiated into the TPN catheter. Every half-hour from time 0, 0.8 mL of blood sample was collected from the femoral catheter and placed in PST^{TM} gel and lithium heparin collection tube (BD-Canada, Mississauga, ON, Canada). Collection tubes were centrifuged at 5,000 RPM for 5 min to separate plasma. Plasma supernatant was collected and pipetted into 2 mL microcentrifuge tubes (Fisher Scientific, Ottawa, ON, Canada), and immediately placed at -80^{oC} for further analyses.

At the end of the 6-hour infusion, piglets were anesthetized via 1.5% isoflurane mixed with oxygen (1.5 mL/min) by mask. A midline incision was made and the abdomen was exposed. The pancreas, liver and kidney were quickly removed, weighed and freeze-clamped in liquid nitrogen. Heart, muscle (bicep femoris), brain, and gut samples were then removed, weighed, freeze-clamped in liquid nitrogen and flash frozen. Urine from the bladder, and bile from the gallbladder were collected in 2mL microcentrifuge tubes and flash frozen in liquid nitrogen. A 30-centimeter section of proximal jejunum was removed from the piglet, flushed with ice cold saline, dissected on ice, and a mucosal sample was scraped, weighed, freeze-clamped and flash frozen. All samples were stored at -80^{o}C until needed for analyses.

2.4 Analytical Procedures

2.4.1 Plasma and Tissue Amino Acid Concentrations and Specific Radioactivity

2.4.1.1 Plasma Amino Acids

Plasma amino acid samples were analyzed by using reverse-phase high performance liquid chromatography (HPLC) following derivatization with phenylisothiocyanate (PITC, Edmans Reagent, Thermo Fisher Scientific, USA) (Bidlingmeyer et al., 1984). Frozen plasma collected at necropsy (Section 2.5) was thawed on ice. Once thawed, 100 μL of plasma supernatant was placed in a 2 mL microcentrifuge tube with the addition of 20 μL of 2.5 μmol/mL norleucine standard as well as 1 mL of 0.5% trifluoroacetic acid (TFA, Sigma-Aldrich, Oakville, Canada) to precipitate plasma proteins. Samples were vortexed and centrifuged at 5000 RPM for 5

min. Samples were then poured into 3 mL test tubes, covered, flash frozen with liquid nitrogen and placed on a freeze dryer overnight at -50^{o}C. The following day, 50 μL of fresh 20:20:60 triethylamine (TEA):methanol:water was added to the test tube. Test tubes were then covered, flash frozen with liquid nitrogen and placed on the freeze dryer for approximately 1 h at -50^{o}C. After 1 h, samples were removed from the freeze dryer and 20 μL of derivatizing solution was added to the samples. The derivatizing solution contained 10% water, 10% TEA, 70% methanol (Fisher Scientific, Ottawa, ON, Canada), 10% PITC. Samples were left at room temperature for 35 min to allow the derivatization with PITC, then flash frozen in liquid nitrogen and placed on a freeze dryer overnight at -50^{o}C. The following day, samples were re-suspended in 200 μL of sample diluent [5 mM Na_2HPO_4, (Sigma-Aldrich, Oakville, Canada) titrated to pH 7.4 with 10% H_3PO_4 and 10% acetonitrile (Fisher Scientific, Ottawa, ON, Canada)], and centrifuged at 5000 RPM for 5 min. Using a glass pipette, avoiding any precipitate, samples were transferred to 0.5 mL microcentrifuge tubes.

A 40 μL sample was injected into a reverse-phase C18 Pico-Tag column (Waters, 60Å, 4 μm 3.9 X 300 mm, Waters Corporation, Milford, MA, USA) connected to a HPLC system. The HPLC system consisted of a Waters 717 plus Auto Sampler Waters 1525 Binary HPLC pumps, Waters 2487 Dual λ absorbance detector and a column heater which maintained the column temperature at 46 ^{o}C. Mobile buffer phase 'A' consisted of 70 mM sodium acetate (Fisher Scientific, Ottawa, ON, Canada), pH was adjusted to 6.55 using 6 M hydrochloric acid (HCl, Caledon Laboratory Chemicals, ON, Canada). and 2.5% acetonitrile. Mobile buffer phase 'B' consisted of 45% acetonitrile, 15% methanol,

and 40% water. Both mobile phases were filtered through a nylon, hydrophilic membrane (47 mm, 0.45 μm) filter to remove any impurities (Canadian Life Science, Peterborough, ON, Canada) and degassed using a vacuum pump as well as a Waters In Line-Degasser AF (Waters Corporation, Milford, MA, USA). The derivatized amino acids were detected at 254 nm. Peaks were integrated using Empower 3 software (Waters Corporation, Milford, MA, USA) and amino acid concentrations were determined by comparing the amino acid area to that of the internal standard, norleucine (Sigma-Aldrich, Oakville, Canada). A fraction collector (Waters Corporation, Milford, MA, USA) was used to collect the eluent that was associated with the methionine peak. A biodegradable liquid scintillant (Scintiverse cocktail, Fisher Scientific, Ottawa, ON, Canada) was used to determine the radioactivity associated with methionine by liquid scintillation counter (PerkinElmer, USA).

2.4.1.2 Tissue Amino Acid Analysis

a. Tissue Preparation

Liver and muscle tissues were completely pulverized using a mortar and pestle under liquid nitrogen. Pulverized samples were placed in 20 mL scintillation vials and stored at -80^{o}C until further analyses. Tissue samples were homogenized in plastic centrifuge tubes with 2% perchloric acid (Fisher Scientific, Ottawa, ON, Canada) at a ratio of 1:4 (weight/volume). After homogenizing for approximately 45 s, samples were centrifuged at 3000 RPM for 15 min. Supernatant was poured and collected into labeled 20 mL scintillation vials (Sigma-Aldrich, Oakville, Canada) and the acid-insoluble

protein pellet was stored at -20^{o}C for further analysis (Section 2.4.1.2.c). This procedure was repeated three times per sample. An internal standard of norleucine was added to the supernatant at a concentration of 25 μmol/mL. Supernatant was mixed and stored at -20^{o}C until further analyses (Section 2.4.1.2.b).

b. Tissue Free Amino Acids

Tissue homogenate supernatant was neutralized in a labeled test tube with 2 M K_2CO_3 (Fisher Scientific, Ottawa, ON, Canada). Samples were vortexed and centrifuged at 5000 RPM for 3 min. Supernatant was poured off into labeled microcentrifuge tube and stored at -20^{o}C after 1 mL of supernatant was transferred to a labeled test tube and derivatized as previously described (Section 2.6.1.1). Amino acid concentrations, including the radioactivity associated with methionine were quantified using HPLC as previously described (Section 2.4.1.1).

c. Tissue Bound Amino Acids

Tissue protein pellets were re-suspended in 1 M NaOH (Fisher Scientific, Ottawa, ON, Canada) and homogenized with a Teflon pestle. This procedure was repeated multiple times until a final volume of 1:9 (weight/volume). Samples were covered, inverted to mix and placed in a water bath at 37^{o}C for approximately 1.5 h to solubilize the protein. After 100 μL was taken to measure total protein, cold 20% perchloric acid (Fisher Scientific, Ottawa, ON, Canada) was added to the samples to re-precipitate protein. The protein concentration is needed to calculate absolute synthesis rate (ASR) of

protein. Samples were placed on ice for 20 min and centrifuged at 4800 RPM for 15 min. The supernatant was discarded and an internal standard of norleucine was added at a concentration of 25 μmol/mL. The pellet was disrupted and transferred to a labeled test tube with 6 M HCl (10 mL/g of tissue). The protein pellet was then hydrolyzed at 110^{o}C for 24 h. Test tubes were removed from the oven and placed in the fume hood to cool overnight. Hydrolysates were mixed and placed in pre-weighed flasks and diluted to a final volume of 25 mL was reached. Contents of the flask were mixed thoroughly and filtered through a 25 mm PTFE 0.45 μm syringe filter (Canadian Life Science, Peterborough, ON, Canada) into a 20 mL scintillation vial and stored at -20^{o}C. Samples (1 mL) were transferred to plastic centrifuge tubes and placed in a drying oven overnight and derivatized, as previously described (Section 2.4.1.1.). Muscle samples were directly hydrolyzed with 6 M HCl after the addition of the internal standard, norleucine, to the protein pellet. A solubilized sample was not needed since ASR cannot be calculated for muscle. The concentrations of methionine and its associated radioactivity were measured using HPLC and fraction collecting, as previously described (Section 2.4.1.1).

2.4.2 Analysis of Transmethylation Products

2.4.2.1 Quantification of Creatine Concentrations and Specific Radioactivity

Tissue creatine concentrations and specific radioactivity were quantified using a modified method by Lamarre (Lamarre et al., 2010). Approximately 200 mg of frozen tissue was weighed into a plastic centrifuge tube and homogenized with 950 μL of 0.5 M Tris Buffer at pH 7.4 (Fisher Scientific, Ottawa, ON, Canada). Samples were transferred

to 1.5 mL centrifuge tubes and 50 μL of trifluoroacetic acid was added (Sigma-Aldrich, Oakville, Canada). Samples were left on ice for 10 min to precipitate protein. Muscle samples required more time to precipitate protein, therefore they were left on ice for 30 min. Samples were centrifuged at 6000 RPM for 10 min at a temperature of 4^{o}C. Supernatant was filtered for solid phase extraction through 40 μm C18 Bond Elut filters (Agilent Technologies, CA, USA) into labeled 0.5 mL microcentrifuge tubes (Fisher Scientific, Ottawa, ON, Canada). Filters were cleaned between each sample once with methanol ((99%) Fisher Scientific, Ottawa, ON, Canada) and twice with HPLC-grade water. A standard curve was quantified using a Waters 2487 Dual λ absorbance detector at 210 nm through a Hypercarb 5μm Carbon 250 column (Waters Corporation, Milford, MA, USA). HPLC mobile phase consisted of 0.1% TFA (Sigma-Aldrich, Oakville, Canada) and 3% methanol (Fisher Scientific, Ottawa, ON, Canada) in HPLC-grade water at an isocratic rate of 1.0 mL/min for 20 min per sample. Creatine peaks were detected using EmPower 3 software and a fraction collector was used to the creatine peak. Radioactivity associated with creatine was determined as previous described (Section 2.4.1.1).

