CREATINE SYNTHESIS AND ARGININE PARTITIONING DURING TOTAL PARENTERAL NUTRITION IN YUCATAN MINIATURE PIGLETS

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Abstract

Arginine is an indispensable amino acid for the neonate. Significant quantities of both arginine and methionine are required for de novo creatine synthesis to satisfy whole body accretion requirements, even when creatine is provided in milk. Neonates that require intravenous feeding (parenteral nutrition, PN) are at risk for arginine deficiency because arginine is synthesized in the small intestine from dietary precursors; as such, arginine deficiency may limit creatine synthesis. L-arginine:glycine amidinotransferase (AGAT) is the first enzyme, and the rate limiting step, involved in creatine biosynthesis and catalyzes the conversion of arginine and glycine to guanidinoacetic acid (GAA) in the kidney. GAA is then methylated by methionine in the liver to produce creatine via guanidinoacetate N-methyltransferase. Our objective was to determine whether a parenteral diet supplemented with GAA or creatine could spare arginine and methionine for protein synthesis and growth. Piglets (6-10 d old, N = 31) were parenterally fed for 5 days one of five diets: 1) low arginine and low methionine (base), 2) base plus GAA, 3) base plus creatine, 4) high arginine and high methionine (Arg&Met), or 5) high methionine and low arginine plus GAA (Met&GAA). On day 6 of the experiment, piglets underwent a 6-hour constant infusion of stable isotopes of arginine, GAA and creatine to measure fractional conversion of arginine to GAA and creatine. In addition, continuous infusions of phenylalanine and tyrosine isotopes were used to measure whole body protein kinetics. At the end of the experiment, the piglets were killed and AGAT activity and GAA and creatine concentrations were measured in tissues. P values < 0.05 were considered statistically significant. In piglets fed the GAA and creatine diets, there was significantly lower kidney AGAT activity compared to the Arg&Met diet ($P < 0.05$), which indicates that GAA and creatine downregulated kidney AGAT and suggests that less arginine was used for GAA synthesis. Pancreatic AGAT activity was also

significantly lower in the creatine group compared to Arg&Met ($P < 0.05$). Whole body nitrogen balance did not differ significantly between groups, however, nitrogen retention was higher in the group fed GAA with excess methionine (Met&GAA), compared to GAA alone ($P < 0.05$) suggesting that methionine in the GAA group was directed towards GAA methylation rather than protein synthesis even when limited amounts of methionine were provided in the diet. This is consistent with the higher weight gain in the Met&GAA group compared to all other groups ($P < 0.05$) except the Arg&Met. A greater amount of arginine was utilized for GAA synthesis in the Arg&Met group compared to the GAA, creatine, and Met&GAA groups ($P < 0.05$), which demonstrated that providing creatine or GAA in the diet spared arginine in those groups. Overall, supplemental GAA in parenterally fed piglets seemed to be effective at sparing arginine for growth, provided that methionine was supplemented in adequate amounts. GAA may be useful as a supplement to parenteral nutrition formulas to preserve arginine for growth by facilitating creatine synthesis.

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Abbreviations

ADP	Adenosine diphosphate
AGAT	L-arginine: glycine amidinotransferase
ANOVA	Analysis of variance
Arg	Arginine
APE	Atom percent excess
ASS	Argininosuccinate synthase
ASL	Argininosuccinate lyase
ATP	Adenosine triphosphate
BW	Body weight
CDP-choline	Cytidine-5′-diphosphocholine
CPS-I	Carbamoylphosphate synthetase-I
DNA	Deoxyribonucleic acid
E	Enrichment
EDTA	Ethylenediaminetetraacetic acid
E_i	Enrichment in the infusate
E_p	Enrichment in plasma
GAA	Guanidinoacetic acid
GAMT	Guanidinoacetate methyltransferase
GNMT	Glycine N-methyltransferase
HPLC	High performance liquid chromatography
I	Isotope infusion rate
IG	Intragastric
IV	Intravenous

MAT	Methionine adenosyltransferase
Met	Methionine
NaF	Sodium fluoride
OAT	Ornithine aminotransferase
OD	Optical density
P-5-C synthase	L -Δ^1 -pyrroline-5-carboxylate synthase
PC	Phosphatidylcholine
PEMT	Phosphatidylethanolamine methyltransferase
Phe	Phenylalanine
PN	Parenteral nutrition
Q	Flux
R_b	Tracer:tracee in natural abundance
R_s	Tracer:tracee in enriched sample
SAH	S-adenosylhomocysteine
SAM	S-adenosylmethioninine
TPN	Total parenteral nutrition
Tyr	Tyrosine

Chapter 1: Literature Review

1.1 Creatine

Creatine has long been known to be an ergogenic sport supplement in high performance and intensity sports, such as football, weight lifting, and rugby (reviewed by Brosnan & Brosnan, 2007). Creatine is a metabolic product of three amino acids, arginine, glycine, and methionine. Creatine can be either obtained from the diet (e.g. from meat, fish, or dairy) or by de novo synthesis from precursor amino acids. The interest in creatine has grown in recent years when it was discovered that it is involved in many diseases of the brain and muscle. Tissues that are major consumers of creatine are skeletal muscles, the heart, the spermatozoa, and the retina of the eye. The brain, brown adipose tissue, seminal vesicles, macrophages, intestines, and endothelial cells also utilize creatine but in intermediate amounts compared to the other tissues. Tissues that utilize very little amounts of creatine include the kidneys, spleen, liver, white adipose tissue, lungs, and red blood cells. Both creatine and creatine phosphate are slowly converted non-enzymatically to creatinine and excreted (reviewed by Wyss & Kaddurah-Daouk, 2000). According to Wyss and Kaddurah-Daouk (2000), it was estimated that a 70 kg male would have 120 g of total body creatine plus creatine phosphate. It was also estimated that ~1.7% of the total creatine pool is lost daily as creatinine in the urine; therefore, this amounts to approximately 2 g of the total creatine pool that needs to be replenished each day. The consumption of "a typical western diet" (da Silva et al., 2009) provides ~50% of the body's total creatine need for an adult. However, vegetarians and vegans acquire little to no creatine from their diet and so the vast majority of it has to be synthesized entirely from dietary precursors (da Silva et al., 2009; Brosnan & Brosnan, 2007).

1.1.1 Creatine/ phosphocreatine as an energy buffer

Creatine and creatine phosphate act as an energy buffer, maintaining constant levels of ATP and ADP during high energy demand.

Creatine + ATP $\longrightarrow$ **Phosphocreatine + ADP**

This reaction is catalyzed by the enzyme creatine kinase (CK) (reviewed by Bessman & Carpenter, 1985). There are different isoenzymes of CK in the body, each of which are unique to specific tissues. The first CK isoenzyme is found in brain cells (B-CK), the second isoenzyme is expressed mostly in muscle cells (M-CK), and the third isoenzyme is specific only to the mitochondria (Mi-CK) (Wallimann et al., 1992). That there are various types of CK only emphasizes the importance of creatine in energy metabolism in the cell (Brosnan & Brosnan, 2007). The CK-phosphocreatine system maintains energy in cells by preventing the uncontrollable increases of ADP concentrations, thus limiting the unnecessary loss of adenosine. Also, it maintains the availability of free protons needed for the regeneration of ATP. As a consequence, the CK-phosphocreatine system maintains constant energy levels during periods of high or fluctuating energy demand in various types of cells (Wallimann et al., 1992).

1.1.2 Creatine as an energy shuttle

Both creatine and phosphocreatine act as an energy shuttle, mobilizing high energy phosphates among different sites in the cell. These sites include the cytosol where energy is utilized and the mitochondria where energy is produced. It was proposed that what actually diffuses back and forth between these two sites is creatine and phosphocreatine, as opposed to ATP and ADP. This is likely due to the fact that ADP and ATP are heavier than creatine and phosphocreatine, and therefore, their mobilization might be more costly to the cell (Wallimann et al., 1992). During physical activity, creatine is released from the muscle fiber where it diffuses to

the mitochondria. After that, ATP is converted to ADP and phosphocreatine is produced where it is readily available to the cell (Bessman & Carpenter, 1985).

1.2 Creatine biosynthesis

Creatine synthesis is a simple process that occurs in two steps (Figure 1.1). In the first step of this reaction, an amidino group is transferred from arginine to glycine via the enzyme L-arginine:glycine amidinotransferase (AGAT) to produce guanidinoacetic acid (GAA) and ornithine in the kidney. GAA is then released into the circulation where it is taken up by the liver. In the second step of the reaction, GAA is methylated by S-adenosyl methionine (SAM) via the enzyme guanidinoacetate N-methyltransferase (GAMT) to produce creatine and S-adenosylhomocysteine (SAH). Creatine is then released by the liver and distributed to tissues that utilize creatine (Wyss & Kaddurah-Daouk, 2000).

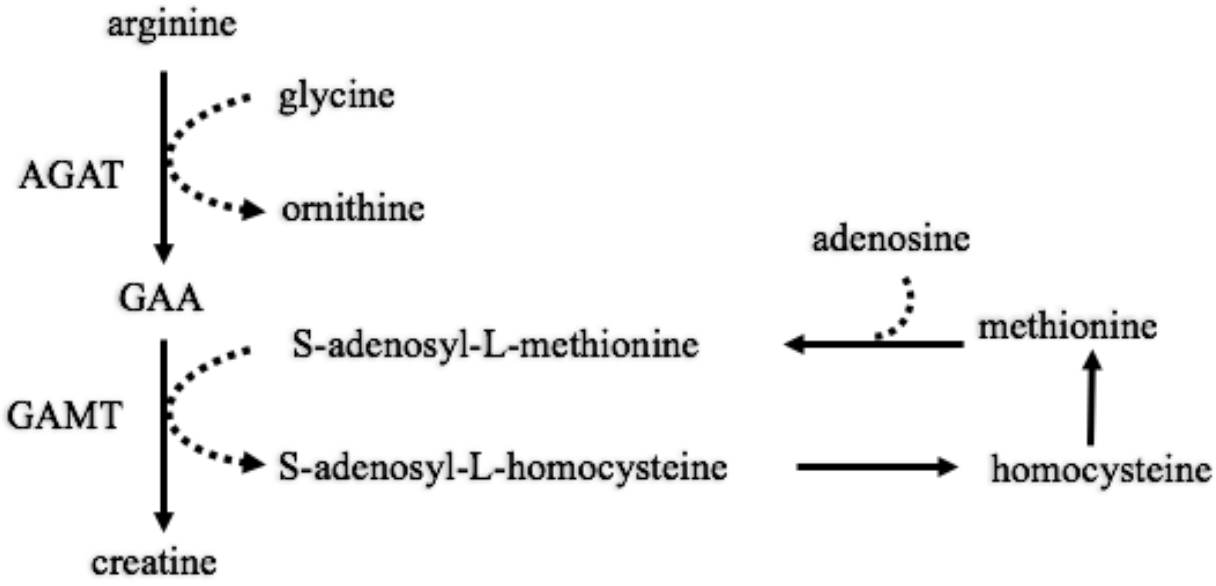

Figure 1.1 Creatine biosynthesis pathway

L- arginine:glycine amidinotransferase (AGAT) catalyzes the reaction that transfers an amidino group from arginine to glycine to produce guanidinoacetic acid (GAA) and ornithine. GAA is then methylated by S-adenosyl-L-methionine (SAM) in the liver via guanidinoacetate N-methyltransferase (GAMT) to produce creatine and S-adenosyl-L-homocysteine (SAH).

1.2.1 AGAT and the role of the kidney and pancreas in GAA synthesis

In vertebrates, the highest expression of AGAT has been reported in the kidney, pancreas, and brain (da Silva et al., 2014). The kidney is suggested to be the most important contributor of GAA production, which is directed towards whole body creatine synthesis (Brosnan & Brosnan, 2007). Nephrectomized rats displayed major reductions in GAA and creatine synthesis when compared to healthy rats, suggesting that the kidney is a key element in total body creatine synthesis (Levillain et al., 1995). The expression of renal AGAT is upregulated by growth and thyroxine hormones, and downregulated by creatine supplementation (Humm et al., 1997).

Edison et al. (2007) examined GAA synthesis in the adult rat and human kidney. In the adult rat kidney, arginine was synthesized in the proximal convoluted tubule from citrulline, and then appeared to undergo a direct conversion to GAA. In that study, the kidney was the main contributor of GAA to whole body creatine synthesis; however, the kidney was directly affected by the dietary intake of creatine, which resulted in a reduction of the synthesis of GAA. Studies in humans have not been as clear. Edison et al. (2007) measured renal blood flow in healthy adults after the consumption of a normal meal (50% carbohydrates, 35% fat, and 15% protein). Results suggested that the human kidney may contribute only 20% of the total GAA needed for creatine synthesis, and that other organs may play a greater role in the synthesis of creatine such as the liver. The mechanism by which AGAT is regulated in other mammals clearly differs by species. In the neonatal piglet, however, AGAT activity was only detected in the kidney and pancreas (Brosnan et al., 2009). Although the pancreas displays 5-fold greater specific activity of AGAT compared to the kidney (Brosnan et al., 2009), the quantitative importance of the pancreas AGAT is not known, considering its small size relative to the kidney. A study by da Silva et al., (2014) measured AGAT activity in the pancreas of rats fed supplemental creatine

and found that creatine supplementation lowered AGAT activity in both the pancreas and the kidney. Although there was a downregulation of AGAT activity in the pancreas, they found elevated GAA concentrations in that tissue when compared to controls. The researchers proposed that high plasma creatine concentrations might have a negative feedback mechanism on the GAA transporter, hence the accumulation of GAA in the tissue. However, unlike the kidney, the pancreas mRNA and protein expression of AGAT was not reduced by creatine supplementation, which suggests that the mechanism by which pancreatic AGAT is regulated is different than in the kidney. Further evidence is required to understand the role of this organ in total body creatine synthesis.

1.2.2 GAMT and the role of the liver in creatine synthesis

GAMT is the second enzyme in the creatine synthesis pathway, which catalyzes the methylation of GAA via S-adenosylmethionine (SAM) to produce creatine and S-adenosylhomocysteine (SAH) (Brosnan et al., 2009). The liver is known to be the major producer of creatine in the body (Wyss & Kaddurah-Daouk, 2000). It is also the main site for the synthesis of SAM which is the major methyl donor in the body (reviewed by Bertolo & McBreairty, 2013). SAM is synthesized by the addition of an adenosine molecule to the amino acid methionine, which then methylates GAA to produce creatine and SAH. Unlike AGAT, creatine supplementation does not affect GAMT activity in piglets (Dinesh et al., 2018) or rats (da Silva et al., 2009). Some research has suggested that human livers might actually synthesize creatine entirely in the liver without relying on the kidney for GAA synthesis. In humans, it was found that AGAT mRNA is highly expressed in the liver. The fact that the urea cycle occurs in the liver may be suggestive that both the AGAT pathway and urea cycle occur hand in hand. Hence, the arginine in the liver has two outcomes: either it is converted to ornithine via arginase

I or converted to GAA and ornithine via AGAT. This process has been coined "the arginine bicycle" by researchers (reviewed by Brosnan & Brosnan, 2004). Evidence from rat studies has also suggested creatine is entirely synthesized in the liver, as immunofluorescent microscopy displayed very high levels of hepatic AGAT in the rat that were comparable with the kidney (McGuire et al., 1986). In contrast, a study by da Silva et al. (2009) tested this hypothesis in isolated rat hepatocytes, and found that there was no synthesis of creatine from its three amino acid precursors. They also found decreased uptake of GAA by the rat liver fed a creatine-supplemented diet compared to control, as well as a decreased renal AGAT activity. These findings suggest that creatine synthesis in the rat is regulated at the renal level. In the piglet, evidence also suggests an interorgan synthesis that involves both the kidney and the liver. The highest GAMT activities were reported in the liver. Moderate to low amounts were detected in the pancreas, kidney, muscles, intestines, and brain (Brosnan et al., 2009). These findings suggest that the liver is the major contributor of creatine synthesis from renal GAA in the piglet. It is clear that creatine synthesis is an interorgan process that occurs via a renal-hepatic axis.

1.3 Methionine requirement in neonates

Methionine is an essential amino acid with many critical roles other than protein synthesis. Methionine serves as a methyl donor in the synthesis of phosphatidylcholine (PC), which is an important component in the structure of biological membranes. Methionine is also involved in the methylation of DNA, in addition to creatine synthesis. Methionine as a methyl donor acts as a precursor for over 200 transmethylation reactions through SAM (Petrossian & Clarke, 2011). Methionine is also the amino acid precursor for cysteine, which is synthesized from homocysteine via transsulfuration (Bertolo & McBreairty, 2013). The amount of cysteine in the diet will alter the dietary methionine requirement. Piglets fed excess dietary cysteine required

less methionine in parenteral and enteral feeding trials (Shoveller et al., 2003b) compared to when fed cysteine-free diets (Shoveller et al., 2003a). Methionine is spared by cysteine, when less is directed towards the transsulfuration pathway (McBreairty et al., 2015). Therefore, more methionine would be available for protein synthesis and other methylation reactions. Methionine can be regenerated in the body through remethylation (via betaine from choline) or through methylneogenesis (via folate) (Bertolo & McBreairty, 2013). Therefore, the status of methionine is highly affected by the availability of the substrates involved in these two pathways.

1.3.1 Transmethylation

The production of SAM requires the adenosylation of methionine via the enzyme methionine adenosyltransferase (MAT) (Bertolo & McBreairty, 2013). The importance of SAM lies in the fact that it is the direct precursor for over 50 different methylation reactions. Hence, methionine deficiency may compromise a wide variety of important methylation reactions. The three most important SAM-dependent methyltransferases (quantitatively) in the body are GAMT, phosphatidylethanolamine methyltransferase (PEMT), and glycine N-methyltransferase (GNMT). PEMT pathway is responsible for approximately one-third of the total endogenous PC synthesis via SAM; the rest of PC is synthesized via the cytidine-5′-diphosphocholine (CDP-choline) pathway. GNMT is involved in the regulation of SAM:SAH ratio which can be used as an indicator for the state of the transmethylation potential, where a lower ratio can lead to impairment of transmethylation reactions. It is also involved in the removal of excess methionine. Both GAMT and PEMT pathways consume the majority of the SAM-derived methyl groups (Williams & Schalinske, 2007).

1.3.2 Remethylation

Once homocysteine is produced via transmethylation, it is either irreversibly converted to cysteine via transsulfuration or regenerated to methionine. The remethylation pathway represents the conversion of homocysteine to methionine via two different routes. In the first route, a methyl group is removed from folate and transferred to homocysteine via the enzyme methionine synthase with cyanocobalamin acting as a coenzyme, to regenerate methionine. The second route of remethylation occurs through the transfer of a methyl group from betaine through betaine:homocysteine methyltransferase to ultimately form methionine. It has been found that methyl donors (choline, betaine, and folate) affect the remethylation rate which is very important during early development due to growth and rapidly expanding tissue pools (Bertolo & McBreairty, 2013). A study by Robinson et al. (2016) removed methyl donors from the diets of enterally-fed neonatal piglets, and reported lower whole body protein synthesis compared to piglets fed a diet sufficient in methyl donors. Therefore, choline, betaine, and folate are important contributors to maintaining the methionine cycle, and deficiencies in these three nutrients may compromise the availability of methionine or increase its requirement.

1.4 Creatine in the neonate

Creatine metabolism in the human neonate has not been studied extensively. However, there are multiple animal studies in which the importance and implications of this metabolite were examined. A study by Ellery et al. (2012) examined the supplementation of creatine in the diet of pregnant spiny mice in a model of asphyxia. Birth asphyxia can cause multiple complications in the neonate and is associated with a high mortality rate. Before birth, asphyxia was induced in the pregnant mice, and then the kidney of the mice pups was examined for signs of acute kidney injury. The creatine-supplemented group showed decreased expression of an

injury marker and normal kidney morphology compared to controls. These data suggest that creatine may have a protective effect in preventing some of the complications of birth asphyxia.

In human infants, it was estimated that nearly 90% of the total body creatine acquired during growth is through de novo synthesis, and that breast milk marginally contributes to total body creatine pools (Edison et al., 2013). Data from piglets seem to support a similar trend. Brosnan and colleagues (2009) estimated that the sow-fed piglet acquires approximately 25% of creatine from sow milk, and 75% has be synthesized de novo. This estimation was calculated based on differences in tissue creatine concentrations at 4 versus 11 days of age in sow-fed piglets, and demonstrates the high need for creatine in growing animals undergoing rapid tissue expansion. It is clear that creatine synthesis is a major metabolic process in the growing neonate that might place significant burden on the precursor amino acids, but this has not been empirically determined.

1.4.1 Creatine deficiency syndrome

The importance of creatine in the neonate was not clearly identified until the characterization of several creatine deficiency syndromes. The deficiencies arise due to mutations in one of the two enzymes involved in the creatine synthesis pathway or in the creatine transporter (reviewed by Leuzzi, 2002). Each of these deficiencies can lead to the depletion of creatine stores in the body. GAMT deficiency is characterized by intellectual disability and severe epilepsy (Leuzzi, 2002). This is likely due to the high concentrations of GAA that accumulate in the brain and surrounding tissues. Treatment with a very high dose of creatine has been successful in reducing the epileptic seizure and other neurological developmental problems, in addition to replenishing creatine stores (Leuzzi, 2002). However, prevention of neurological consequences was only successful when intervention was administered early in life and before

the onset of symptoms (Leuzzi, 2002). AGAT deficiency results in no net synthesis of GAA in the body, hence, no creatine synthesis. AGAT deficiency shares some characteristics with GAMT deficiency, but AGAT deficiency has a much broader range of developmental and neurological symptoms. Similar to GAMT deficiency, pre-symptomatic intervention with creatine seemed to be successful. Of all of the inborn errors, creatine transporter deficiency is the most severe and the most difficult to treat. These patients have normal GAA blood profiles but abnormally high creatine concentrations. The lack of the creatine transporter means that creatine cannot be moved between cells (Leuzzi, 2002). It is not known why creatine is essential for the normal function of the brain. Nonetheless, it has been hypothesized that creatine might act as a neuromodulator or have "neuroprotective effects" (Brosnan & Brosnan, 2007). Some of these protective effects of creatine might include the stimulation of the nerve cells to uptake glutamate near the synapse, as well as the maintenance of calcium homeostasis in the brain (Ferrante et al., 2000). Rodent studies have demonstrated that under normal conditions, the creatine transporter SLC6A8 is expressed in the endothelial microcapillary cells of the blood brain barrier (BBB); however, there is no expression of the transporter in the astrocytes associated with the BBB (Reviewed by El‐Daher & Braissant, 2016). This suggests that there is little access to peripherally-supplied creatine by the brain, and supports the idea that the brain relies on its own synthesis of creatine. Both AGAT and GAMT expression have been demonstrated in various structures in the brain, but not within the same cell type. It may be that GAA is transported within the brain from AGAT-expressing to GAMT-expressing cells, to complete the synthesis of creatine. In rodents, this process relied on the SLC6A8 creatine transporter for the movement of GAA (El‐Daher & Braissant, 2016). If this is the case in humans, it would partially explain the severe pathology that occurs with creatine transporter deficiency, despite normal activities of

AGAT and GAMT. (Reviewed by El‐Daher & Braissant, 2016). Most of this work has been conducted in mature rodent models. In one study of neonatal piglets however, no AGAT activity was detected in the brain (Brosnan et al., 2009), which may be a developmental or a species difference in the reliance on the periphery for the supply of creatine for the brain. It is clearly evident that creatine is essential in the normal development of the neonate.

1.5 Arginine synthesis in the neonate

Arginine is considered an essential amino acid in neonates, and conditionally essential in adults during situations of stress, such as surgery (Brunton et al., 1999; Yu et al., 2001). It is involved not only in protein synthesis but in other non-protein roles as well. For example, arginine is a substrate for the synthesis of nitric oxide, which acts as a vasodilator and immune modulator. It is also necessary for the detoxification of ammonia through the urea cycle. Arginine is also involved in the synthesis of polyamines, which are important components in cellular expression during development and in cell protection during oxidative stress (reviewed by Flynn et al., 2002). It was speculated that both human and sow milk are deficient in arginine; therefore, the neonate must synthesize the majority of its requirement (Davis et al., 1994; Brunton et al., 1999). For example, the suckling piglet must synthesize approximately 60% of the whole body arginine requirement, with only 40% provided by the sow's milk (Wu & Knabe, 1994). Flynn et al., (2000) compared plasma arginine concentration of sow-fed piglets ages 1-3 and 7-21 days old. They found that plasma arginine concentrations decreased dramatically between the ages of 3 to 14 days old. There was also a significant increase of plasma ammonia in the 7- and 14-day-old piglets compared to the 1-3-day-old piglets. This might suggest that sow milk is deficient in arginine and that the piglet cannot synthesize sufficient amounts to meet its own requirement. This view was further supported in a later study by Kim et al. (2004) who

measured the effects of supplementing arginine to milk-fed piglets, and found that the piglets' growth was significantly increased compared to their non-supplemented controls.