2.4.2.2 Quantification of Phosphatidylcholine Concentrations and Specific Radioactivity

Total lipid was extracted from liver tissue using the Folch method (Folch et al., 1957). Approximately 300 mg of frozen liver tissue was weighed and transferred to a plastic centrifuge tube with the addition of 900 μL of 50 mM NaCl (Fisher Scientific,

Ottawa, ON, Canada). Tissue was homogenized for 45 s and transferred to acid-washed glass tubes. (Glass tubes were acid-washed in concentrated H_2SO_4, (Fisher Scientific, Ottawa, ON, Canada) for 24 h, then rinsed in running water overnight). A 2:1 chloroform:methanol (Fisher Scientific, Ottawa, ON, Canada) solution was prepared and 6 mL was added to each sample and extensively vortexed. Samples were left at 4^{o}C overnight. Tissue samples were centrifuge at 3000 RPM for 10 min to allow for separation of lipids and aqueous phase. The lipid/chloroform layer was extracted and placed in a pre-weighed glass culture tube; the extracted lipids were dried under nitrogen gas. Glass culture tubes were weighed to determine the total fat within each sample. Extracted lipids were re-suspended by adding 300 μL of 99% isopropanol (Fisher Scientific, Ottawa, ON, Canada), vortexed and transferred to 1.5 mL microcentrifuge tubes to be stored at -80^{o}C until further analyses. Samples were spotted onto a K6 Silica Gel-G60 thin-layer chromatography (TLC) plate (EMD, Millipore Corporation, Billerica, MA, USA) in 5 μL increments until a total volume of 20 μL was reached. A chloroform:methanol:acetic acid:water (25:15:4:2) mobile phase was prepared and added to a mobilization chamber and complete saturation required approximately 1 h. Plates were removed when the mobile phase reached the top of the plate and transferred to an iodine visualization chamber for the appearance of phosphatidylcholine bands. Phosphatidylcholine was scraped using a razor blade and transferred to acid-washed glass tubes with the addition of 400 μL of 70% perchloric acid (Fisher Scientific, Ottawa, ON, Canada). Samples were placed into a heating oven at 180^{o}C for 2 h until samples became clear and removed to sit at room temperature. Samples were centrifuge at 5000 RPM for 2

min and 100 μL was transferred to a glass culture tube where it was diluted with 200 μL 70% perchloric acid (Fisher Scientific, Ottawa, ON, Canada). Total phosphate measurement was quantified using a modified method from Bartlett (Bartlett, 1959) and a standard phosphate stock solution. Samples and standards were prepared by adding 50 μL of 5 % ammonium molybdate (Fisher Scientific, Ottawa, ON, Canada) and 50 μL of 1-amino-2-naphthol-6-sulphonic acid (ANSA) solution and vortexed. Sample and standard volumes were matched by the addition of 500 μL of deionized water to each sample. Samples and standards were placed in a water bath at 100^{o}C for 12 min to allow for reaction and colour change. Samples were removed, cooled to room temperature and absorbance of both samples and standards were quantified at 815 nm using a dual-absorbance spectrophotometer. After reading the absorbance, 600 μL of the sample was removed from the cuvette and placed in a 20 mL scintillation vial. Radioactivity associated with phosphatidylcholine was determined as previously described (Section 2.4.1.1).

2.4.2.3 Quantification of DNA Concentration and Specific Radioactivity

Approximately 300 mg of liver tissue was weighted in a plastic centrifuge tube and homogenized for 30 s in 4.0 mL of buffer (50 mM Tris, 100 mM EDTA, 100 mM NaCl, 1% SDS) (Fisher Scientific, Ottawa, ON, Canada; Sigma-Aldrich, Oakville, Canada) and 450 μL of proteinase K (Sigma-Aldrich, Oakville, Canada) solution. Tissue homogenates were transferred to 15 mL glass test tubes and placed in the water bath at 56^{o}C for 12 h overnight. Phenol solutions were prepared by melting 150 g of pure phenol

(Sigma-Aldrich, Oakville, Canada) at 56^{o}C for 1 h and then adding an equal amount of 0.20 M solution of Tris (Fisher Scientific, Ottawa, ON, Canada). Aliquots were placed into 50 mL plastic test tubes and stored at -20^{o}C until needed. Tissue samples were removed from the water bath and an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) (Fisher Scientific, Ottawa, ON, Canada) was added to the samples, vortexed and centrifuged at 8000 RPM for 10 min until a phenol and aqueous layer formed. Aqueous layer was extracted and placed in a new 15 mL test tube. This process was repeated once more until no protein inference remains between the phenol and aqueous phases. An equal volume of choloroform:isoamyl alcohol ((24:1) Fisher Scientific, Ottawa, ON, Canada) was added and centrifuged at 5000 RPM for 15 min to recover the aqueous phase and transferred to a new 15 mL test tube. An equal volume of 99% isopropanol (Fisher Scientific, Ottawa, ON, Canada) and 1/10 the volume of 1.0 M sodium acetate (Sigma-Aldrich, Oakville, Canada) was added and centrifuged at 5000 RPM for 15 min to precipitate the DNA. The pellet was rinsed twice with 70% ethanol (Fisher Scientific, Ottawa, ON, Canada) and once in 95% ethanol. The pellet was removed from the 15 mL test tube and transferred to a 1.5 mL microcentrifuge tube to dry for approximately 20 min. After the pellet has completely dried, 1.0 mL of TE buffer (10 mM Tris, 0.1 mM EDTA) (Fisher Scientific, Ottawa, ON, Canada) was added to re-suspend the DNA in solution. The pellet was then dissolved in a rocking water bath at 56^{o}C for 20 min, removed and stored at 4^{o}C overnight. The DNA concentration was determined using Thermo Scientific Nano Drop 2000 spectrophotometer (Thermo Fisher Scientific, Canada) and radioactivity associated with DNA was determined as previously described (Section 2.4.1.1).

2.4.2.4 Quantification of SAM & SAH Concentrations and Specific Radioactivity Associated with SAM

Liver tissue SAM and SAH concentrations were quantified using a modified method by Ratnam and colleagues (Ratnam et al., 2006). Approximately 200 mg of frozen liver tissue were weighed in a plastic centrifuge tube and homogenized for 45 seconds in 1 mL of ice cold 8% trichloroacetic acid (Fisher Scientific, Ottawa, ON, Canada). Homogenized samples were transferred to 1.5 mL microcentrifuge tubes (Fisher Scientific, Ottawa, ON, Canada) and centrifuged at 12,000 RPM for 5 min. Supernatant was extracted and filter through 13 mm PTFE 0.45 μm syringe filter (Canadian Life Science, Peterborough, ON, Canada) into labeled 0.5 mL microcentrifuge tubes (Fisher Scientific, Ottawa, ON, Canada). A standard curve was quantified using a Waters 2487 Dual λ absorbance detector at 258 nm through a VYDAC HPLC C18 column (Waters Corporation, Milford, MA, USA). Mobile phase 'A' consisted of 50 mM NaH_2PO_4 (Sigma-Aldrich, Oakville, Canada) and 10 mM heptane sulfonic acid at pH 3.2 (Fisher Scientific, Ottawa, ON, Canada) in HPLC-grade water. Mobile phase 'B' consisted of 100% acetonitrile (Fisher Scientific, Ottawa, ON, Canada). Both mobile phases were filtered through a 47 mm nylon, hydrophilic 0.45 μm filter to remove any impurities (Canadian Life Science, Peterborough, ON, Canada) and degassed using a Waters In Line-Degasser AF. 50 μL of sample was injected and buffers infused at a rate of 1.0 mL/min for 30 min. SAM peaks were detected and a fraction collector ((Waters Fraction Collector III) Waters Corporation, Milford, MA, USA) was used to collect the sample

that was associated with the SAM peak. Radioactivity associated with SAM was determined as previously described (Section 2.4.1.1).

2.4.3 Analysis of Plasma Creatine

Plasma creatine concentrations and specific radioactivity was quantified using a modified method by Lamarre (Lamarre et al., 2010). Plasma, 50 mM Tris buffer (Fisher Scientific, Ottawa, ON, Canada) and 97% TFA (Fisher Scientific, Ottawa, ON, Canada) (3:2:0.5) were mixed in a 1.5 mL microcentrifuge tube (Fisher Scientific, Ottawa, ON, Canada), vortexed and placed on ice for 10 min. Samples were centrifuged at 6000 RPM for 10 min. Creatine concentrations and radioactivity was determined as previously described (Section 2.4.2.1).

2.4.4 Hepatic GAMT Activity

Liver GAMT activity was quantified using a modified method by Ogawa (Ogawa et al., 1983). Fresh liver tissue was manually homogenized, 1:5 (w/v) in a sucrose HEPES buffer (0.25 M sucrose, 1 mM EDTA, and 10 mM Hepes) (Sigma-Aldrich, Oakville, Canada) and centrifuged at 35,000 RPM at 4^{o}C for 60 min. Supernatant was used to determine hepatic GAMT activity. To start, 10 µL of supernatant was combined with 350 µL of Tris β-mercaptoethanol buffer (100 mM Tris and 20 mM β-mercaptoethanol) (Fisher Scientific, Ottawa, ON, Canada; Sigma-Aldrich, Oakville, Canada) and 620 µL of deionized water, then 10 µL of SAM solution (4.4 mg of SAM in 500 µL deionized water + 500 µL Tris β-mercaptoethanol buffer) (Fisher Scientific, Ottawa, ON, Canada; Sigma-

Aldrich, Oakville, Canada) was added and samples were incubated at room temperature for 10 min. The reaction took place when 10 μL of GAA solution (23.42 mg GAA in 5 mL deionized water + 5 mL L Tris β-mercaptoethanol buffer) (Fisher Scientific, Ottawa, ON, Canada; Sigma-Aldrich, Oakville, Canada) was added and samples were incubated at 37^{o}C for 20 min. After 20 min incubation at 37^{o}C, the reaction was stopped when 750 μL of 15% trichloroacetic acid was added, vortexed, and placed on ice for 10 min. Then 720 μL of 1 M Tris buffer was added to neutralize the reaction. Measuring creatine production to quantify hepatic GAMT activity was determined by derivatizing 400 μL of supernatant as described below (Section 2.4.4.1).

2.4.4.1 Derivatization using Ninhydrin

Hepatic GAMT activity was determined using a modified method by Buchberger and Ferdig (Buchberger & Ferdig, 2004). Samples, 1.3 M KOH, and 0.9% Ninhydrin (4:3:1.5) were mixed and incubated at room temperature in a dark environment for 15 min. After incubation, 100 μL of 5% ascorbic acid (Sigma-Aldrich, Oakville, Canada) and 5 M phosphoric acid (Fisher Scientific, Ottawa, ON, Canada) were added and samples were incubated at 90^{o}C for 30 min. After incubation, samples were cooled to room temperature and filtered using a 13 mm PTFE 0.45 μm syringe filter (Canadian Life Science, Peterborough, ON, Canada) into a labeled 0.5 mL microcentrifuge tube (Fisher Scientific, Ottawa, ON, Canada). Samples (50 μL) were injected on a YMC-Pack Pro C18 column (150 X 4.6 mm I.D., 3 μm) (YMC America, Inc., Allentown, PA, USA) and creatine was separated using reverse-phase HPLC. Samples were run for 32 min with a

flow rate of 1 mL/min. Mobile phase 'A' consisted of 50 mM formic acid (Sigma-Aldrich, Oakville, Canada) and mobile phase 'B' consisted of 100% methanol (Fisher Scientific, Ottawa, ON, Canada). Samples were detected by a Waters 474 scanning fluorescence detector at 470 nm emission and 390 nm excitation (Waters Corporation, Milford, MA, USA). A standard curve was used for quantification of creatine peaks and peaks were identified using EmPower 3 software (Waters Corporation, Milford, MA, USA).