In neonates, de novo arginine synthesis occurs in the enterocytes of the small intestine. However, once the animal transitions from the neonatal stage towards weaning, the synthesis of arginine shifts to involve both the small intestine and the kidneys (Wu & Knabe, 1995). In older animals, the main route of de novo arginine synthesis is through L -Δ^1 -pyrroline-5-carboxylate synthase (P-5-C synthase) from glutamate which results in the synthesis of P-5-C. P-5-C is then converted to ornithine and subsequently citrulline which is released into the circulation and used to synthesize arginine in the kidney. In neonates, however, the activity of P-5-C synthase is low and located almost exclusively in the small intestines and thymus. Hence, there is negligible synthesis of citrulline or arginine from glutamate in the neonatal gut (reviewed by Bertolo & Burrin, 2008). In contrast, proline is the primary precursor for arginine synthesis in intestinal enterocytes (Bertolo et al., 2003). It is interesting to note that human and porcine milk have relatively low concentrations of preformed arginine, but quite high concentrations of proline relative to the needs of the neonate (Brunton et al., 1999).

1.5.1 Arginine synthesis in the neonate and total parenteral nutrition

Total parenteral nutrition (TPN) is used widely in the clinical care of newborns, and particularly preterm infants. TPN involves the infusion of nutrients directly into the systemic circulation, by-passing first pass intestinal metabolism. The amino acid profile of the early parenteral nutrition (PN) solutions used the composition of human milk as a template. However, it became apparent quickly that the arginine concentration was too low to meet the metabolic needs of infants. TPN-fed neonates showed symptoms of arginine deficiency as evidenced by high plasma ammonia concentrations (Batshaw et al., 1984; Thomas et al., 1982). In most cases,

the hyperammonemia was reversed by the IV administration of arginine (Batshaw et al., 1984). Neonatal piglets also showed a similar response to limited arginine in the diet. A study by Brunton et al. (1999) provided neonatal piglets with arginine-free TPN, which resulted in an extremely rapid rise of plasma ammonia concentrations over hours, despite adequate proline in the PN solution. Piglets that were enterally-fed the same formulation did not develop hyperammonemia unless proline was also deleted; enteral diets devoid of arginine and proline exhibited a rapid rise in plasma ammonia, demonstrating the importance of first-pass intestinal metabolism of proline in maintaining arginine synthesis. The study also demonstrated that glutamate, which was included in the formulations, was not an adequate precursor for arginine synthesis when both arginine and proline were absent from the diet. The researchers concluded that the neonatal piglet cannot synthesize adequate arginine and proline to meet whole body requirements; therefore, both of these amino acids should be treated as co-indispensable. A subsequent study by Bertolo et al. (2003) found that almost 50% of arginine synthesis from proline occurred in the small intestine in 1-3-day-old intragastrically fed piglets; therefore, both arginine and proline are also considered co-indispensable in neonates. They also determined that both arginine and proline become essential in situations where the gut is compromised, such as during TPN feeding or intestinal disease. As a consequence, the requirement for arginine would be much higher when first-pass metabolism is bypassed (Bertolo et al., 2003). In human neonates, there is also evidence to support the use of proline as the main precursor for arginine synthesis in the small intestine. Tomlinson et al. (2011) quantified arginine synthesis in healthy preterm neonates fed breast milk or formula using multiple stable isotopes. They found that the majority of the arginine was synthesized from proline, and there was no net synthesis of arginine

from glutamate, confirming the findings in neonatal piglets and validating the piglet as a model for the infant with respect to arginine metabolism.

During TPN, the gastrointestinal tract is bypassed, therefore lacking stimulation and inhibiting its natural function (Dinesh et al., 2014). As a consequence, the small intestine is more susceptible to inflammation and atrophy (Brunton et al., 2012; Dinesh et al., 2014). A number of studies have demonstrated an association between arginine status and the risk for intestinal complications following premature birth. In particular, low plasma arginine in premature neonates receiving TPN is associated with necrotizing enterocolitis (NEC) (Becker et al., 2000). NEC is a devastating condition caused by an infection of the intestine, characterized by inflammation, perforation, bleeding, and necrosis of a part or all of the gastrointestinal tract, and is associated with a high mortality rate (Azcarate-Peril et al., 2011; Llanos et al., 2002). The association between arginine status and the risk of developing NEC is complex, but may be influenced by the link between arginine and overall gut health. Studies using TPN-fed piglets have clearly demonstrated gut atrophy which may be a cause or consequence of diminished arginine synthesis. A study by Dinesh and colleagues (2014) reported that TPN-fed neonatal piglets demonstrated gut atrophy that was partially avoided with the enteral supplementation of arginine alone. The IV supplementation of arginine, however, did not improve the morphology of the small intestine. Furthermore, Urschel et al. (2007) found that greater arginine synthesis occurred in TPN-fed piglets that were treated with hormone GLP-2 compared to a saline control. The authors attributed the arginine synthesis to enhanced small intestinal morphology induced by GLP-2; however, it was not sufficient enough to improve the whole body status of arginine. This emphasizes the importance of arginine in maintaining the structure and function of the gut.

It is clear that the use of TPN will compromise the synthesis of arginine in the gut, initially by the loss of first pass metabolism, and then further by intestinal atrophy and the reduction in metabolic mass of the gut. Poor arginine status may lead to devastating outcomes such as NEC, but subtle deficiencies in arginine could contribute to slower growth and may impact de novo creatine synthesis. An important question is how arginine is partitioned between protein synthesis and growth and other fates such as creatine synthesis, particularly when its availability is limited by intestinal injury or parenteral feeding.

1.6 Creatine synthesis and precursor amino acids

1.6.1 Arginine

The idea that creatine synthesis placed a burden on whole body arginine availability was initially proposed by Visek (1986). He argued that in adults, the equivalent of 70% of the dietary arginine intake is directed towards creatine synthesis, and that dietary intake alone is not satisfactory to meet the body's requirement for other metabolic functions of arginine. This view was also put forward earlier by another researcher, Walser in 1983. Walser proposed that adults consuming a typical Western diet do not meet the requirement for arginine. However, it is now known that arginine is not an essential amino acid in healthy adults (Dioguardi, 2011). The previous two studies gave rise to the question whether young animals can synthesize enough arginine to meet their needs. The requirement of arginine for younger people would be even higher due to rapid growth and expanding tissues (Visek, 1986). Furthermore, this also raises the issue that dietary arginine might not be sufficient to support maximal growth in rapidly growing neonates.

In neonatal piglets, it was estimated that the arginine required for creatine synthesis is equivalent to approximately 20% of the dietary intake of arginine (Brosnan et al., 2009). It is

important to note that creatine synthesis generates ornithine, which could be used to regenerate arginine in the urea cycle. But it is not known whether the ornithine produced by AGAT in the kidney is converted back to arginine. Ornithine has the potential to be oxidized via ornithine aminotransferase (OAT) or converted to citrulline via carbamoyl phosphate synthetase-I (CPS-I) and then to arginine. However, the distribution of CPS-I enzyme is limited to the intestines and liver, and OAT has been found to be highly expressed in the kidney. Therefore, it is more likely that ornithine produced via renal AGAT is fully catabolized (reviewed by Brosnan et al., 2011). In situations where the small intestine is compromised, the risk of arginine deficiency increases, thus placing a greater burden on a dietary source of arginine. Therefore, in these situations, the status of arginine may be limiting growth and ultimately, creatine synthesis.

1.6.2 Methionine

SAM, as the major methyl donor, requires a constant supply of labile methyl groups through two routes: through the availability of labile methyl groups from dietary methionine, betaine, or choline or through a process called methylneogenesis (Brosnan et al., 2011). The latter route is dependent on folate; therefore, a deficiency of folate might compromise remethylation back to methionine (Bertolo & McBreairty, 2013). Clearly, the demand for methylation reactions is greatest in growing animals. Brosnan et al. (2009) have estimated that in growing sow-fed neonatal piglets, the equivalent of 35% of the dietary intake of methionine is utilized in creatine synthesis. Additionally, it was estimated that up to 77% of all of the labile methyl groups used in methylation reactions by the piglet are directed towards creatine synthesis. As such, creatine synthesis has the potential to be a burden on the availability of methionine, and in turn limit other important metabolic functions of methionine such as protein synthesis.

1.7 The concept of GAA and creatine sparing effects in animals

The concept of amino acid sparing by the supplementation of GAA or creatine to the diet for growth was proposed very early in the literature. Fisher et al. (1956) compared three different diets to measure which one would result in greater growth in chicks. They supplemented a 25% casein diet with arginine, methionine, and glycine and compared it with a regular chick diet and a creatine-supplemented diet. They found that there was greater growth in chicks supplemented with arginine and glycine alone compared to the control diet, and that methionine had no effect on growth. However, creatine supplementation appeared to support an increased growth response when compared to control diet only. Furthermore, Austic & Nesheim (1972) measured arginine needs in chicks and found that most of the commercially available casein diets were deficient in arginine, and negatively affected growth. However, when the diets were supplemented with creatine, the chicks showed a positive growth response in spite of insufficient arginine. These two studies confirmed the importance of arginine for growth as well as the sparing effects of creatine on arginine availability.

There have been few studies in pigs that investigated the sparing effects of creatine. One study by Guzik et al. (2000) tested the effects of supplementing creatine to newly weaned pigs. They found that creatine supplementation did not affect growth or tissue creatine concentration regardless of the amount of creatine added to the diet. The lack of response may be attributed to the inclusion of adequate amino acids in the diet to support optimal growth and creatine synthesis; thus there was no sparing benefit to additional creatine. Moreover, O'Quinn and colleagues (2000) measured the effects of creatine supplementation in growing finishing pigs. They found that creatine supplementation for 10 days did not affect the growth of these pigs.

Again, this might be attributed to the fact that their diets were not marginal in the precursor amino acids of creatine and consequently, no positive effect of creatine was observed.

GAA has also been tested as a replacement for arginine in the agricultural industry. A recent study by Dilger et al. (2013) examined the supplementation of GAA to broiler chicks. GAA added to a diet marginal in arginine resulted in greater weight gain and growth than when GAA was supplemented to a diet adequate in arginine. This study was in agreement with a similar study by Almquist et al. (1941) where they tested the dietary supplementation of GAA in a typical chick diet. They found that GAA supplementation led to greater liver and muscle creatine concentrations compared to supplementing creatine alone. The sparing effects of GAA was also evaluated in swine. A recent study by McBreairty et al. (2015) found that supplementing GAA to a regular grower diet for weaned pigs increased tissue creatine concentrations compared to a diet supplemented with creatine, but with no growth effect. Nonetheless, it should be noted that excess amounts of GAA in the diet might actually lead to a depletion of methyl group supply, as more methionine is required to methylate GAA via hepatic GAMT (McBreairty et al., 2015). Methionine balance should always be taken into consideration when supplementing GAA to ensure that other methylation reactions are not compromised. The long term effects of supplementing GAA in growing animals have not been investigated; however, studies in humans have shown that supplementing GAA in healthy men for a period of 8 weeks resulted in increased brain creatine concentrations with no neurological side effects (Ostojic et al., 2017). GAA is more stable than creatine in feed additives with much lower costs (Michiels et al., 2012), however, GAA was found to be slightly less soluble than creatine in aqueous solutions (Vraneš et al., 2017). As the popularity of individual nutrient supplements grows, it remains an important question to understand how changes in one nutrient or metabolite,

such as GAA or creatine, might affect the use or requirement of others, particularly in rapidly growing neonates.

1.8 Yucatan miniature piglets as a model for the human neonate

Premature infants, depending on their gestational age at birth, are often born with immature organs and physiological functions, therefore complicating the treatment approaches that ensure the neonate's survival. Because this vulnerable population of patients vary in birth conditions, there was a need to establish a neonatal animal model to investigate various interventions (Wykes et al., 1993). This animal model would allow researchers to understand how to efficiently support normal growth and development in this group while employing invasive procedures. As such, the neonatal piglet has become a well-established model for the human neonate (Wykes et al., 1993; Shulman, 1993). Neonatal piglets are considered an accelerated growth model for humans. Piglets grow more rapidly than humans, which enables researchers to detect physiological changes within shorter time periods, when different treatments are administered (Shulman, 1993). Both the neonatal piglet and human neonate show similar characteristics in terms of development, physiology, anatomy, and essential amino acid requirements (Wykes et al., 1993). For example, intestinal function and response to environmental stressors is very similar to the human infant, in addition to similar cardiovascular physiology (Shulman, 1993; Azcarate-Peril et al., 2011). Moreover, advantages of using miniature piglets as a model includes their smaller size, easy housing, as well as their docile temperament and acceptance of handling (Panepinto et al., 1978; Shulman, 1993). Both arginine (Brunton et al., 2003) and methionine (Shoveller et al., 2003a; Shoveller et al., 2003b) requirements have been studied in neonatal piglets. Hence, the neonatal piglet represents an

excellent comparative model for studying the metabolic pathways of both of these amino acids in the human neonate.