2.5 Calculations

Specific radioactivity (SRA) of transmethylated products, methionine and SAM were calculated as DPM/nmol. Plateaus were determined by visual inspection and verified by a non-significant linear regression of the data set. For all infusions, the mean of plasma methionine SRA at plateau was calculated using data collected between 4 and 6 h of the L-[methyl-^{3}H] methionine infusion. Methionine flux was calculated using the following equation:

Flux (μmol/kg/h) = L-[methyl-^{3}H] Methionine infusion rate (DPM/kg/h)/Mean methionine SRA of methionine at plateau (DPM/μmol)

Fractional Synthesis Rate (FSR) of protein and transmethylation products were calculated using the following equation:

Fractional Synthesis Rate (%/h) = $SRA_{\text{of product}}$ / $SRA_{\text{of precursor}}$ * 100/time

SRA of hepatic SAM was used as a $SRA_{precursor}$ for calculations of FSR of transmethylation products, but SRA of the tissue-free methionine was used as $SRA_{precursor}$

to calculate the FSR of tissue specific protein synthesis. Time refers to the duration of labelling in hours; time of L-[methyl-^{3}H] methionine infusion.

Proportion of the ^{3}H-methyl products remaining in the liver at the end of the study was calculated using the following equation:

$$\% = (DPM_{\text{of product}}/\text{g of tissue}) / (DPM_{\text{sum of all measured products}}/\text{g of tissue})*100$$

2.6 Statistical Analyses

Data on piglets were analyzed using n=6 and n=7 (Base + GAA), unless otherwise stated. Data were analyzed by one-way ANOVA with Tukey's multiple comparisons post-hoc test using GraphPad Prism 7 (Graph Pad Software, San Diego, CA, USA). The alpha level was set at 0.05. All data were reported as mean ± standard deviation.

3. Results

The study's two major outcomes were to measure whole body amino acid and protein turnover with the use of stable isotopes (performed by another graduate student; Alaa Al-Jaroudi) and tissue-specific transmethylation and protein synthesis (the subject of this thesis). The study design and outcomes were similar to previous research completed in our laboratory for an intragastric (IG) feeding model. Based on sample size calculation using data from the previous study, we calculated that a sample size of n=6 was sufficient for the current study. Due to suspected sickness in one of our piglets in Base + GAA treatment group, we added another piglet to this treatment group. This resulted in a sample size of n=7 for the Base + GAA treatment group to ensure we had at least 6 healthy piglets in each treatment group for all outcomes.

3.1 Growth and Weight Analysis of Piglets

3.1.1 Piglet Weight Gain

Piglets receiving Adequate Met + GAA treatment had significantly greater weight gain during 5 d of treatment ($p < 0.05$) compared to piglets receiving Base, Base + GAA and Base + CRE diet throughout the 5 days of treatment diets (Figure 3.1). At necropsy on Day 7, no differences were found for organ weights across treatment groups (Table 3.1).

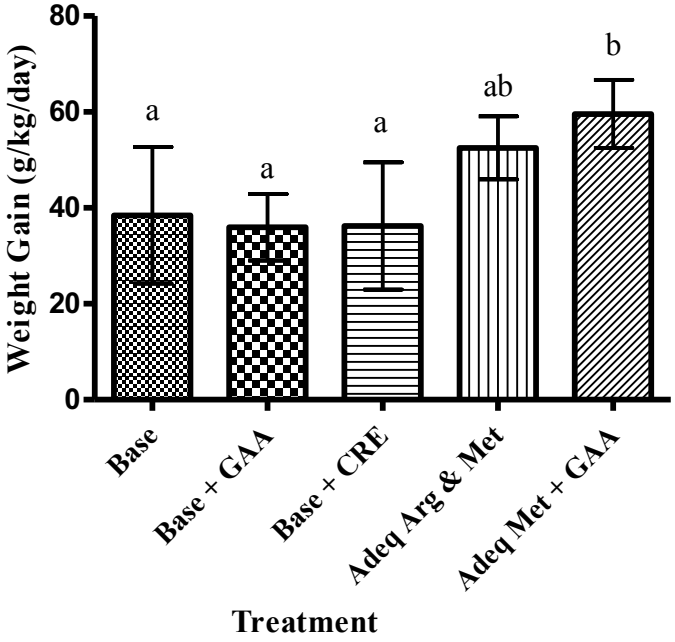

Figure 3.1: Effects of Diet on Piglet Weight Gain over 5 d of Treatment

Bars represent means (± standard deviations). For piglet weight analysis (n=6) for all treatment groups. One-way ANOVA followed by Tukey's multiple comparisons ($p < 0.05$) were used to test for differences among diets. Significant differences among groups are shown by the different letters above each bar.

Adeq, Adequate; Arg, Arginine; CRE, Creatine; GAA, Guanidinoacetic acid; Met, Methionine

Table 3.1: Weight of Piglet Organs after 5 d of Treatment Diet

	Treatment				
	Base	**Base + GAA**	**Base + CRE**	**Adequate Arg & Met**	**Adequate Met + GAA**
Piglet Organ					
Liver	46.0±2.5	44.8±3.3	45.1±1.2	40.7±5.4	46.7±7.0
Kidney*	4.3±0.3	4.6±0.9	4.5±0.6	3.9±0.2	4.4±0.4
Heart	6.6±0.6	6.3±0.4	6.7±0.7	6.3±0.2	6.5±0.4
Brain	14.6±1.6	14.0±2.7	15.5±3.9	13.7±1.0	14.2±1.7

Data represent means ± standard deviations (g/kg of BW). One-way ANOVA followed by Tukey's multiple comparisons ($p < 0.05$) were used to test for differences among diets; there were no differences among treatments.

* Represents the weight of the left kidney.

Arg, Arginine; CRE, Creatine; GAA, Guanidinoacetic acid; Met, Methionine

3.1.2 72-Hour Urinary Nitrogen Retention

Nitrogen retention was completed by another graduate student, but are presented here for reference. Urine was collected every 24 h for 3 consecutive days. As shown below in Figure 3.2, piglets receiving Adequate Met + GAA had significantly greater 72-hour nitrogen retention (%) compared to piglets receiving a treatment diet of Base + GAA ($p < 0.05$).

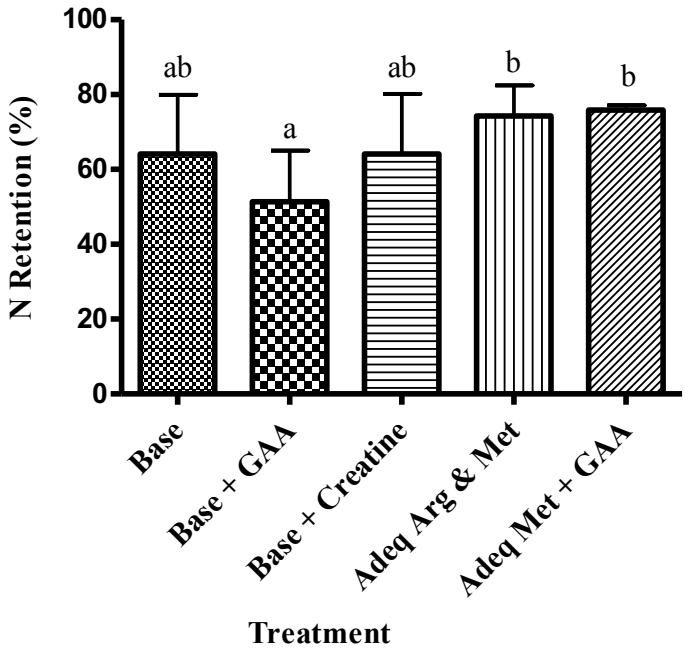

Figure 3.2: 72-Hour Percent Nitrogen (N) Retention Across all Treatment Groups

Bars represent means ± standard deviations for % N retention. For % N retention analysis (n=5) for Base + GAA and Met+GAA. One-way ANOVA followed by Tukey's multiple comparisons ($p < 0.05$) were used to test for differences among diets. Significant differences among groups are shown by the different letters above each bar.

Adeq, Adequate; Arg, Arginine; GAA, Guanidinoacetic acid; Met, Methionine; N, Nitrogen

3.2 Plasma Metabolite Analysis

3.2.1 Plasma Amino Acid and Creatine Concentrations

Piglets receiving Adequate Arg & Met, and Adequate Met + GAA had significantly higher ($p < 0.05$) plasma methionine concentrations than all other treatment

groups, reflecting dietary intake. In the Adequate Arg & Met group, plasma arginine and ornithine concentrations were found to be significantly greater ($p < 0.05$) but plasma glutamine and alanine concentrations were found to be significantly lower ($p < 0.05$) compared to all other treatments (Table 3.2). Piglets receiving supplemental creatine (CRE) had higher plasma concentrations of tyrosine ($p < 0.05$) compared to piglets fed Base + GAA, Adequate Arg & Met, and Adequate Met + GAA diets. Plasma glycine was significantly higher ($p < 0.05$) in piglets receiving Adequate Arg & Met compared to piglets receiving Base + GAA. Piglets supplemented with creatine had significantly greater ($p < 0.05$) plasma concentrations of serine compared to those receiving Adequate Met + GAA. A diet high in arginine and methionine also led to a lower plasma creatine concentration ($p < 0.05$) compared to all other treatments. Unexpectedly, piglets receiving supplemental creatine did not show elevated levels of plasma creatine, compared to all other treatments.