1.8.1 Neonatal TPN piglet model

The neonatal TPN piglet model was first established and validated by Wykes and colleagues in 1993. They compared 1-3-day-old TPN-fed piglets to sow-fed piglets. The formulation of TPN was based on the compositions of sow milk, published requirements (National Research Council, 1988), and knowledge of piglet gastrointestinal metabolism. The results of their study showed that the TPN-fed piglets had comparable growth rates to the sow-fed group, and similar blood biochemistry profiles. Furthermore, the piglets were able to efficiently use the amino acids in the TPN formula as evidenced by a positive nitrogen balance. Additionally, this model is also considered clinically relevant, because the TPN formula was comparable to that used in clinical settings for infants. Therefore, the TPN-fed piglet model is an ideal model to demonstrate the effects of TPN manipulation, while ensuring the survival of the animal for the duration of the study.

1.9 Whole body stable isotope kinetics

Isotopes, whether stable or radioactive, have been used to measure the metabolic fate of different nutrients in the body. The use of radioisotopes is not favoured by researchers because in some cases, it is not considered ethical for use in human studies (Thompson et al., 1989). In animal studies, the use of both stable and radioisotopes is very popular with favour towards the latter due to lower analytical costs; however, in recent years, the use of mass spectrometry in research has grown exponentially, resulting in lower analytical costs. The advantages to stable isotopes are that they are generally safer to use, and also permit multiple investigations in one

animal. The use of isotopically labeled amino acids also allows for the quantification of the rate conversion of amino acids to other products in an *in vivo* state.

To measure the rate of conversion of amino acids, the flux of the amino acid and its products have to be measured through a representative body pool, most commonly plasma. We assume that the amino acid pool is well mixed (Welle, 1999; Wolfe, 1984). We also assume that the body "does not discriminate" (Clarke & Bier, 1982) between the tracer infused and the amino acid of interest; the fate of both the tracer and tracee has to be the same. Additionally, we assume that the tracer infused in the body pool does not re-enter the pool (Vella & Rizza, 2009). According to Figure 1.2, in a single amino acid pool model, at Time 0 the amount of amino acids entering the pool should be equal to the amount leaving the pool when the body is in equilibrium. Once the labeled amino acid appears intravenously at Time 1 (either from enteral or parenteral administration), the amount of label appearing in the pool is greater than the amount leaving the pool. After some time with continuous infusion of the labeled amino acid at Time 2, the amount of the label entering the pool would equal the amount of the label leaving the pool. Thus, we can say that the label has reached isotopic equilibrium or plateau; the ratio of tracer to tracee in the plasma is constant (Welle, 1999; Wolfe, 1984).

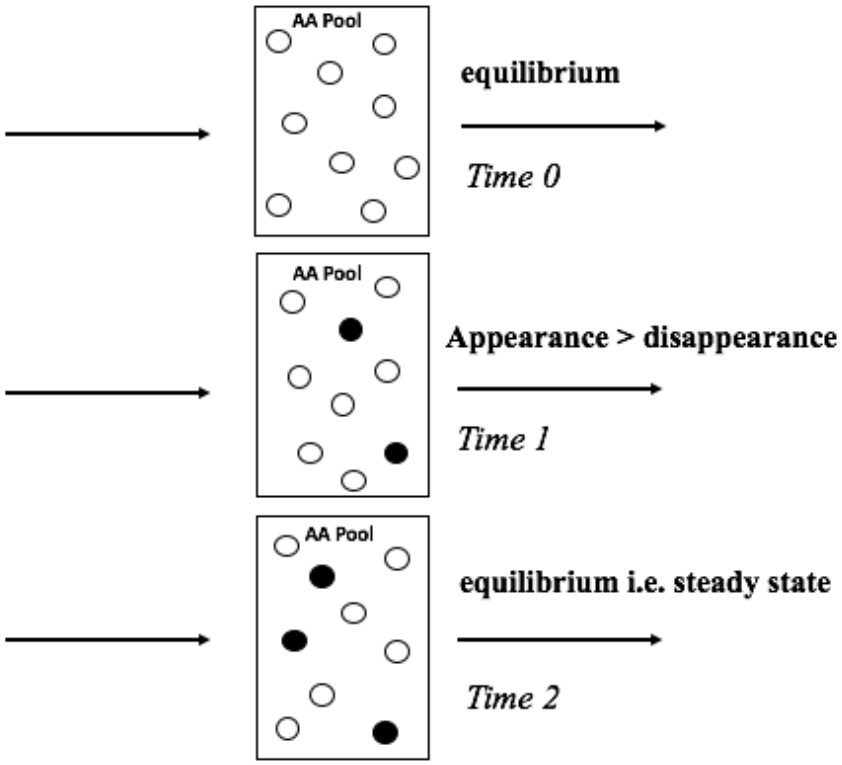

Figure 1.2 Single amino acid pool. (Inspired by Wolfe, 1984)

Isotopically labeled amino acids can also be used to measure protein dynamics in the body. The use of phenylalanine to measure its kinetics and protein turnover was not popular until the late 1980's (reviewed by Matthews, 2007). As an essential amino acid, phenylalanine can only be obtained from the diet or from protein breakdown. Phenylalanine has two fates in the body. It is either hydroxylated to tyrosine as the first step towards disposal of excess phenylalanine, or it is incorporated into proteins as phenylalanine or tyrosine; however, it cannot be stored (Wolfe, 1984; Welle, 1999). The greater the rate of oxidation of phenylalanine (as determined by hydroxylation into tyrosine), the lower the rate of incorporation of phenylalanine into protein; hence, less protein accretion would be expected (Clarke & Bier, 1982). Clarke and Bier (1982) were the first to establish that the hydroxylation of phenylalanine to tyrosine and the flux can both be measured using continuous infusion of L-[ring-$^{2}H_5$]-phenylalanine and L-[1-^{13}C]-tyrosine. Thompson et al. (1989) used the same principle, however, they modified it by priming the pools of both [$^{2}H_4$] tyrosine and [$^{2}H_5$] phenylalanine to achieve isotopic plateau more quickly, followed by continuous infusion of the isotopes. According to Thompson et al. (1989), the hydroxylation of phenylalanine to tyrosine can be quantified provided that tyrosine flux is measured using a distinguishable tracer, and the enrichment of tyrosine coming from a tracer of phenylalanine can also be measured (Short et al., 1999).

The same principle of Thompson et al. (1989) can be applied to measure other amino acids and their respective products, such as the conversion of arginine to GAA and creatine. It would require the infusion of uniquely labeled isotopes of GAA and creatine to measure their fluxes and conversion rates.

Because the conversion of phenylalanine to tyrosine can be used to estimate phenylalanine oxidation, one advantage of using the model described above is that it eliminates

the need to sample the label from breath (to determine amino acid oxidation) or urine and reduces the sampling to blood only (Thompson et al., 1989). One limitation of this model is the overestimation of whole body protein synthesis. It has been determined that the plasma enrichment of phenylalanine and tyrosine is almost 25% greater than the intracellular enrichment. The main hydroxylation site of phenylalanine is the liver; therefore, a better estimate of the enrichment would be provided by sampling at the site, or through the measurement of specific liver-derived plasma proteins such as Apo-B100. Moreover, measuring whole body protein synthesis may not provide enough detailed information about the protein status of the body and may not be sensitive enough to capture organ-specific differences in protein kinetics. Finally, some studies have reported that the use of specific phenylalanine tracers in metabolic studies might produce a kinetic isotope effect depending on the site of the label on the molecule. If the label is located on the aromatic group, it may alter the rate of the hydroxylation reaction. Reports in the literature varied between [ring-$^{2}H_{5}$] phenylalanine slowing phenylalanine hydroxylation or increasing the rate of reaction, when compared to phenylalanine labeled with [1- ^{13}C] position, or with a ^{15}N label and result in either an increase or decrease in the plasma enrichment (Matthews, 2007). A study done by Krempf et al. (1990) examined the fluxes of three different tracers of phenylalanine infused concurrently through the venous (IV) and intragastric (IG) routes in fasted subjects, L-[ring-$^{2}H_{5}$]-phenylalanine, L-[1- ^{13}C]-phenylalanine, and L-[^{15}N]-phenylalanine. They found that the IG fluxes were generally higher than the IV. In addition, the fluxes of the IV infused tracers did not differ regardless of label; however, L-[ring-$^{2}H_{5}$]-phenylalanine had a significantly greater flux rate compared to the [1- ^{13}C] and [^{15}N] tracers when infused through IG. The researchers attributed this observation to possible "proton exchange" in the intestines. Another study done by Marchini et al. (1993) measured

phenylalanine hydroxylation in fasted and fed healthy men by the simultaneous primed infusion of L-[ring-$^{2}H_5$]-phenylalanine, L-[1- ^{13}C]-phenylalanine, and L-[ring-$^{2}H_4$]-tyrosine. They found that L-[ring-$^{2}H_5$]-phenylalanine had a significantly decreased oxidation rate in comparison to L-[1- ^{13}C]-phenylalanine in both the fed and fasted state. They also argued that the use of L-[ring-$^{2}H_5$]-phenylalanine might underestimate the *in vivo* hydroxylation of phenylalanine and recommended that a correction factor should be used to better approximate the hydroxylation rate.

1.10 Research rationale

Both arginine and methionine are indispensable amino acids in the neonate. Our laboratory has previously demonstrated that when arginine or methionine is limiting, creatine synthesis is impaired. Since a fraction of creatine and creatine phosphate are irreversibly converted to creatinine (~1.7%/day), there is a need to replace this lost pool (Wyss & Kaddurah-Daouk, 2000). Furthermore, it was estimated that creatine synthesis consumes an equivalent of 20% of the dietary intake of arginine (Brosnan et al., 2009) and as a consequence, with limited dietary supply, less arginine may be available for protein synthesis and ultimately, growth. In addition, TPN feeding inhibits the gut from producing arginine in the neonate, thus, increasing the risk of deficiency due to gut atrophy (Dinesh et al., 2014). Taken together, this information suggests that arginine deficiency may limit creatine synthesis. Finally, in a previous study from our laboratory, it was demonstrated that dietary arginine supplemented to TPN-fed piglets predicted tissue creatine concentrations (Gagnon, unpublished data). Therefore, using a TPN-fed piglet model, the current study addressed the question of how arginine is partitioned between protein and creatine synthesis when limited in the diet, and whether GAA or creatine effectively

spares arginine for growth. It was anticipated that arginine deficient diets will compromise creatine synthesis, and that supplementing GAA or creatine would spare arginine and/or methionine for protein synthesis and other metabolic functions.

1.11 Hypothesis

A parenteral diet supplemented with creatine or its precursor GAA will spare arginine and/or methionine for protein synthesis and growth in neonatal piglets.

1.12 Specific objectives

In this study, our objectives were:

- to assess the capacity of the kidney and the pancreas to synthesize GAA via the enzyme AGAT
- to measure the efficiency of dietary amino acid utilization as indicated by nitrogen retention
- to measure the concentrations of GAA and creatine in various tissues
- to utilize stable isotopes to measure the partitioning of arginine into GAA and creatine under conditions of limited and excess arginine, and supplemental GAA and creatine
- to measure whole body protein dynamics using tracers of phenylalanine and tyrosine

Chapter 2: Materials and Methods

2.1 Animal Experimentation and Surgical Procedures

Yucatan miniature piglets were used in this study (n= 31). 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. Animals were obtained from a breeding colony at Memorial University of Newfoundland, St. John's, NL, Canada. On the initial day of the study (day 0), piglets were transported from the breeding colony via Animal Care Services staff to the animal research facility (Biotechnology Building, Memorial University of Newfoundland). Piglets were weighed upon arrival and given an intramuscular injection of 22 mg.kg body weight^{-1} (BW) ketamine hydrochloride (Bimeda Canada, Cambridge, ON, Canada) and 0.5 mg.kg BW^{-1} acepromazine (Vetoquinol, Quebec, Canada) mixed in one syringe to induce anesthesia. An intramuscular injection of buprenorphine hydrochloride (Temgesic, Reckitt Benckiser Healthcare, Berkshire, UK) at a dose of 0.03 mg.kg BW^{-1} was also administered intramuscularly for analgesic effects. Once the pre-anesthetic had taken effect, the piglets were cleaned with Prepodyne solution (West Pentone, Quebec, Canada), 70% isopropanol, and Prepodyne scrub (West Pentone, Quebec, Canada). The pigs were then placed on a surgical table, and general anesthesia was induced using 1.5% isoflurane anesthesia (Abbott Laboratories Inc., Markham, ON, Canada) mixed with oxygen (1.5 ml.min^{-1}). Under sterile conditions, a catheter (described below) was implanted in the left external jugular vein and advanced to the superior vena cava. Another catheter was implanted in the left femoral vein and advanced to the inferior vena cava. The jugular catheter was used for intravenous diet infusion and femoral catheter was used for blood sampling. After the surgery was completed, 0.5 mL of the antibiotic Borgal (trimethoprim 40 mg.ml^{-1} and sulfadoxine 200 mg.ml^{-1}; Intervet

Canada Ltd, Kirkland, QC, Canada) diluted to 10 mL with normal saline was injected in both of the catheters to prevent infection, along with 5 mL of heparinized saline to prevent the formation of clots in the catheters. Antibacterial Hibitane ointment (chlorhexidine acetate 1% w/w veterinary ointment; Wyeth, Kirkland, QC, Canada) was administered to the incision sites to prevent infection and aid in recovery. Piglets were then moved to a housing room where they remained for the rest of the study. The temperature of the room was kept at 28°C with a 12-hour light and 12-hour dark cycle. The piglets were placed in metabolic cages. A tether and swivel system (Lomir Biomedical, Montreal, Canada) was used that attached to a jacket which was fitted securely on the piglet. This enabled the safe infusion of the intravenous treatments while allowing the piglets to move freely inside the cage. Heat lamps attached to the cages provided supplemental heat. The piglets were housed individually, but were able to see and socialize with each other.