Table 3.2: Plasma Metabolite Concentrations after 5 d of Treatment Diet

	Treatment				
	Base	Base + GAA	Base + Creatine	Adequate Arg & Met	Adequate Met + GAA
Non-Essential Amino Acids					
Alanine	**685 ± 112^{a}**	**619 ± 94^{a}**	**665 ± 59^{a}**	**326 ± 146^{b}**	**710 ± 37^{a}**
Hydroxyproline	56 ± 5	48 ± 7	51 ± 4	70 ± 29	60 ± 4
Proline	613 ±143	580 ± 63	632 ± 68	674 ± 270	633 ± 42
Tyrosine	**192 ± 43^{ab}**	**129 ± 24^{b}**	**234 ± 46^{a}**	**158 ± 63^{b}**	**146 ± 32^{b}**
Glutamine	**369 ± 45^{a}**	**294 ± 77^{a}**	**361 ± 55^{a}**	**155 ± 63^{b}**	**325 ± 92^{a}**
Glutamate	159 ± 21	148 ± 35	142 ± 15	97 ± 38	125 ± 21
Citrulline	**114 ± 23^{ad}**	**116 ± 18^{a}**	**123 ± 23^{ab}**	**79 ± 37^{c}**	**83 ± 20^{cd}**
Ornithine	**43 ± 11^{ab}**	**45 ± 10^{ab}**	**57 ± 12^{b}**	**168 ± 67^{c}**	**45 ± 6^{a}**
Essential Amino Acids					
Arginine	**105 ± 14^{a}**	**100 ± 14^{a}**	**122 ± 25^{a}**	**381 ± 146^{b}**	**86 ± 7^{a}**
Histidine	141± 23	119 ± 11	137 ± 59	108 ± 46	105 ±23
Isoleucine	217 ± 23	210 ± 45	202 ± 43	175 ± 67	181 ±18
Leucine	416 ± 76	388 ± 90	424 ± 80	405 ± 160	429 ± 45
Lysine	437 ± 132	415 ± 86	514 ± 131	471 ± 162	399 ± 58
Phenylalanine	192 ± 26	179 ± 21	178 ± 13	209 ± 80	197 ± 55
Threonine	345 ± 73	330 ± 43	356 ± 52	361 ± 143	338 ± 40
Tryptophan	132 ± 18	115 ± 18	120 ± 23	130 ± 51	106 ± 14
Valine	438 ± 38	397 ± 70	417 ± 91	380 ± 146	368 ± 48
Sulfur Amino Acid Metabolites					
Glycine	1037 ± 113^{ab}	924 ± 88^{a}	1122 ± 145^{b}	1134 ± 438^{b}	1078 ± 98^{ab}
Methionine	**66 ± 15^{a}**	**72 ± 22^{a}**	**81 ± 18^{a}**	**233 ± 99^{b}**	**220 ± 43^{b}**
Serine	**684 ± 128^{a}**	**525 ± 94^{ab}**	**712 ± 76^{a}**	**512 ± 204^{b}**	**518 ± 76^{b}**
Taurine	217 ± 39	185 ± 33	192 ± 37	192 ± 76	214 ± 19
Other Metabolites					
Creatine	**300 ± 141^{a}**	**251 ± 59^{a}**	**256 ± 35^{a}**	**74 ± 46^{b}**	**139 ± 87^{a}**

Data represent means ± standard deviations in μmol/L. One-way ANOVA followed by Tukey's multiple comparisons ($p < 0.05$) were used to test for differences among diets. Means within rows not sharing a superscript letter were different.

Arg, Arginine; GAA, Guanidinoacetic acid; Met, Methionine

3.2.2 Plasma SRA of Creatine

Plasma SRA of creatine was significantly greater ($p < 0.05$) in piglets supplemented with adequate methionine and arginine compared to piglets receiving all low methionine diets (Figure 3.3). Moreover, piglets supplemented with GAA in the presence of adequate methionine had higher ($p < 0.05$) plasma SRA of creatine, but only compared to piglets supplemented with creatine.

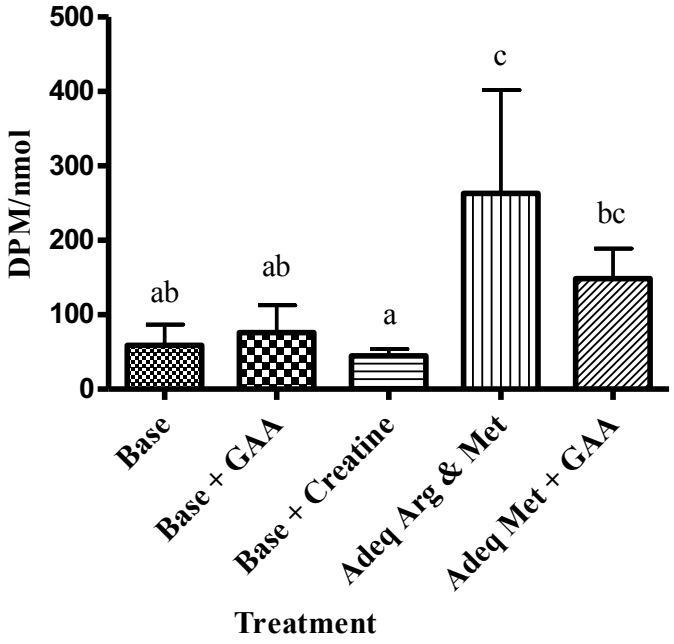

Figure 3.3: Plasma SRA of Creatine After 5 d of Treatment Diet

Bars represent means ± standard deviations in DPM/nmol. One-way ANOVA followed by Tukey's multiple comparisons ($p < 0.05$) were used to test for differences among diets. Significant differences among groups are shown by the different letters above each bar. Adeq, Adequate; Arg, Arginine; DPM, Disintegrations per minute; GAA, Guanidinoacetic acid; Met, Methionine

3.3 Hepatic Free Amino Acids Concentrations

Analysis of hepatic free amino acids revealed that hepatic concentration (μmol/g of tissue) of methionine was significantly greater in piglets receiving Adequate Arg & Met and Adequate Met + GAA compared to all other treatments (Table 3.3) ($p < 0.05$). Hepatic free arginine concentrations were significantly greater in piglets receiving Adequate Arg & Met, but only compared to piglets receiving a treatment diet of Base + GAA diet ($p < 0.05$). Moreover, hepatic phenylalanine and ornithine concentrations were significantly greater in piglets receiving Adequate Arg & Met diet compared to piglets receiving a Base, Base + GAA and Base + Creatine diet ($p < 0.05$). Piglets receiving Adequate Met + GAA had significantly lower concentrations of ornithine, compared to all other treatments, while hepatic proline concentrations were shown to be significantly greater compared to piglets receiving a diet of Base + GAA diet ($p < 0.05$).

Table 3.3: Hepatic Free Amino Acid Concentrations after 5 d of Treatment Diet

	Treatment				
	Base	Base + GAA	Base + Creatine	Adequate Arg & Met	Adequate Met + GAA
Non-Essential Amino Acids					
Alanine	1056 ± 398	1516 ± 844	1437 ± 751	1267 ± 249	1728 ± 578
Aspartate	710 ± 153	712 ± 97	552 ± 337	693 ± 67	849 ± 105
Hydroxyproline	**1773 ± 119^{a}**	**1662 ± 186^{a}**	**1316 ± 325^{ab}**	**1043 ± 318^{b}**	**1234 ± 219^{b}**
Proline	**575 ± 134^{ab}**	**914 ± 299^{a}**	**995 ± 205^{ab}**	**1107 ± 291^{ab}**	**1484 ± 704^{b}**
Tyrosine	162 ± 57	191 ± 166	218 ± 71	165 ± 29	175 ± 67
Glutamine	**1631 ± 346^{a}**	**1658 ± 240^{a}**	**1739 ± 291^{a}**	**873 ± 153^{b}**	**1550 ± 207^{a}**
Glutamate	1253 ± 98	1232 ± 165	953 ± 419	1177 ± 84	1246 ± 175
Citrulline	1499 ± 688	1233 ± 441	1072 ± 624	667 ± 434	1027 ± 620
Ornithine	**637 ± 142^{a}**	**575 ± 129^{a}**	**624 ± 101^{a}**	**860 ± 132^{b}**	**282 ± 103^{c}**
Essential Amino Acids					
Arginine	**126 ± 81^{ab}**	**71 ± 18^{a}**	**98 ± 40^{ab}**	**171 ± 49^{b}**	**148 ± 52^{ab}**
Histidine	189 ± 32	232 ± 24	183 ± 44	376 ± 250	411 ± 200
Isoleucine	228 ± 25	230 ± 29	229 ± 23	194 ± 25	195 ± 18
Leucine	449 ± 43	462 ± 29	466 ± 45	464 ± 59	471 ± 57
Lysine	1491 ± 611	1351 ± 497	1373 ± 550	733 ± 78	723 ± 180
Phenylalanine	**150 ± 18^{a}**	**158 ± 18^{a}**	**164 ± 33^{a}**	**224 ± 38^{b}**	**182 ± 30^{ab}**
Threonine	1099 ± 634	595 ± 426	782 ± 225	537 ± 190	726 ± 675
Valine	403 ± 47	416 ± 64	437 ± 99	375 ± 101	397 ± 100
Sulfur Amino Acid Metabolites					
Glycine	3734 ± 413	3449 ± 203	4113 ± 932	3765 ± 326	3606 ± 350
Methionine	**55 ± 14^{a}**	**49 ± 9^{a}**	**40 ± 15^{a}**	**119 ± 24^{b}**	**126 ± 29^{b}**
Serine	**3349 ± 323^{a}**	**2724 ± 724^{ab}**	**3399 ± 409^{a}**	**2301 ± 251^{b}**	**2131 ± 277^{b}**
Taurine	3753 ± 309	4376 ± 1362	4747 ± 1671	5955 ± 1904	5957 ± 1208

Data represent means ± standard deviations in nmol/g tissue. One-way ANOVA followed by Tukey's multiple comparisons ($p < 0.05$) were used to test for differences among diets. Means within a row not sharing a superscript letter were different.

Arg, Arginine; GAA, Guanidinoacetic acid; Met, Methionine

3.4 Hepatic Metabolite Concentrations

Other hepatic metabolite concentrations were analyzed (Table 3.4). There were no significant differences in hepatic creatine or phosphatidylcholine concentrations ($p < 0.05$) among diets. However, hepatic SAM concentrations were significantly greater in piglets receiving a diet Adequate Arg & Met and Adequate Met + GAA diets compared to all other treatments ($p < 0.05$). Hepatic TG were greater in piglets fed Adequate Met + GAA compared to piglets fed a Base diet supplemented with creatine ($p < 0.05$).