The study protocol is presented in Figure 2.1. For the first two days of study, the piglets were provided with a nutritionally complete parenteral adaptation diet to aid in recovery (Appendix I). This adaptation diet was infused at 50% of the target rate (13.5 ml.kg BW^{-1} $.h^{-1}$) on the day of surgery. The following morning, the rate was increased to 75% of target, and advanced to 100% by the evening of day 1. The IV diets were delivered as a constant infusion via pressure-sensitive, hospital-grade peristaltic pumps that were accurate to 1 mL per hour (Baxter Healthcare Corporation, Deerfield, USA). The piglets were monitored daily for signs of pain or infection. Buprenorphine hydrochloride (0.015 mg.kg BW^{-1}) was administered in the evening post-surgery as well as the next morning. At the end of day 2, piglets were randomized to one of the five parenteral experimental diets (Table 2.1). Blood samples were taken daily (0.7 ml) to measure plasma ammonia using an ammonia assay kit (Sigma-Aldrich, Oakville, Canada).

The piglets were weighed daily, and the rate of diet infused was adjusted accordingly. On day 6 of the experiment, a 6-hour constant infusion of stable isotopes was performed to determine the kinetics of amino acids, creatine and GAA, as well as whole body protein dynamics. On day 7, a 6-hour constant infusion of L-[methyl-^{3}H]-methionine was performed followed by necropsy with tissue collection. All tissues collected were freeze-clamped using liquid nitrogen, and stored at -80˚C for later analysis. The results of the radioisotope infusion study on day 7 are not presented in this thesis and constitute the MSc thesis work of Scott Bray (S.B.). All of the work involved in carrying out the procedures and animal care described above were shared equally by A.A. and S.B.

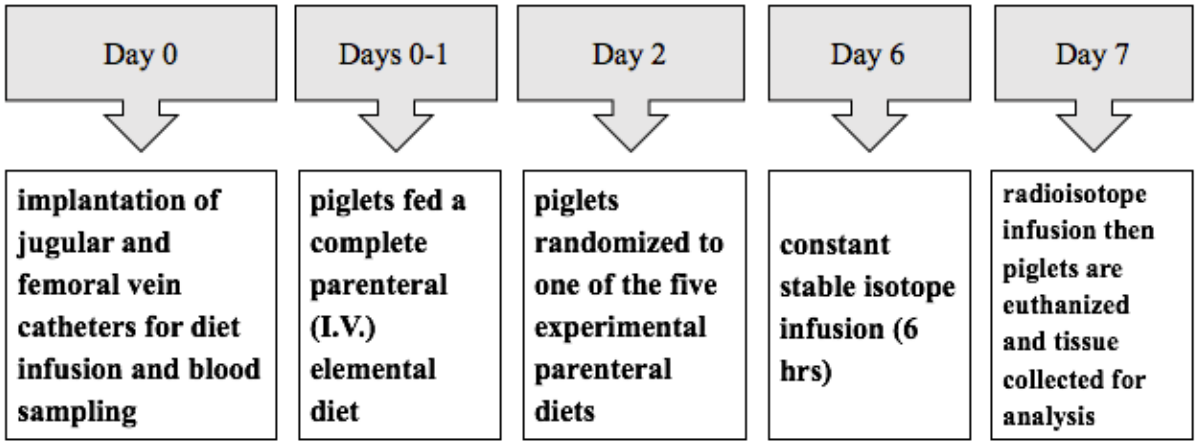

Figure 2.1 The timeline of the experiment.

2.2 Catheters

Silastic catheters were assembled in our laboratory. Jugular catheters were made of silastic tubing (i.d. 0.76 mm, o.d. 1.65 mm) attached to a lever lock cannula (Becton Dickinson & Co., New Jersey, USA). For the femoral catheters, the tubing (i.d. 0.63 mm, o.d. 1.19 mm) was attached to a blunt cannula (Becton Dickinson & Co., New Jersey, USA). All catheters were sterilized using an ethylene oxide gas sterilizer (Anderson Products, North Carolina, USA).

2.3 Parenteral Diets

All diets provided 1.1 MJ non protein energy.kg BW^{-1} and the equivalent of ~15.5 g.kg $BW^{-1}.day^{-1}$ delivered as crystalline amino acids (Table 2.1).

Table 2.1 The amino acid composition of the experimental parenteral diet

Amino Acid	Base	GAA	Creatine	Arg&Met	Met&GAA
	g.kg BW^{-1}.day^{-1}				
Alanine	2.59	2.37	2.37	0.00	2.13
Arginine	0.550	0.550	0.550	1.800	0.550
Aspartate	0.942	0.942	0.942	0.628	0.942
Cysteine	0.224	0.224	0.224	0.224	0.224
Glutamate	1.63	1.63	1.63	1.63	1.63
Glycine	0.357	0.357	0.357	0.357	0.357
Histidine	0.479	0.479	0.479	0.479	0.479
Isoleucine	0.718	0.718	0.718	0.718	0.718
Leucine	1.62	1.62	1.62	1.62	1.62
Lysine-HCl	1.29	1.29	1.29	1.29	1.29
Methionine	0.100	0.100	0.100	0.500	0.500
Phenylalanine	0.610	0.610	0.610	0.610	0.610
Proline	1.29	1.29	1.29	1.29	1.29
Serine	0.868	0.868	0.868	0.868	0.868
Taurine	0.075	0.075	0.075	0.075	0.075
Tryptophan	0.329	0.329	0.329	0.329	0.329
Tyrosine	0.117	0.117	0.117	0.117	0.117
Valine	0.823	0.823	0.823	0.823	0.823
Threonine	0.823	0.823	0.823	0.823	0.823
Creatine monohydrate	0.00	0.00	0.118	0.00	0.00
GAA	0.00	0.093	0.00	0.00	0.093
Glycyl-L-tyrosine dihydrate*	0.385	0.385	0.385	0.385	0.385
Total amino acids	15.8	15.7	15.7	14.7	15.8

* Glycyl-L-tyrosine provided 0.293 g.kg BW^{-1}.day^{-1} of tyrosine and 0.121 g.kg BW^{-1}.day^{-1} of glycine.

2.3.1 Adaptation parenteral nutrition

The adaptation PN composition was modified from the work of Wykes et al. (1993) (Appendix I). The diet was prepared in the laboratory using crystalline L-amino acids (Ajinomoto North America Inc., North Carolina, USA; Evonik Industries AG, Hanau-Wolfgang, Germany; Sigma, St. Louis, MO, USA; USB Corporations, Cleveland, OH, USA; Merck Schuchardt OHG, Hohenbrunn, Germany). The amino acids were weighed individually, mixed well, and then dissolved slowly in 4 L of pyrogen-free water, heated to 55-60°C, and maintained under a blanket of nitrogen gas to prevent the oxidation of amino acids. Once the amino acids were completely dissolved, D-glucose and major minerals (Appendix III) were added. More pyrogen-free water was added to the solution to bring it up to the final volume. The diet solution was sterilized by working in a laminar airflow cabinet while cold filtering through a 0.22 μm filter (AcroPak, Pall Corporation, Switzerland) into sterile IV bags (Baxter Corporation, Mississauga, ON, Canada). The diet-filled bags were stored at 4˚C away from light, until needed.

2.3.2 Trace elements

The trace elements were estimated based on the work of Wykes et al. (1993) (Appendix II). The individual trace elements were weighed out and mixed with pyrogen-free water. The mixture was then filtered through a sterile 0.22 μm syringe filter (Millipore Ireland Ltd., Ireland) into a sterile IV bag (Baxter Corporation, Mississauga, ON, Canada). The trace elements solution was kept refrigerated until needed.

2.3.3 Multivitamins

Injectable vitamins were a commercial product (Infuvite Pediatric-Multiple Vitamins, Baxter Health Corporation, Illinois, USA). The product was provided as two separate vials that required mixing just prior to adding to the PN diet (Table 2.1). Just prior to using the diet-filled

IV bags, 3 mL of vitamin preparation, 3 mL of trace elements, 1 mL of injectable iron (IPCO, Saskatoon, Canada; 3 mg/kg BW), and 145 mL of 20% Intralipid emulsion (Fresenius Kabi, Uppsala, Sweden) were added to 750 mL of prepared diet and inverted repeatedly to ensure proper mixing.

2.3.4 Experimental parenteral diets

The experimental diets were prepared by using the same procedures as described in 2.3.1 for the adaptation diet. The composition of the experimental diets was based on the adaptation diet, with modifications (Table 2.1). To ensure that all diets were isonitrogenous, the concentrations of alanine and aspartate were adjusted accordingly (Table 2.1).

Base diet (Base): The base diet was marginal in both arginine (0.55 g.kg $BW^{-1}.day^{-1}$) and methionine (0.10 g.kg $BW^{-1}.day^{-1}$) and was considered as the negative control. Based on the work by Shoveller et al. (2003b), the methionine requirement in PN-fed piglets was 0.18 g.kg $BW^{-1}.day^{-1}$ (when cysteine was included in excess). Therefore, this diet provided ~55% of the methionine requirement for PN-fed piglets. The arginine concentration chosen for this study was previously demonstrated to be low enough to impair protein synthesis in piglets without causing other adverse outcomes such a hyperammonemia or death (Brunton et al., 2003); as such, this should allow us to detect differences among treatment groups and the negative control, when creatine or its precursors are added to the diet.

Base + GAA diet (GAA): The purpose of this diet was to test if a dietary source of GAA could spare arginine for protein synthesis. The piglets in this diet group received 0.093 g.kg $BW^{-1}.day^{-1}$. If converted entirely to creatine, this amount would supply enough creatine to meet the neonatal piglets' daily accretion rate (Brosnan et al., 2009); therefore, we could determine

whether less arginine is converted to GAA when it is supplied in the diet, making it more available for protein synthesis.

Base + creatine diet (Creatine): The purpose of this diet was to test if creatine supplementation would spare both arginine and methionine for growth and protein synthesis. This treatment allowed us to determine whether less arginine is directed towards GAA synthesis and whether methionine is spared from GAA methylation, because preformed creatine was supplied in the diet. The piglets in this group received 0.118 g creatine.kg BW^{-1}.day^{-1}, which was estimated to meet 100% of the daily creatine accretion rate in neonatal piglets (Brosnan et al., 2009).

Adequate arginine and methionine diet (Arg&Met): This diet served as a positive control and provided a direct comparison to the Base diet. The arginine concentration in this diet provided 1.8 g.kg BW^{-1}.day^{-1}, and was chosen based on a previous study that predicted greater than 1.2 g.kg BW^{-1}.day^{-1} is required to satisfy the whole body metabolic needs of arginine (Brunton et al., 2003). The methionine concentration in this diet supplied 0.5 g.kg BW^{-1}.day^{-1} which was almost triple the requirement of methionine determined for PN-fed piglets and double that for enterally fed piglets (Shoveller et al., 2003b).

Low arginine + GAA + adequate methionine diet (Met&GAA): The composition of this diet allowed for direct comparison to the Base + GAA diet. Excess methionine was supplied to support maximal conversion of GAA to creatine. We also predicted that excess GAA could contribute to methionine deficiency, because large amounts of methionine would be required to methylate GAA via hepatic GAMT. Therefore, this diet group acted as a direct comparison to the Base + GAA diet to elucidate whether methionine deficiency contributed to impaired protein synthesis.