Table 3.4: Hepatic Metabolite Concentrations after 5 d of Treatment Diet

	Treatment				
	Base	Base + GAA	Base + Creatine	Adequate Arg & Met	Adequate Met + GAA
Hepatic Metabolites					
Creatine	1.5 ± 0.8	1.6 ± 0.4	1.2 ± 0.2	0.9 ± 0.2	2.2 ± 1.66
PC	26.8 ± 4.1	30.0 ± 6.7	27.8 ± 8.6	30.3 ± 4.8	29.8 ± 6.5
DNA	2.8 ± 0.6	2.9 ± 0.4	2.8 ± 0.4	2.8 ± 1.9	2.3 ± 0.3
SAM	**0.05 ± 0.03^{b}**	**0.06 ± 0.03^{b}**	**0.06 ± 0.05^{b}**	**0.5 ± 0.06^{a}**	**0.4 ± 0.2^{b}**
SAH	0.02 ± 0.01	0.03 ± 0.01	0.02 ± 0.01	0.04 ± 0.01	0.04 ± 0.01
TG	**0.21 ± 0.06^{ab}**	**0.18 ± 0.04^{ab}**	**0.12 ± 0.04^{a}**	**0.17 ± 0.06^{ab}**	**0.24 ± 0.10^{b}**
Cholesterol	1.0 ± 0.2	1.1 ± 0.2	1.1 ± 0.2	1.1 ± 0.2	1.0 ± 0.2
SAM:SAH	**2.2 ± 1.0^{a}**	**1.8 ± 0.9^{a}**	**2.4 ± 1.6^{a}**	**13.3 ± 2.8^{b}**	**11.5 ± 1.9^{b}**

Data represent means ± standard deviations in μmol/g of tissue for all metabolites except TG and cholesterol (mg/g) and DNA (10^3 ng/μL). One-way ANOVA followed by Tukey's multiple comparisons ($p < 0.05$) were used to test for differences among diets. Means not sharing a superscript letter within a row were different.

DNA, deoxyribonucleic acid; GAA, Guanidinoacetic acid; PC, Phosphatidylcholine; SAM, S-adenosyl methionine; SAH, S-adenosyl homocysteine; TG, triglyceride

3.5 Muscle Free Amino Acids & Metabolite Concentrations

Data for muscle metabolites (Table 3.5) were not different among treatment groups.

Table 3.5: Muscle Free Amino Acid & Metabolite Concentrations after 5 d of Treatment Diet

	Treatment				
	Base	Base + GAA	Base + Creatine	Adequate Arg & Met	Adequate Met + GAA
Muscle Metabolites					
Methionine	0.4 ± 0.2	0.3 ± 0.2	0.1 ± 0.04	0.4 ± 0.3	0.4 ± 0.2
Glycine	3.1 ± 0.6	3.1 ± 0.48	3.5 ± 0.41	3.8 ± 0.56	3.7 ± 1.2
Taurine	4.6 ± 1.0	4.2 ± 1.3	4.9 ± 1.2	5.5 ± 0.8	4.4 ± 1.0
Creatine	18.1 ± 4.4	15.4 ± 5.9	20.5 ± 1.9	20.5 ± 2.7	20.2 ± 3.9

Data represent means ± standard deviations in μmol/g of tissue. One-way ANOVA followed by Tukey's multiple comparisons ($p < 0.05$) were used to test for differences among diets; data were not different.

Arg, Arginine; GAA, Guanidinoacetic acid; Met, Methionine

3.6 Methionine Kinetics

3.6.1 Plasma Methionine Kinetics

3.6.1.1 Plasma Methionine SRA

Plasma methionine SRA at steady state was determined using the plasma SRA of methionine for and last five plasma samples from the 6-hour L-[methyl-^{3}H] methionine infusion. Steady state plasma methionine SRA (DPM/μmol) was significantly greater ($p <$

0.05) in piglets receiving adequate methionine compared to all other treatments (Figure 3.4).

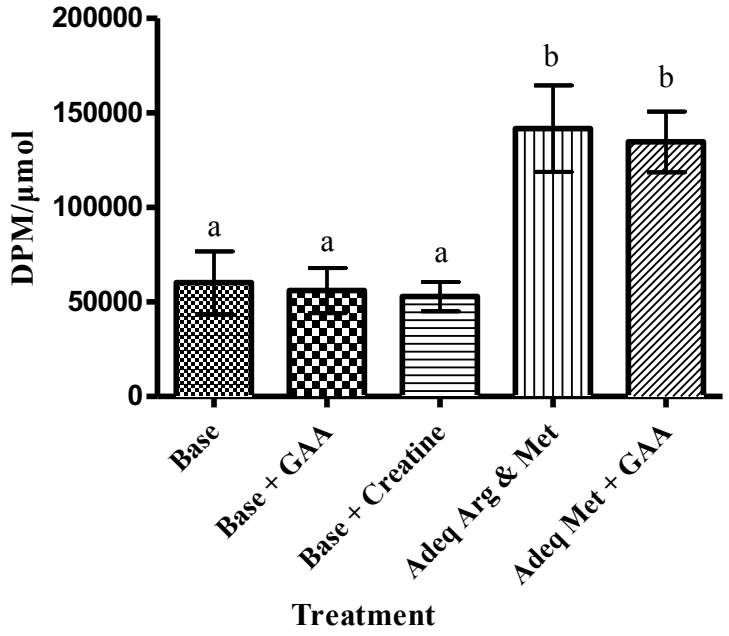

Figure 3.4: Steady State Plasma Methionine SRA on Day 7

Bars represent means ± standard deviations in DPM/nmol. One-way ANOVA followed by Tukey's multiple comparisons ($p < 0.05$) were used to test for differences among diets. Significant differences among groups are shown by the different letters above each column.

Adeq, Adequate; Arg, Arginine; DPM, disintegrations per minute; GAA, Guanidinoacetic acid; Met, Methionine

3.6.1.2 Plasma Methionine Flux

Plasma L-[methyl-^{3}H]-methionine flux was significantly lower in piglets receiving adequate methionine diets compared to low methionine diets (Figure 3.5).

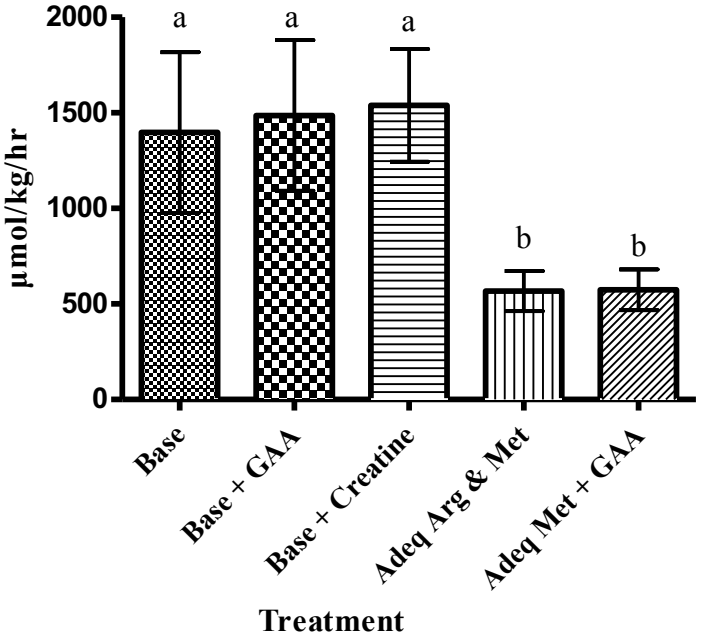

Figure 3.5: Plasma L-[Methyl-^{3}H]-Methionine Flux on Day 7 Infusion

Bars represent means ± standard deviations in µmol/kg/h. One-way ANOVA followed by Tukey's multiple comparisons ($p < 0.05$) were used to test for differences among diets. Significant differences between groups are shown by the different letters above each column.

Adeq, Adequate; Arg, Arginine; GAA, Guanidinoacetic acid; Met, Methionine

3.6.2 Hepatic Methionine Kinetics

3.6.2.1 Hepatic SRA of Methionine and Transmethylated Products

Hepatic SRA of transmethylated products was analyzed using the L-[methyl-^{3}H] methionine tracer (Table 3.6). Hepatic free methionine SRA was significantly greater ($p < 0.05$) in piglets receiving Adequate Arg & Met and Adequate Met + GAA diet compared to all other treatments. Hepatic protein-bound methionine SRA was significantly greater ($p < 0.05$) in piglets receiving supplemental creatine compared to those receiving Base + GAA and Adequate Arg & Met treatments. On the other hand, hepatic creatine SRA was significantly greater ($p < 0.05$) in piglets receiving Adequate Met + GAA compared to piglets receiving Base + Creatine. Hepatic SRA of PC and SAM were both significantly greater in piglets supplemented with adequate methionine in comparison to low methionine treatments ($p < 0.05$).

Table 3.6: Hepatic SRA of Transmethylated Products

	Treatment				
	Base	Base + GAA	Base + Creatine	Adequate Arg & Met	Adequate Met + GAA
Hepatic Product					
Free Methionine	**0.4 ± 0.1^{a}**	**0.5 ± 0.2^{a}**	**0.5 ± 0.3^{a}**	**1.5 ± 0.4^{b}**	**1.3 ± 0.4^{b}**
Bound Methionine	**8.6 ± 4.6^{ab}**	**7.2 ± 2.1^{a}**	**15.4 ± 5.1^{b}**	**8.1 ± 4.4^{a}**	**11.1 ± 3.2^{ab}**
Creatine	**10.9 ± 5.3^{ab}**	**11.5 ± 2.2^{ab}**	**6.7 ± 1.3^{a}**	**9.3 ± 2.2^{ab}**	**17.1 ± 9.1^{b}**
PC	**5.1 ± 3.8^{a}**	**3.4 ± 1.1^{a}**	**5.6 ± 2.4^{a}**	**16.1 ± 3.4^{b}**	**14.0 ± 2.7^{b}**
DNA	0.7 ± 0.1	0.7 ± 0.2	0.7 ± 0.2	0.8 ± 0.3	1.1 ± 0.5
SAM	**0.5 ± 0.3^{a}**	**0.5 ± 0.1^{a}**	**0.4 ± 0.2^{a}**	**3.1 ± 0.6^{b}**	**2.6 ± 0.4^{b}**

Data represent means ± standard deviations in DPM/nmol, except for DNA (DPM/μg). Bound methionine analysis (n=5). One-way ANOVA followed by Tukey's multiple comparisons ($p < 0.05$) were used to test for differences among diets. Means not sharing a superscript letter within a row were different.

Arg, Arginine; DNA, deoxyribonucleic acid; GAA, Guanidinoacetic acid; Met, Methionine; PC, Phosphatidylcholine; SAM, S-adenosyl methionine.

3.6.2.2 Hepatic Fractional Synthesis Rate (FSR) of Transmethylated Products

Hepatic FSR of protein was significantly greater in piglets supplemented with creatine compared to all treatments ($p < 0.05$), except Base Diet (Table 3.7). The hepatic FSR of transmethylated products were calculated using the SRA of SAM as the precursor. The FSR of creatine and PC synthesis was significantly greater in piglets fed diets supplemented with adequate methionine compared to low methionine treatments ($p < 0.05$).