2.4 Analytical methods

2.4.1 In vitro AGAT activity

The AGAT activity in both the kidney and the pancreatic tissues was measured in vitro. The method was adapted from the method by Van Pilsum et al. (1970). In this reaction, the ornithine produced in the presence of arginine and glycine reacts with ninhydrin to form a yellow-orange color. Sodium fluoride (NaF) is used to inhibit ornithine production by arginase in the reaction, so the ornithine produced can be used to estimate the activity of AGAT. Frozen pulverized tissue was diluted with 50 mM potassium phosphate buffer (pH 7.4) (1:5, w/v). The samples were then homogenized for 25 s at 50% speed. Next, the samples were diluted to 1:20 (v/v) with the same buffer, resulting in a 1% homogenate. Four more additional buffers were prepared using the same potassium phosphate buffer (20 mL each) as follows: -Arg/-Gly (0.0285 g NaF), -Arg/+Gly (0.069 g Gly and 0.0285 g NaF), +Arg/-Gly (0.1045 g arginine and 0.0285 g NaF), and +Arg/+Gly (0.1045 g Arg, 0.069 g Gly, and 0.0285 g NaF). 0.5 mL of samples were added to 0.5 mL of each of the buffers and incubated in a shaking water bath at 37˚C for 60 min. After 60 min, the reactions were stopped with 1.5 mL of 0.6 M perchloric acid. Next, the samples were centrifuged at 4˚C using a speed of 10621 *g* for 5 minutes. The samples were then placed on ice. A ninhydrin solution (6 g ninhydrin mixed in 100 mL 1-propanol) was prepared and 1.5 mL was added to each sample and to a series of ornithine standards. The samples were mixed well and transferred to a 92˚C water bath for 25 min, and then left to cool. The resulting colour was read using a microplate spectrophotometer (BioTeK, Vermont, USA) at a wave length of 505 nm. A calibration curve was constructed to calculate the unknown concentration of ornithine.

The concentration of ornithine in each sample was calculated based on the following equation:

1. ΔOD due to AGAT = ΔOD (+Arg/+Gly) – [ΔOD (+Arg/-Gly) + ΔOD (-Arg/+Gly) - ΔOD (-Arg/-Gly)]
2. nmol ornithine in the unknown sample = [(ΔOD due to AGAT – b) / m]
3. nmol ornithine. min^{-1}. g $kidney^{-1}$ = nmol ornithine in unknown sample / (60 minutes x g tissue in assay medium)

Where OD is the optical density, b is the y-intercept and m is the slope from the standard curve equation.

2.4.2 Nitrogen balance

Starting on day 4 of the experiment, the diet bag was weighed. ~5 mL of 18.4 M sulphuric acid was added to the clean urine collecting flask, and the flask was weighed. After 24 h, the diet pump was stopped and the diet bag was weighed again. The urine flask was also weighed and a urine sample was collected and stored in -20˚C for later analysis. The procedure was repeated for two subsequent days, to provide total of 3 urine samples, each a sample from 24 h pooled urine. Stool was not collected because PN-treated animals stopped defecating by day 4. Accurate records of quantity of diet infusion were maintained and were used to calculate the actual nitrogen intake.

The urine samples were analyzed using a LECO system (FP-528) (LECO Corp., Michigan, USA). Frozen urine samples were thawed on ice. 1 mL of the thawed sample was neutralized using 6 M NaOH. The neutralized samples were centrifuged (Eppendorf centrifuge 5804R) at 10621 *g* for 5 minutes to remove any solids. Six 50 mg samples of $Na_2EDTA•2H_2O$ were used as a standard. 100 µL of urine was weighed into a Leco tin foil cone and sealed. The

samples were then placed onto the loading head of the instrument and purged of all atmospheric gases. The samples were then dropped into an 850˚C furnace, which combusts the samples in the presence of pure oxygen. This combustion yields in CO_2, H_2O, NO_x, and N_2. All of these products were then passed through a furnace filter and a thermoelectric cooler where they were collected in a ballast apparatus, then homogenized via passive mixing. A 3 cc aliquot of the mixed gases was passed through hot copper for the removal of oxygen and for the conversion of NO_x to N_2. After that, the aliquot is passed through Lecosorb and Anhydrone for the removal of CO_2 and water. The reaction results in nitrogen that was measured by thermal conductivity cells (Leco, 2008).

2.4.2.1 Nitrogen balance calculations

Nitrogen Excreted ($g.kg\ BW^{-1}.day^{-1}$) = [urine output ($g.kg\ BW^{-1}.day^{-1}$) x urine nitrogen concentration ($mg.g^{-1}$)] / 1000

Nitrogen Balance ($g.kg\ BW^{-1}.day^{-1}$) = nitrogen intake ($g.kg\ BW^{-1}.day^{-1}$) – nitrogen excreted ($g.kg\ BW^{-1}.day^{-1}$)

% Nitrogen Retention = [nitrogen balance ($g.kg\ BW^{-1}.day^{-1}$) / nitrogen intake ($g.kg\ BW^{-1}.day^{-1}$)] x 100

2.4.3 GAA and creatine tissue concentrations

The analysis of tissue GAA and creatine concentrations was based on the method by Buchberger and Ferdig (2004). This method was applied to kidney, pancreas, and brain tissues. 2 mL of 1 M perchloric acid was added to 0.5 g of frozen pulverized tissues. The tissue was then homogenized at 50% speed for 25 seconds. After that, the homogenate was centrifuged at 4˚C using a speed of 17131 *g* for 20 minutes. The supernatant was then transferred to new tubes and neutralized (pH 6.5 – 7) using 50% K_2CO_3 and 20% KOH. The solution was centrifuged again at

4˚C and 10621 g for 10 minutes. 400 µL was aliquoted for derivatization with ninhydrin. 300 µL of 1.5 M KOH was added to 400 µL of sample followed by 150 µL of 0.9% ninhydrin. The sample was mixed well and left to incubate at room temperature for 15 minutes. After that, 5% ascorbic acid followed by 5 M phosphoric acid was added to the sample then incubated in a 90˚C water bath for 30 minutes. Next, the samples were left to cool down and then filtered through a 0.45 µm PTFE syringe filter (Canadian Life Science, Peterborough, Canada). The filtered samples were injected into a YMC-Pack Pro C18 column (150 X 4.6 mm I.D, 3 µm; YMC America, Inc, Allentown, PA, USA). The derivatized GAA and creatine were separated via reverse-phase HPLC using a dual buffer system (Mobile phase A: 50 mM formic acid and mobile phase B: 100% methanol). Peaks were detected by Waters 474 scanning fluorescence detector at 470 nm emission and 390 nm excitation. The run time for each sample was 37 minutes with a flow rate of 0.5 ml.minute^{-1}. A standard curve was used for quantification of the peak areas which was determined using Empower Software (Waters, version 3, 2010, Waters Corporation, Woburn, MA, USA).

2.4.4 *In vivo* stable isotope kinetics

2.4.4.1 Isotope preparation and procedures

On the morning of day 6, piglets were weighed and 0.7 mL blood sample was drawn into a K_2EDTA vacutainer tube (4 ml; 13 x 75 mm), and centrifuged for 5 minutes at 1300 g (VWR Clinical 200, Hermle Labortechnik, Wehingen, Germany). A priming infusion was given as a bolus into the jugular catheter and contained the following isotopes: L-arginine-$^{13}C_6$-HCl, guanidineacetic acid-2,2-D_2, creatine-D_3-H_2O (methyl D_3), L-[ring-D_5]-phenylalanine, L-[ring-D_4]-tyrosine, L-[ring-3,5-D_2]-tyrosine. Immediately following, a constant infusion was initiated

through the same catheter. The constant infusion contained all of the isotopes, except for L-[ring-3,5-D_2]-tyrosine. Isotope prime and constant infusion doses are presented in Table 2.2. The test diets were continued during the isotopes infusion experiment, and the amino acid compositions of the test diets were adjusted to accommodate the contribution of the isotopically labeled amino acids. The constant infusion continued for 6 hours, and blood samples were drawn from the femoral catheter every half hour (Figure 2.2). All blood samples were centrifuged immediately and plasma was collected in Eppendorf tubes and stored at -80 ˚C for later analysis. The plasma samples were analyzed at the Analytical Facility for Bioactive Molecules (AFBM) of the Centre for the Study of Complex Childhood Diseases (CSCCD) at the Hospital for Sick Children, Toronto, Ontario.

Table 2.2 The compositions of the prime and constant doses for stable isotope infusion

Isotope	Prime *mg.kg BW^{-1}*	Constant *mg.kg $BW^{-1}.h^{-1}$*	Products/Outcome
L-arginine-$^{13}C_6$-HCl	3.64	6.07	Arginine flux, GAA_{M+1}, $Creatine_{M+1}$
Guanidineacetic-2,2-D_2	0.61	1.02	GAA_{M+2} flux, $Creatine_{M+2}$, rate of GAA synthesis from arginine
Creatine-D3-H_2O (Methyl D3)	1.5	1.5	$Creatine_{M+3}$ flux , rate of synthesis from arginine and GAA
L-[ring-D_5]-Phenylalanine	1.09	3.4	Tyr_{M+4}, Phe flux, Phe hydroxylation to Tyr (surrogate for Phe oxidation)
L-[ring-D4]-tyrosine	0.51	NA	Tyr_{M+4} (priming the product pool)
L-[ring-3,5-D2]-tyrosine	0.5	1.65	Tyr_{M+2} flux

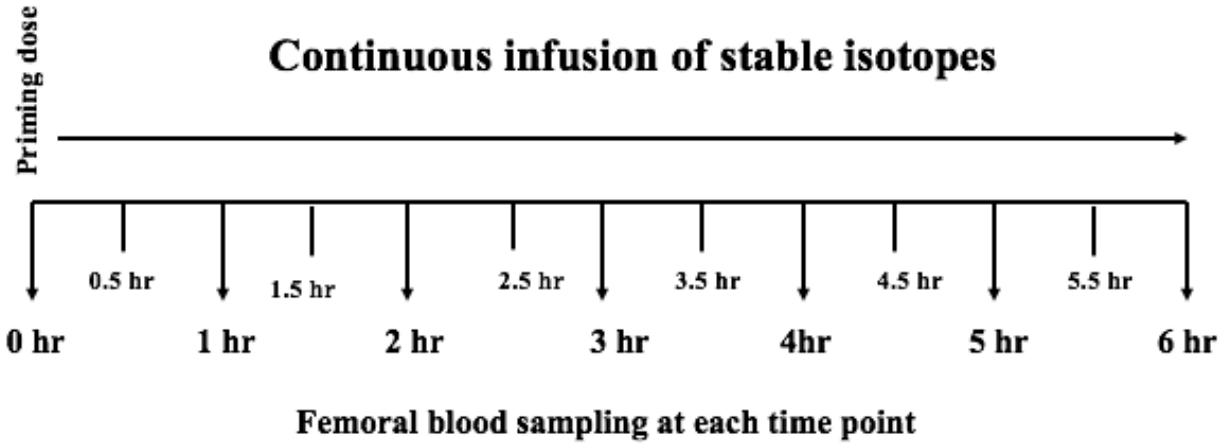

Figure 2.2 Stable isotope infusion and blood sampling protocol

2.4.4.2 Sample preparation

Plasma samples were thawed on ice and 200 µL of methanol was added to 25 µL plasma and centrifuged at 4˚C and 17949 *g* (Eppendorf Canada, Mississauga, ON, Canada). The supernatant was transferred to glass tubes, covered with parafilm, frozen in liquid nitrogen then kept at -80˚C for 20 minutes. The samples were then placed in a freeze dryer overnight (Thermo Savant, Canada). The next day, the samples were removed and stored at -80˚C until shipped on dry ice to the facility in Toronto. When samples arrived, they were reconstituted with 1 mL of 10/90 water/acetonitrile 5 mM ammonium formate at a pH of 3.2.

LC-MS-MS Analysis

Sample analysis was performed with an injection volume of 5 µL on a Sciex QTrap 5500 mass spectrometer (Framingham, Massachusetts, USA) and Agilent 1290 HPLC system (Agilent Technologies: Santa Clara, California, USA). Chromatography was run at a flow rate of 500 $\mu l.minute^{-1}$ on a Kinetex HILIC column (4.6 x 50 mm, 2.6 µm) (Phenomenex, Torrance, California, USA) with a gradient starting at 5% A (90/10 water/acetonitrile 5 mM ammonium formate pH 3.2), and 95% B (10/90 water/acetonitrile 5 mM ammonium formate pH 3.2) ramped to 100% A at 5 minutes and returning to initial conditions at 6 minutes for re-equilibration and a total run time of 10 minutes.