Table 3.7: Hepatic FSR of Protein and Transmethylated Products

	Treatment				
	Base	Base + GAA	Base + Creatine	Adequate Arg & Met	Adequate Met + GAA
Hepatic Product					
Protein	**1.78 ± 0.72^{ab}**	**0.60 ± 0.41^{a}**	**3.27 ± 1.66^{b}**	**0.91 ± 0.50^{a}**	**1.21 ± 0.82^{a}**
Creatine	**11.85 ± 2.84^{a}**	**12.21 ± 5.82^{a}**	**10.48 ± 5.68^{a}**	**28.07 ± 1.86^{b}**	**27.69 ± 2.09^{b}**
PC	**0.87 ± 0.75^{a}**	**0.75 ± 0.52^{a}**	**1.14 ± 0.83^{a}**	**4.18 ± 0.55^{b}**	**3.69 ± 0.63^{b}**
DNA	**0.05 ± 0.01^{a}**	**0.06 ± 0.03^{a}**	**0.05 ± 0.02^{a}**	**0.08 ± 0.03^{ab}**	**0.11 ± 0.05^{b}**

Data represent means ± standard deviations in %/h. Hepatic FSR of Protein (n=5) for Base, Base + GAA and Base + Creatine. One-way ANOVA followed by Tukey's multiple comparisons ($p < 0.05$) were used to test for differences among diets. Means not sharing a superscript letter within a row were different.

Arg, Arginine; DNA, deoxyribonucleic acid; FSR, fractional synthesis rate; GAA, Guanidinoacetic acid; Met, Methionine; PC, Phosphatidylcholine.

3.6.2.3 Hepatic Distribution of Transmethylated Products

After the 6-hour infusion, newly transmethylated hepatic PC from L-[methyl-^{3}H] methionine was significantly greater ($p < 0.05$) in piglets receiving diets with adequate dietary methionine compared to other groups (Table 3.8). In piglets receiving low methionine diets, hepatic SAM derived from L-[methyl-^{3}H] methionine was significantly lower compared to piglets receiving adequate dietary methionine.

Table 3.8: ^{3}H-methyl Products Remaining in the Liver After 6 h of L-[methyl-^{3}H] Methionine Infusion

	Treatment				
	Base	Base + GAA	Base + Creatine	Adequate Arg & Met	Adequate Met + GAA
Creatine	108.6 ± 52.7	114.7 ± 22.4	66.9 ± 13	92.6 ± 22	171 ± 91.4
DNA	8.5 ± 0.8	8.5 ± 1.8	8.4 ± 1.2	7.2 ± 3.7	10.7 ± 3.6
PC	**135.7 ± 85.8^{a}**	**107.2 ± 59^{a}**	**142.3 ± 45^{a}**	**491.2 ± 156^{b}**	**425.1 ± 116.6^{b}**
Tissue Bound	85.6 ± 45.7	71.5 ± 24.1	153.8 ± 50.5	80.5 ± 43.9	111.1 ± 32.4
Tissue Free	4.5 ± 1.2	4.7 ± 1.8	4.7 ± 2.6	14.6 ± 4.0	13.1 ± 4.1
SAM	**4.9 ± 2.6^{a}**	**4.6 ± 1.4^{a}**	**4.3 ± 1.7^{a}**	**31.3 ± 5.5^{b}**	**26.0 ± 4.4^{b}**

Data represent means ± standard deviations in x 1000 DPM/g. One-way ANOVA followed by Tukey's multiple comparisons ($p < 0.05$) were used to test for differences among diets. Means not sharing a superscript letter within a row were different.
Arg, Arginine; DNA, deoxyribonucleic acid; DPM, disintegrations per minute; GAA, Guanidinoacetic acid; Met, Methionine; PC, Phosphatidylcholine; SAM, S-adenosyl methionine.

Across all treatment groups, approximately 25% of dietary methionine was incorporated into protein and approximately 75% was used for transmethylation reactions (Table 3.9). Interestingly, when adequate methionine and arginine were fed, almost 70% of dietary methionine was used for synthesis of hepatic PC, while only ~12% was used for incorporation into protein. On the other hand, when piglets received supplemental

creatine, approximately 40% of methionine was used for incorporation into protein, despite receiving limited dietary methionine.

Table 3.9: Proportion of ^{3}H-methyl Products Remaining in the Liver After 6 h of L-[methyl-^{3}H] Methionine Infusion

	Treatment				
	Base	Base + GAA	Base + Creatine	Adequate Arg & Met	Adequate Met + GAA
Creatine	**31.2 ± 15.1^{ab}**	**39.5 ± 7.7^{a}**	**18.9 ± 3.7^{b}**	**13.4 ± 3.2^{b}**	**23.9 ± 12.8^{ab}**
DNA	**2.5 ± 0.2^{a}**	**2.9 ± 0.6^{a}**	**2.4 ± 0.3^{a}**	**1.0 ± 0.5^{b}**	**1.5 ± 0.5^{b}**
PC	**39.0 ± 24.7^{ab}**	**37.0 ± 20.4^{a}**	**40.1 ± 12.7^{ab}**	**71.1 ± 22.6^{b}**	**59.4 ± 16.3^{ab}**
Tissue Bound	**24.6 ± 13.2^{a}**	**24.7 ± 8.3^{ab}**	**43.4 ± 14.2^{b}**	**11.4 ± 4.9^{a}**	**15.5 ± 4.5^{a}**
Tissue Free	1.3 ± 0.4	1.6 ± 0.6	1.3 ± 0.7	2.1 ± 0.6	1.8 ± 0.6
SAM	**1.4 ± 0.7^{a}**	**1.6 ± 0.5^{a}**	**1.2 ± 0.5^{a}**	**4.5 ± 0.8^{b}**	**3.6 ± 0.6^{b}**
Total Measured ^{3}H-Products	**347.8 ± 118.0^{a}**	**290.1 ± 75.6^{a}**	**354.7 ± 71.7^{a}**	**691.6 ± 151.9^{b}**	**715.7 ± 117.3^{b}**

Data represent means in (% of total measured ^{3}H-Products). Total measured ^{3}H-products represented in (x1000 DPM/g) (± standard deviations). One-way ANOVA followed by Tukey's multiple comparisons ($p < 0.05$) were used to test for differences between diets. Means not sharing a superscript letter within a row were different.

Arg, Arginine; DNA, deoxyribonucleic acid; GAA, Guanidinoacetic acid; Met, Methionine; PC, Phosphatidylcholine; SAM, S-adenosyl methionine.

3.6.3 Muscle Methionine Kinetics

3.6.3.1 SRA of Methionine and Creatine in Muscle

Piglets receiving adequate arginine and methionine had significantly greater ($p < 0.05$) SRA of free methionine in the muscle compared to piglets receiving low arginine and methionine Base diet after 5 days of treatment. Moreover, piglets receiving adequate arginine and methionine had significantly greater ($p < 0.05$) creatine SRA in muscle compared to piglets receiving low arginine and methionine, as well as piglets receiving supplemental creatine (Table 3.10); piglets with supplemental creatine also had lower muscle SRA than Adequate Met + GAA and Base + GAA diets.

Table 3.10: SRA of Methionine and Creatine in Muscle

	Treatment				
	Base	Base + GAA	Base + Creatine	Adequate Arg & Met	Adequate Met + GAA
Muscle Metabolites					
Free Methionine	**11.1 ± 9.7^{a}**	**14.1 ± 10.7^{ab}**	**21.3 ± 12.2^{ab}**	**33.1 ± 16.8^{b}**	25.8 ± 12.3^{ab}
Bound Methionine	1.7 ± 0.4	1.6 ± 0.4	1.8 ± 0.3	1.3 ± 0.4	1.4 ± 0.3
Creatine	**1.3 ± 0.3^{ab}**	**2.1 ± 0.7^{bc}**	**0.9 ± 0.3^{a}**	**2.6 ± 0.5^{c}**	**2.0 ± 0.6^{bc}**

Data represent means standard deviations in DPM/nmol. One-way ANOVA followed by Tukey's multiple comparisons ($p < 0.05$) were used to test for differences among diets. Means not sharing a superscript letter within a row were different.

Arg, Arginine; GAA, Guanidinoacetic acid; Met, Methionine.

3.6.3.2 Fractional Synthesis Rate of Muscle Protein Synthesis

Fractional synthesis rate of protein synthesis in the muscle was not different among treatment groups after 5 d of treatment (Figure 3.6).

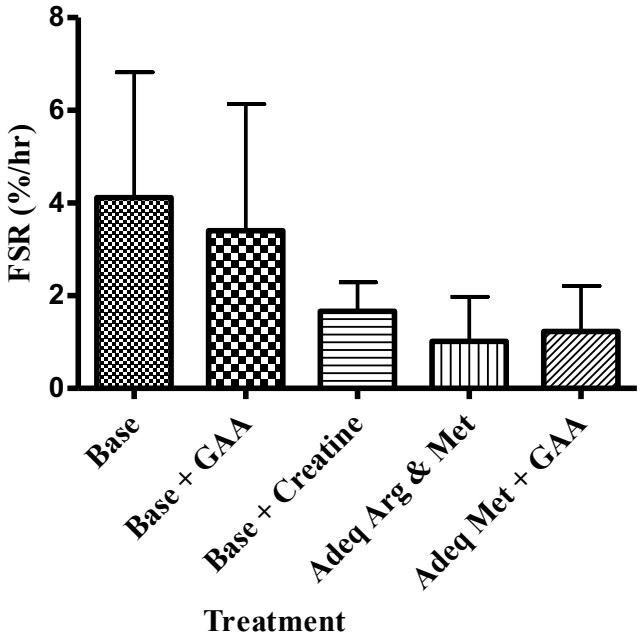

Figure 3.6: Fractional Synthesis Rate (FSR) of Muscle Protein (%/h)

Bars represent means ± standard deviations in %/h. One-way ANOVA followed by Tukey's multiple comparisons ($p < 0.05$) were used to test for differences among diets. No differences among groups were observed.

Adeq, Adequate; Arg, Arginine; GAA, Guanidinoacetic acid; Met, Methionine

3.7 Kidney Creatine and GAA Concentrations

Kidney concentrations of creatine were significantly higher ($p < 0.05$) in piglets receiving Adequate Met + GAA compared to piglets receiving Adequate Arg & Met

(Table 3.11). Kidney concentrations of GAA were greatest in Adequate Arg & Met piglets ($p < 0.05$) compared to all other treatments. Moreover, piglets receiving the Base + GAA diet had significantly greater renal GAA concentrations compared to piglets supplemented with creatine ($p < 0.05$).