Acquisition was performed in positive mode ESI with a source temperature of 600°C and an ion spray voltage setting of 5300. MRM mass transitions (m/z) were as follows: arginine 175.1-70.0, arginine (M+6) 181.1-74.0, creatine 132.1-90.0, creatine (M+1) 133.1-90.0, creatine (M+2) 134.1-92.0, creatine (M+3) 135.1-93.0, guanidinoacetate 118.1-76.0, guanidinoacetate (M+1) 119.1-76.0, guanidinoacetate (M+2) 120.1-78.0, phenylalanine 166.1-120.0, phenylalanine (M+5) 171.1-125.0, tyrosine(a) 182.1-136.0, tyrosine(b) 182.1-91.0, tyrosine

(M+1) 183.1-91.0, tyrosine (M+2) 184.1-138.0, tyrosine (M+4) 186.1-140.0. Peak integration and data analysis were performed using Analyst 1.6.3 software spectrometer (Sciex, Framingham, Massachusetts, USA). Area ratios were calculated by plotting peak areas of the labelled mass transition/peak areas of the unlabeled mass transition for each analyte.

2.4.4.3 Calculations

Isotope enrichment

Enrichment (atom percent excess, APE) was calculated using the following equation (Wilson et al., 2000):

$$APE = [(R_s - R_b) / (1 + R_s) - R_b] \times 100$$

Where R_s is tracer:tracee in enriched sample; R_b is the tracer:tracee in natural abundance

Amino acid flux

The whole body amino acid flux was needed to calculate molar conversions. The flux was calculated using the following equation (Tomlinson et al., 2011):

$$\text{(Flux)}\ (\mu mol.\ kg\ BW^{-1}.h^{-1}) = I \times [(E_i/E_p)-1]$$

Where I is the isotope infusion rate; E_i is the enrichment in the infusate; E_p is the enrichment in plasma.

Precursor to product conversion rate

Precursor to product conversion rate was calculated based on the following equation (Tomlinson et al., 2011):

$$Q_{precursor>product}\ (\mu mol.\ kg\ BW^{-1}.h^{-1}) = (E_{product}/\ E_{precursor}) \times Q_{product}$$

Where Q is the flux; E is the enrichment.

Whole body protein dynamics

Whole body protein dynamics was calculated based on the following equations (de Betue et al., 2017):

a. protein synthesis:

Whole Body Protein Synthesis $(\mu mol.\ kg\ BW^{-1}.h^{-1})$ = Q_{Phe} – (Phe to Tyr conversion rate)

b. protein breakdown:

Whole Body Protein Breakdown $(\mu mol.\ kg\ BW^{-1}.h^{-1})$ = Q_{Phe} – Phe infusion rate

c. protein accretion:

Whole Body Protein Accretion $(\mu mol.\ kg\ BW^{-1}.h^{-1})$ = Protein Synthesis – Protein Breakdown

2.5 Statistical analysis

All resulted were calculated using one-way ANOVA followed by Tukey's multiple comparison test via GraphPad Prism 7 (GraphPad Softwear Inc., CA, USA). P values less than 0.05 were considered statistically significant.

Chapter 3: Results

The study was divided into two sets of outcomes. The first set of outcomes was to investigate the whole body protein dynamics and the whole body partitioning of arginine into GAA and creatine using a stable isotope technique, which is included in this thesis. The second set of outcomes was to describe tissue-specific methionine metabolism, and comprised the MSc thesis of S.B. (not reported here).

In total, 31 piglets were used for this study, which allowed a sample size of 6 piglets per treatment group. All piglets survived for the duration of the study. However, one of the piglets in the GAA group developed gastric ulcers that were discovered during necropsy. Therefore, one extra piglet was added to the GAA group to ensure that the outcomes of the study had complete data sets. After the analyses were completed, the affected piglet's results were within the range of its treatment group, so the data were included resulting in n=7 for GAA.

3.1 Weight gain

The mean body weights of the piglets among treatment groups were not significantly different at the start of the study or at necropsy. All piglets gained weight during the study (Fig. 3.1). The greatest weight gain was exhibited by the Met&GAA group which was significantly greater than piglets on the arginine and methionine deficient treatments (Base, GAA, and Creatine), with the Arg&Met group intermediate.

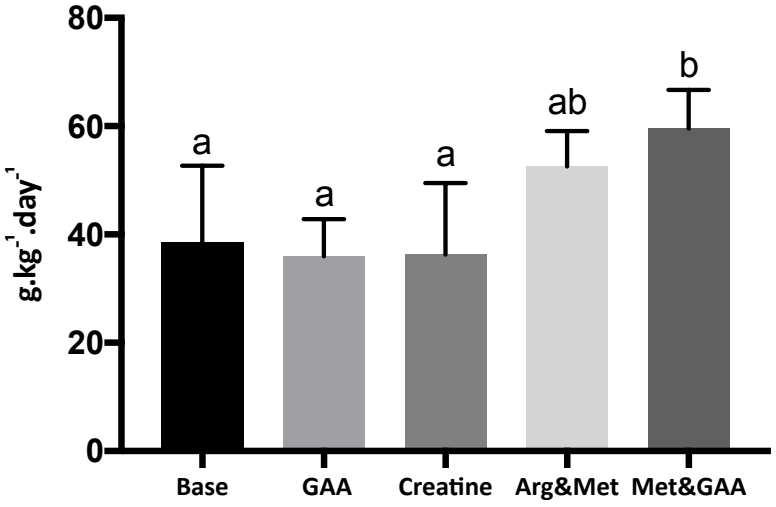

Figure 3.1: **Daily weight gain across diet treatments.**

Values are means ± SD; n=6 per group, except for GAA (n=7). Data were analyzed using a one-way ANOVA followed by a Tukey's multiple comparisons test; different letters indicate statistical differences ($P < 0.05$).

3.2 Nitrogen balance

Whole body nitrogen balance did not differ significantly between groups when expressed as $g.kg^{-1}.day^{-1}$ (Figure 3.2). However, when expressed as percent of nitrogen retained (Figure 3.3), significant differences were observed.

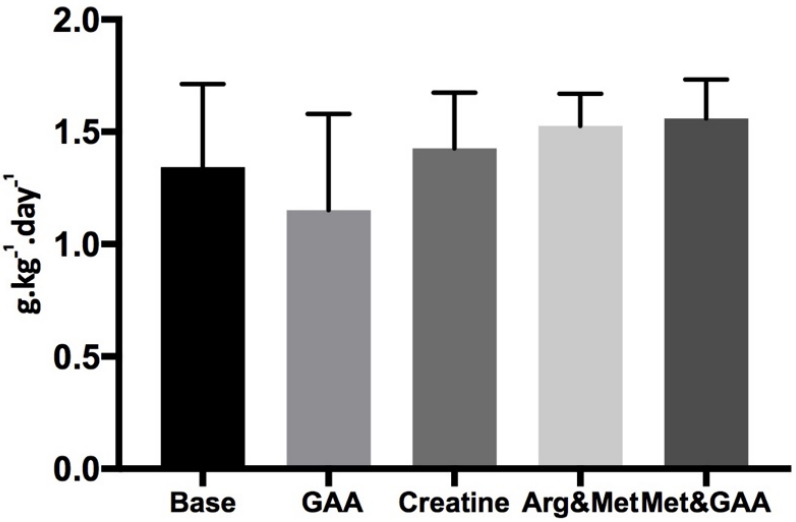

Figure 3.2 Nitrogen balance across diet treatments.

Values are means ± SD; n=6 per group, except for GAA (n=7). Data were analyzed using a one-way ANOVA followed by a Tukey's multiple comparisons test; different letters indicate statistical differences ($P< 0.05$).

There was significantly greater percent nitrogen retention in the Met&GAA compared to the GAA group only. The supplementation of creatine or excess arginine and methionine did not affect percent nitrogen retention.

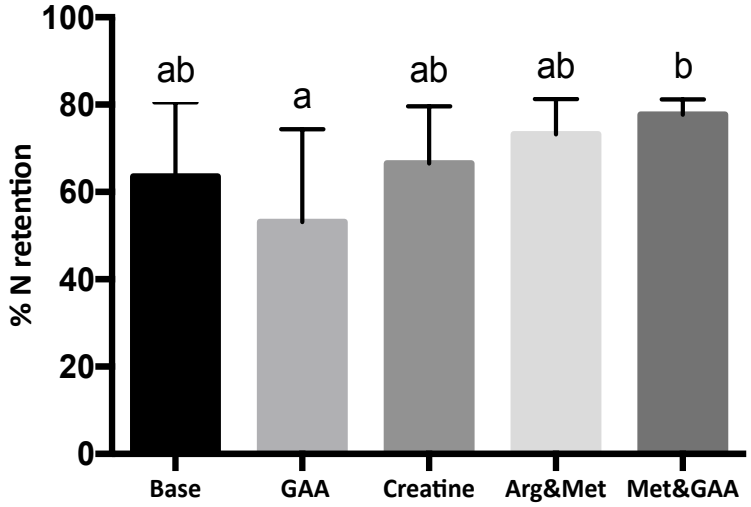

Figure 3.3 Percent nitrogen retention across diet treatments.

Values are means ± SD; n=6 per group, except for GAA (n=7). Data were analyzed using a one-way ANOVA followed by a Tukey's multiple comparisons test; different letters indicate statistical differences ($P < 0.05$).

3.3 In vitro AGAT activity

AGAT activity in the kidney (Figure 3.4) was significantly greater in the Arg&Met group compared to the GAA and Creatine groups. The supplementation of creatine or its precursor GAA with low arginine appeared to negatively downregulate AGAT activity in the kidney compared to when arginine or methionine is supplemented in excess.

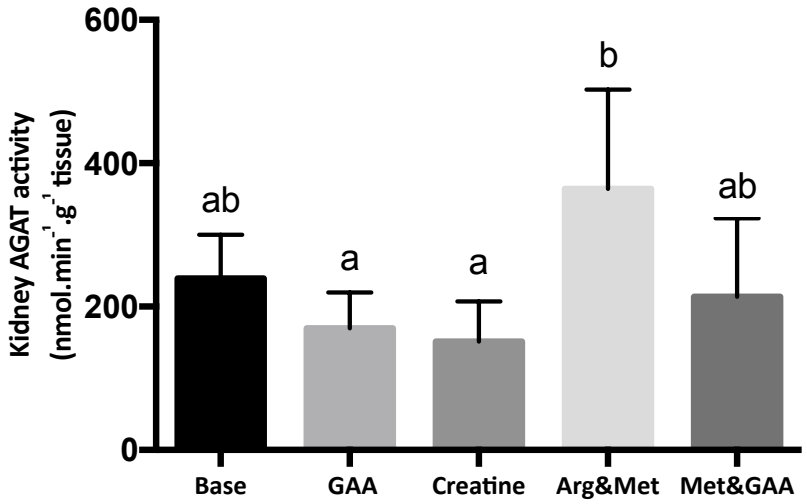

Figure 3.4 AGAT activity in kidney across diet treatments

Values are means ± SD; n=6 per group, except for GAA (n=7). Data were analyzed using a one-way ANOVA followed by a Tukey's multiple comparisons test; different letters indicate statistical differences ($P < 0.05$).

AGAT activity in the pancreas (Figure 3.5) followed a similar trend as in the kidney (Figure 3.4); however, only the creatine group was significantly lower than the Arg&Met group, with no other differences. It appeared that supplementing creatine also negatively downregulated AGAT in the pancreas compared to when arginine and methionine were supplemented in excess.

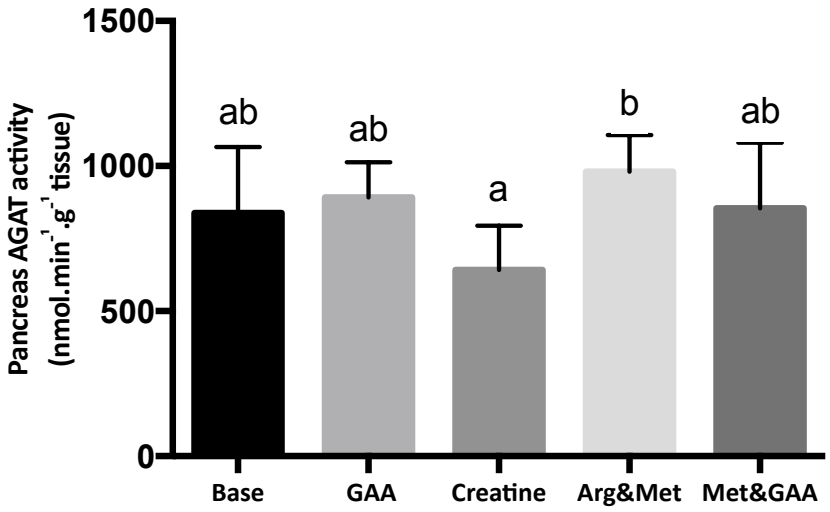

Figure 3.5 AGAT activity in pancreas across diet treatments.