Table 3.11: Kidney Metabolite Concentrations after 5 d of Treatment

	Treatment				
	Base	Base + GAA	Base + Creatine	Adequate Arg & Met	Adequate Met + GAA
Kidney Metabolites					
Creatine*	**7.11 ± 3.68^{ab}**	**6.02 ± 1.73^{ab}**	**7.50 ± 1.39^{ab}**	**4.12 ± 1.14^{a}**	**8.59 ± 2.68^{b}**
GAA*	**1.81 ± 0.21^{ab}**	**2.83 ± 1.00^{b}**	**1.29 ± 0.23^{a}**	**4.13 ± 0.70^{c}**	**1.95 ± 0.71^{ab}**

Data represent means ± standard deviations in μmol/g of tissue. One-way ANOVA followed by Tukey's multiple comparisons ($p < 0.05$) were used to test for differences among diets. Means not sharing a superscript letter within a row were different.

Arg, Arginine; GAA, Guanidinoacetic acid; Met, Methionine

* Analyses performed by another graduate student, Alaa Al-Jaroudi

3.8 Kidney & Hepatic Enzymatic Activity

Hepatic GAMT activity was not different among treatment groups (Table 3.12). Renal AGAT activity was significantly greater ($p < 0.05$) in piglets receiving the treatment Adequate Arg & Met compared to piglets receiving Base + GAA, Base + Creatine and Adequate Met + GAA diets. Moreover, AGAT activity in the pancreas was

also significantly higher ($p < 0.05$) in piglets receiving Adequate Arg & Met compared to piglets supplemented with creatine.

Table 3.12: Enzyme Activities of AGAT and GAMT after 5 d of Treatment

	Treatment				
	Base	Base + GAA	Base + Creatine	Adequate Arg & Met	Adequate Met + GAA
Enzymatic Activity					
Kidney AGAT*	**239 ± 61^{ab}**	**170 ± 50^{a}**	**151 ± 56^{a}**	**364 ± 139^{b}**	**214 ± 109^{a}**
Pancreas AGAT*	**838 ± 228^{ab}**	**891 ± 122^{ab}**	**642 ± 152^{a}**	**980 ± 127^{b}**	**855 ± 226^{ab}**
Hepatic GAMT	61.9 ± 23.3	48.4 ± 16.9	38.3 ± 26.8	59.0 ± 28.4	74.9 ± 37.4

Data represent means ± standard deviations in nmol/min/g. One-way ANOVA followed by Tukey's multiple comparisons ($p < 0.05$) were used to test for differences among diets. Means not sharing a superscript letter within a row were different.

AGAT, arginine:glycine amidinotransferase; Arg, Arginine; GAA, Guanidinoacetic acid; GAMT, guanidinoacetate methyltransferase; Met, Methionine.

***** Analyses performed by another graduate student, Alaa Al-Jaroudi

4. Discussion

The methionine requirement during TPN has been demonstrated to be 70% of the enteral requirement in the presence of excess cysteine (Shoveller et al., 2003b). Moreover, arginine concentrations in parenteral solutions range from as high as 12.3 g/100 g amino acids to as low as 4.7 g/100 g amino acids (Vlaardingerbroek et al., 2011). PN solutions vary in amino acid concentrations due to using different reference proteins, but also may be due to confounding research on the optimum concentrations for maximum growth in the neonate. The amount of amino acid necessary for maximum growth is also affected by the amino acid demands for synthesis of alternative products. For example, in the growing neonatal piglet, the equivalent of approximately 35% of dietary methionine and 20% of arginine is required for de novo creatine synthesis (Brosnan et al., 2009). To demonstrate the sparing effect of creatine and GAA during TPN, methionine and arginine needed to be marginally supplied in the diet so that synthesis of metabolic products are not already maximized and spared amino acids have no effect. We hypothesized that supplemental creatine and GAA could spare methionine and/or arginine for other transmethylation functions and for protein synthesis. The main objectives of this study were to quantify the partitioning of methionine among transmethylation reactions, and to further quantify the sparing effect of creatine and its precursor GAA on these methylation pathways and protein synthesis during TPN.

4.1 Rationale of Treatment Groups

Shoveller and colleagues have previously demonstrated that the neonatal methionine requirement is estimated at 0.18 g/kg/day during TPN, in the presence of

cysteine (Shoveller et al., 2003b). Our group chose to supply 0.10 g/kg/day in our Base diet, which supplies approximately 55% of the methionine requirement when cysteine is present. This inadequate supply of methionine allowed us to evaluate and better demonstrate the effects of spared methionine from supplemental creatine. The oral arginine requirement for the neonatal piglet has been estimated at approximately 0.38 g/kg/day (National Research Council, 1998). Whereas, the TPN requirement for dietary arginine has been estimated at approximately 1.0 g/kg/day (unpublished observation); this higher requirement with TPN is due to TPN-induced gut atrophy which diminished de novo arginine synthesis (Bertolo et al., 2003). We chose to supply 0.55 g/kg/day of arginine in our Base diet, which is 55% of the estimated TPN requirement. This inadequate supply of dietary arginine allowed our group to evaluate and better demonstrate, like methionine, the sparing effect of supplemental creatine and its precursor GAA during TPN.

Supplemental GAA and creatine were supplied in the diet at levels based on previous findings by Brosnan and colleagues (Brosnan et al., 2009). They demonstrated that suckling piglets aged 4 to 11 days have a total body creatine accretion rate of approximately 0.103 g/kg/day. Therefore, our diets were supplemented with an equivalent amount of creatine as monohydrate and our piglets received 0.112 g/kg/day via TPN. GAA was provided to achieve the same accretion rate on a molar basis. Therefore, GAA was supplemented in the TPN diet so our piglets received 0.093 g/kg/day of GAA. However, because GAA that is provided to the liver is rapidly converted to creatine without feedback regulation at GAMT (McBreairty et al., 2013), we needed to ensure adequate dietary methionine was included in the diet to methylate supplemental GAA and

meet needs for protein synthesis. Therefore, a second GAA supplemented group was employed with a surplus of methionine.

As a positive control, one group of piglets needed to receive adequate amounts of arginine and methionine to ensure that all products of these amino acids were accommodated, including maximizing creatine and protein syntheses. Therefore, dietary arginine and methionine were adequately supplied at a rate of 1.8 and 0.5 g/kg/day, respectively, which equates to approximately twice the respective oral requirements.

4.2 Creatine Synthesis and TPN

The large demands for arginine and methionine for the synthesis of creatine potentially places a substantial burden on the growth of the neonate (Brosnan et al., 2009). Indeed, when our piglets received adequate dietary methionine and arginine, the hepatic FSR of creatine was approximately 175% greater than in those piglets receiving an inadequate supply of arginine and methionine. Moreover, piglets receiving supplemental GAA with adequate amounts of methionine demonstrated similar hepatic FSR of creatine as in pigs receiving adequate dietary methionine and arginine. However, the hepatic FSR of creatine was lower in piglets supplemented with GAA receiving low methionine, demonstrating that creatine synthesis during GAA supplementation is induced only when adequate dietary methionine is provided to furnish the higher transmethylation requirements. This facilitated creatine synthesis could have spared arginine and/or methionine for growth since we also determined that piglets receiving supplemental GAA with adequate methionine had 50% greater weight gain in comparison to piglets receiving low methionine and arginine diets, despite no differences in organ

weights between treatments. Moreover, 72-hour nitrogen retention in GAA plus methionine piglets was similar to those receiving adequate arginine and methionine, suggesting GAA can spare arginine for growth. On the other hand, our Base and Base + Creatine group were also deficient in methionine, but did not differ in 72-hour nitrogen retention from those receiving adequate methionine. These findings warrant further investigation, as one would expect a lower nitrogen retention when receiving inadequate arginine and methionine in the diet. For our Adequate met + GAA group, the increases in hepatic creatine synthesis and overall growth suggest that GAA is the driving force of creatine synthesis, but only when adequate dietary methionine is available, while also sparing arginine for growth.

In the kidney, arginine transfers an amidino group to glycine to form GAA and ornithine via the enzyme AGAT (Brosnan & Brosnan, 2016). When supplying adequate arginine to the diet AGAT activity was significantly upregulated. As previously mentioned, we also demonstrated that supplying GAA to the diet in the presence of adequate methionine downregulated AGAT and GAA synthesis, but upregulated creatine synthesis via GAMT, and can therefore spare arginine for protein synthesis. On the contrary, our FSR data did not support greater arginine incorporation into protein. The FSR of muscle protein synthesis showed no differences among treatments. And hepatic FSR was not different than the Base diet for either GAA group, whether supplemented with additional methionine or not. The only group with higher hepatic protein FSR was in piglets supplemented with creatine, which at first glance suggests methionine might have been spared by creatine to synthesize protein. However, the positive control group with higher methionine levels did not have higher hepatic protein FSR. These findings warrant

further investigation as one would presume a higher nitrogen retention and weight gain would equate to a higher rate of protein synthesis. On the other hand, because a methionine tracer was used with varying methionine intakes, perhaps an alternative tracer could have been utilized to more accurately measure protein synthesis. In future studies, a methionine tracer should be used to quantify transmethylation reactions and another tracer, phenylalanine, should be used to quantify protein synthesis.

4.3 Methionine Metabolism and TPN

Methionine is the single essential sulfur-containing amino acid and plays a major role directly and indirectly in many transmethylation reactions in the neonate (Finkelstein et al., 1988). In the cell, methionine is adenylated to SAM via the enzyme MAT and transfers its methyl group via many methyltransferases. Indeed, when our piglets received adequate dietary methionine the hepatic concentration and SRA of SAM was significantly greater in comparison to those receiving inadequate methionine. Whether this significant increase in the SRA of SAM suggests an increase in overall transmethylation was not determined, although total methyl tracer in creatine, PC and DNA (Table 3.8) suggests that higher dietary methionine led to more methylation of these major transmethylation products. Another indicator commonly used as a transmethylation index is the SAM:SAH ratio (Rowling et al., 2002). A lower SAM:SAH ratio suggests a higher transmethylation rate. Our results would suggest that piglets receiving inadequate dietary methionine had a higher rate of transmethylation. This is because the SAM:SAH ratio is likely dictated by the dietary supply of methionine. Therefore, it is difficult to compare the SAM:SAH ratio of piglets receiving variable amounts of dietary methionine and this indicator was in

direct contrast to the tracer incorporation data into the two most abundant transmethylated products, creatine and PC.

PC is synthesized via two major pathways: the CDP-choline pathway, also known as the Kennedy pathway and the PEMT pathway (Cole et al., 2012). PEMT activity is localized to the liver and contributes approximately 30% of total PC and is SAM-dependent (Reo et al., 2002; Sundler & Akesson, 1975). Our treatments demonstrated that during TPN, PC synthesis was approximately 400% greater in piglets receiving adequate methionine in comparison to those groups receiving inadequate dietary methionine. It is possible that this finding indicates that the synthesis of PC via PEMT relies on the supply of dietary methionine. Although the synthesis of PC was significantly upregulated for piglets receiving adequate methionine in comparison to those receiving inadequate dietary methionine, we did not observe a difference in total hepatic PC concentrations between treatments. A definitive reason for this finding cannot be explained but there are several possible explanations. Firstly, as previously explained, PC synthesis via the PEMT pathway only contributes approximately 30% of the total PC pool and the other 70% of PC synthesis via the Kennedy Pathway was not investigated (Cole et al., 2012). It is possible that the Kennedy and PEMT pathways work synergistically to establish the required amount of PC needed by neonates (Vance et al., 2007). Secondly, the piglets received TPN for 7 days which contains Intralipid (Appendix IV). Intralipid is high in PC and so these piglets may have been receiving adequate PC via TPN leading to a maximal concentration of PC in the liver, thereby masking potential effects of our treatments.

4.4 Methionine Partitioning and TPN

Methionine plays a major role in transmethylation reactions which include, but are not limited to, creatine biosynthesis, DNA methylation, and phosphatidylcholine synthesis (Bertolo & McBreairty, 2013). Quantification of partitioning of methionine among these major transmethylation reactions and to protein synthesis was a major objective in our study. Estimates of the distribution of methionine among the many transmethylation reactions vary widely in the literature with few data in neonates (Bertolo & McBreairty, 2013; Mudd & Poole, 1975; Noga & Vance, 2003). It has been previously found that variations in dietary methionine change the partitioning of available methionine (Bertolo & McBreairty, 2013). Indeed, similar to orally fed piglets, variations in parenterally fed methionine in our treatment groups changed the partitioning of available methionine. When piglets received adequate dietary methionine, a large portion (i.e. ~60%) of dietary methionine was used for PC synthesis and only ~15% for creatine synthesis. On the other hand, when piglets received inadequate dietary methionine during TPN, available methionine was equally distributed between PC and creatine synthesis, at approximately 35% each. These findings are similar to those of previous work in our laboratory. When piglets receive inadequate dietary methionine, a large portion of available methionine is used for creatine synthesis, which seems to be prioritized over PC when methionine is limiting. When excess dietary methionine is fed, a larger percentage of methionine is partitioned to PC synthesis.

Interestingly, supplementation with creatine or GAA also altered the partitioning of available methionine. In piglets fed inadequate dietary methionine, supplemental

creatine led to a lower percentage of methionine used for creatine synthesis, concomitant with an increase in methionine incorporation into protein. This was hypothesized, as providing dietary creatine was expected to decrease the need for creatine synthesis and spare methionine for other products. These findings paralleled the hepatic FSR data for creatine and protein syntheses.

On the other hand, when our piglets received supplemental GAA with adequate dietary methionine, we observed an increase in partitioning of available methionine for creatine synthesis, with almost a 2-fold higher partitioning of available methionine to creatine compared to piglets receiving adequate arginine and methionine. However, the response was not observed when comparing GAA supplementation in piglets receiving inadequate dietary methionine, where ~one-third of methionine ended up in creatine in both groups. These findings further demonstrate that GAA is the driving force of creatine synthesis during TPN, but only when adequate methionine is provided

4.5 Amino Acid Metabolism and TPN

In other studies, GAA has been shown to be as effective as creatine in sparing arginine for growth, especially when arginine is deficient in the diet (Almquist et al., 1941; Dilger et al., 2013). Whether GAA can spare arginine for growth and other metabolic processes during TPN has yet to be explored. Plasma concentrations of metabolites were analyzed and compared across treatment groups. No surprisingly, we found that piglets supplemented with inadequate arginine had lower plasma arginine and ornithine concentrations, and higher citrulline concentrations, than piglets receiving

adequate dietary arginine. The significant decrease in plasma ornithine indicates that an inadequate supply of dietary arginine impaired the urea cycle for normal function. Plasma glutamine was significantly elevated in piglets receiving inadequate dietary arginine, likely to remove excess ammonia which accumulates with arginine deficiency and a slower urea cycle (Brunton et al., 1999); glutamine is readily synthesized from ammonia and glutamate via glutamine synthase. These same trends were observed when analyzing the hepatic free amino acid concentrations. Interestingly, among the inadequate arginine groups, GAA or creatine did not change this pattern, suggesting that neither GAA nor creatine spared arginine for the urea cycle.

4.6 Future Directions and Conclusion

The varying concentrations of amino acids in commercially available TPN can be attributed to the different reference proteins used, but also to confusing evidence in the literature as to the optimal amino acids profile required for TPN-fed neonates. Therefore, it is important to understand the metabolic roles of methionine and arginine in the neonatal model, as well as the effectiveness of using primary products of these amino acids (i.e., creatine and GAA) to spare them for protein synthesis and growth. In this study, we intended to quantify the partitioning of methionine to protein and major transmethylation reactions and to determine the sparing effect of creatine and its precursor GAA on these pathways.

In the neonate, TPN causes atrophy of the gut which diminishes arginine synthesis and compromises arginine metabolism. Providing GAA, a precursor of creatine and

product of arginine metabolism, during TPN seems to relieve this stress and promote growth in the neonatal piglet model. Although the present study produced confounding results between growth and protein synthesis, there appears to be strong evidence to support the finding that GAA is the force driving creatine synthesis. We conclude that GAA supplementation during TPN can drive creatine synthesis and spare arginine for growth, but only when adequate dietary methionine is provided to ensure enough labile methyl groups are available for creatine synthesis. A potential strategy to further increase growth and ensure proper remethylation is to provide other dietary remethylation precursors during TPN in excess, such as betaine, choline or folate. This will further increase methionine availability for creatine synthesis and methionine incorporation into protein.

Higher PC synthesis was expected in our piglets receiving adequate dietary methionine, as was demonstrated by the greater hepatic FSR of PC in groups fed more methionine. To our surprise, however, hepatic concentrations of total PC did not differ between treatments. Although, because PC is synthesized via two major pathways, the PEMT and CDP-Choline pathway, future studies should measure both major PC synthesis pathways to determine if this higher PC synthesis via PEMT actually translates to more total PC for the piglet. Measuring activity of both pathways will allow us to determine if both pathways work synergistically during TPN. Using isotope kinetics approaches with a labelled choline can help determine the activity of the CDP-choline pathway.

In conclusion, TPN has provided many low birthweight infants with an increased chance of survival. It has re-innovated how gut and intestinal disease are treated. Ensuring that the optimum nutritional intake is received during these times of distress is a major objective of such research. The first step in reaching this goal is ensuring the amino acid requirements of the neonate are met and that these requirements include all products of amino acids, and not just protein. Elevated concentrations of GAA may be particularly neurotoxic in infants. These finding arises from the comparison of outcomes seen in infants suffering from AGAT deficiency and GAMT deficiency with elevated concentrations of GAA. Before moving forward in the clinical setting, GAA's possible neurotoxic effects should be established or disputed. With that being said, results from this study suggest that supplemental GAA could be considered as a novel TPN ingredient to increase the synthesis of creatine and spare arginine for growth.

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Appendices

Appendix I: D-Glucose & Major Minerals in Complete Elemental and Treatment Diets

	g/L
D-Glucose	90.3
Trihydrate K_2HPO_4	1.57
Monobasic KH_2PO_4	0.85
Potassium Acetate	1.09
NaCl	2.17
$MgSO_4$	0.78
$ZuSO_4$	0.089
Calcium Gluconate	6.41

Appendix II: Trace Mineral Concentrations in Complete Elemental and Treatment Diets

Elemental Name	Supplied Mineral Formula	Dose (mg/kg BW/day)	Amount Supplied (g/L diet)
Zinc	$ZnSO_4.7H_2O$	10.09	40.78
Copper	$CuSO_4.5H_2O$	0.86	3.12
Manganese	$MnSO_4.H_2O$	0.66	1.89
Chromium	$CrCl_3.6H_2O$	0.01	0.05
Selenium	SeO_2	0.05	0.06
Iodine	NaI	0.02	0.02

Appendix III: Commercial Pediatric IV-Multi-Vitamin Mixture

Vial 1 Vitamin

	Amount per 4 mL	Dose (per kg BW/day)
Ascorbic Acid (Vitamin C)	80 mg	17.41
Palmitate (Vitamin A)	2300 IU	500.48
Cholecalciferol (Vitamin D_3)	400 IU	87.04
Thiamine (Vitamin B_1/hydrochloride form)	1.2 mg	0.26
Riboflavin (Vitamin B_2/riboflavin-5-phosphate sodium)	1.4 mg	0.30
Pyridoxine HCl (Vitamin B_6)	1 mg	0.22
Niacinamide	17 mg	3.70
Dexapanthenol (d-panthenyl alcohol)	5 mg	1.1
dl-α-tocopherol acetate (Vitamin E)	7 IU	1.52
Vitamin K_1	0.2 mg	0.04

Inactive Ingredients: 50 mg polysorbate 80, sodium hydroxide and/or hydrochloric acid to adjust pH.

Vial 2 Vitamin

	Amount per 2 mL	Dose (per kg BW/day)
Biotin	140 μg	4.35
Folic Acid	20 μg	30.46
Cyanocobalamin (Vitamin B_{12})	1 μg	0.22

Inactive Ingredients: 75 mg mannitol, citric acid and/or sodium citrate to adjust pH.

Procedure: Vial 1 and 2 were mixed just prior to delivery to animals. To deliver vitamin mixture, a total of 3 mL was added to each 750 mL diet bag.

Appendix IV: Commercial IV Fat Emulsion (Baxter Intralipid 20%)

	Percent (%)
Soybean oil*	20
Egg yolk Phospholipids**	1.2
Glycerin	2.25

***** Consists of linoleic acid (44-62%), oleic acid (19-30%), palmitic acid (7-14%), α-linolenic acid (4-11%) and stearic acid (1.4-5.5%).

****** Consists of phosphatidylcholine and phosphatidylethanolamine.

Appendix V: Composition of the Prime and Constant Dose for Stable Isotopes

(Tracer study conducted by A. Al Jaroudi)

Stable Isotope	Prime (mg/kg)	Constant (mg/kg/h)
L-arg-$^{13}C_6$-HCl	3.64	6.07
Guanidineacetic-2,2-D_2	0.61	1.02
Creatine-D3-H_2O (Methyl D3)	1.50	1.5
L-[ring-D_5]-Phenylalanine	1.09	3.4
L-[ring D4]-tyrosine	0.51	NA
L-[ring 3, 5 D2]-tyrosine	0.50	1.65