Values are means ± SD; n=6 per group, except for GAA (n=7). Data were analyzed using a one-way ANOVA followed by a Tukey's multiple comparisons test; different letters indicate statistical differences ($P < 0.05$).

3.4 GAA and creatine tissue concentrations

3.4.1 Tissue GAA concentrations

Brain GAA concentrations were not different among treatment diets (Figure 3.6).

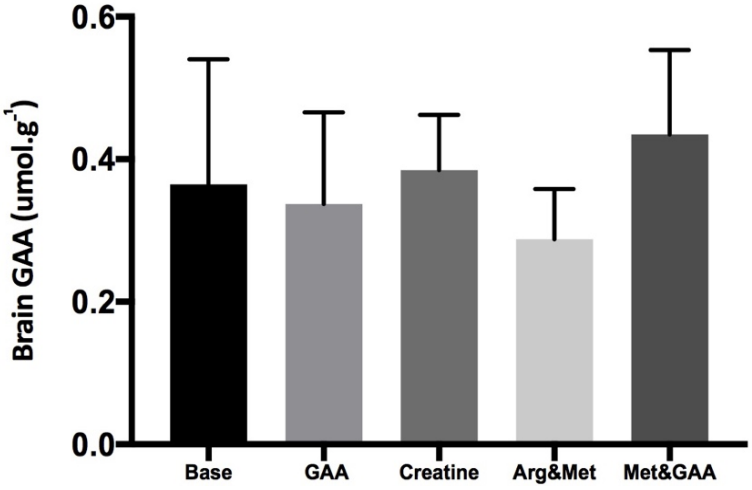

Figure 3.6 Brain GAA concentration across diet treatments.

Values are means ± SD; n=6 per group, except for GAA (n=7). Data were analyzed using a one-way ANOVA followed by a Tukey's multiple comparison test; different letters indicate statistical differences ($P < 0.05$).

Kidney GAA concentration was highest in the Arg&Met group, and was significantly greater than all other groups, including the groups that were provided with supplemental GAA (Figure 3.7).

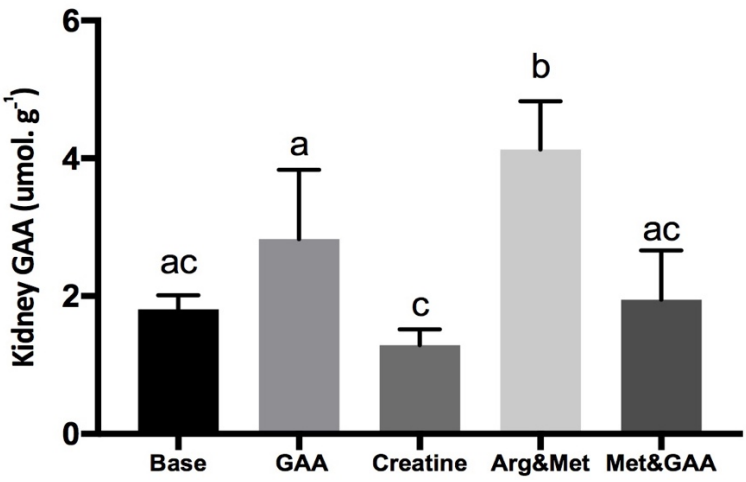

Figure 3.7 Kidney GAA concentration across diet treatments.

Values are means ± SD; n=6 per group, except for GAA (n=7). Data were analyzed using a one-way ANOVA followed by a Tukey's multiple comparisons test; different letters indicate statistical differences ($P < 0.05$).

Similar to the results in the kidney, the pancreas GAA concentration was highest in the Arg&Met group and was significantly greater than all other groups (Figure 3.8).

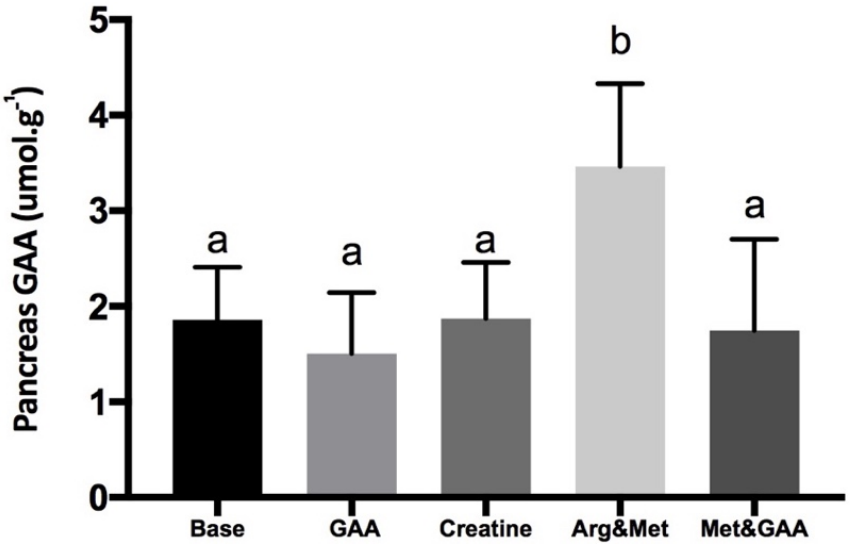

Figure 3.8 Pancreas GAA concentrations across diet treatments.

Values are means ± SD; n=6 per group, except for GAA (n=7). Data were analyzed using a one-way ANOVA followed by a Tukey's multiple comparisons test; different letters indicate statistical differences ($P < 0.05$).

3.4.2 Tissue creatine concentrations

Similar to the GAA concentration in the brain, the brain creatine concentrations also did not differ in response to treatment (Figure 3.9).

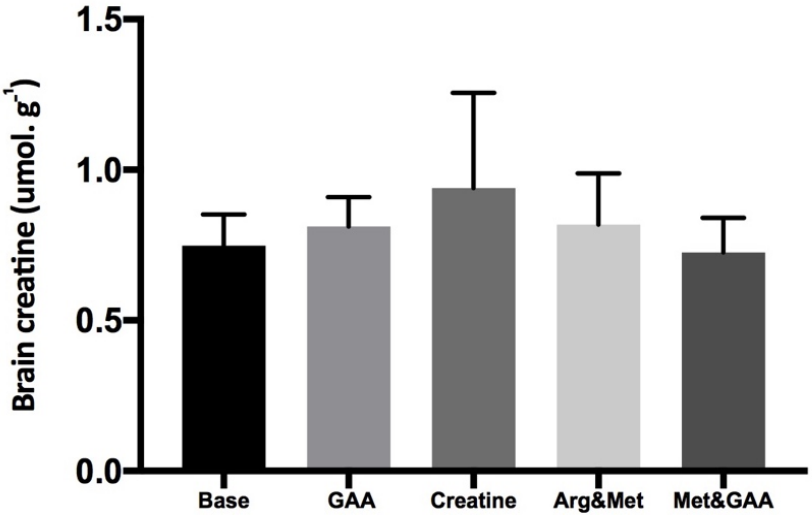

Figure 3.9 Brain creatine concentrations across diet treatments.

Values are means ± SD; n=6 per group, except for GAA (n=7). Data were analyzed using a one-way ANOVA followed by a Tukey's multiple comparison test; different letters indicate statistical differences ($P < 0.05$).

There was significantly greater kidney creatine concentration in the Met&GAA group compared to the Arg&Met group, with no other differences detected (Figure 3.10).

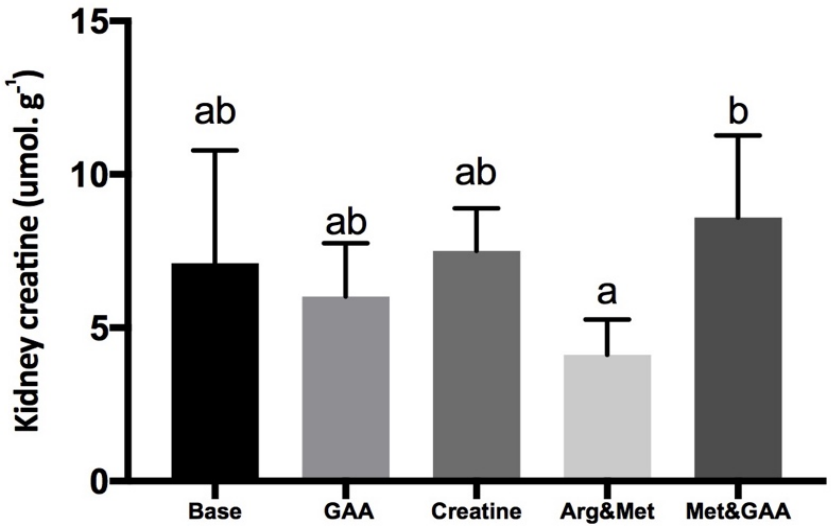

Figure 3.10 Kidney creatine concentrations across diet treatments.

Values are means ± SD; n=6 per group, except for GAA (n=7). Data were analyzed using a one-way ANOVA followed by a Tukey's multiple comparisons test; different letters indicate statistical differences ($P < 0.05$).

There were no differences in pancreas creatine concentration across all diet treatments (Figure 3.11).

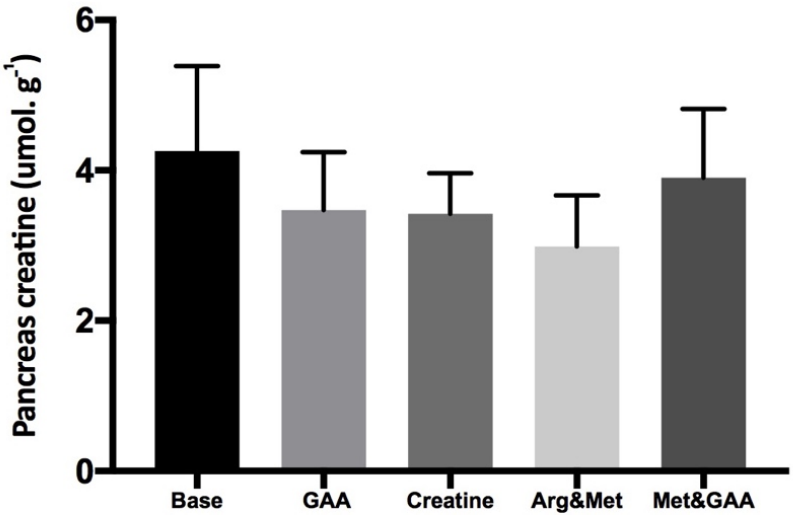

Figure 3.11 Pancreas creatine concentrations across diet treatments.

Values are means ± SD; n=6 per group, except for GAA (n=7). Data were analyzed using a one-way ANOVA followed by a Tukey's multiple comparisons test; different letters indicate statistical differences ($P < 0.05$).

3.5 *In vivo* stable isotope kinetics

3.5.1 Isotope enrichment

All of the infusion concentrations of stable isotopes of amino acids, GAA and creatine were successful at producing measurable enrichments in their respective plasma pools as well as their respective products. Measurable enrichment was not detected in two piglets in the base group (for creatine M+1) and in the Met&GAA group (for creatine M+3) (Table 3.1). To determine steady state, plasma tracer:tracee ratios were plotted over time and plateaus were visually identified and confirmed by a non-significant linear regression. The mean of these plateau values was used to calculate the enrichment of each metabolite isotope.

Table 3.1 Isotope enrichment by diet treatment

Isotope	Base	GAA	Creatine	Arg&Met	Met&GAA
	Atom percent excess				
Arg (M+6)	8.21±0.730^{ab}	8.50±0.480^{b}	7.66±0.538^{a}	8.09±0.316^{ab}	8.68±0.304^{b}
GAA (M+1)	4.85±1.14^{ab}	3.96±1.28^{a}	5.84±1.25^{b}	6.29±0.663^{b}	5.06±0.885^{ab}
Creatine (M+1)	0.983±0.190^{a}	0.628±0.304^{a}	0.867±0.160^{a}	2.17±0.352^{b}	0.935±0.239^{a}
GAA (M+2)	25.5±4.47^{a}	27.2±5.66^{a}	37.8±2.28^{b}	29.9±6.70^{ab}	30.7±6.10^{ab}
Creatine (M+2)	7.84±1.47^{ac}	6.62±2.55^{a}	9.21±1.05^{ac}	13.6±1.46^{b}	9.72±1.45^{c}
Creatine (M+3)	16.5±2.71^{a}	15.0±3.07^{a}	16.1±3.12^{ab}	21.8±2.38^{b}	16.3±3.13^{a}
Phe (M+5)	7.87±0.463	7.87±0.654	8.00±0.384	7.71±0.604	7.85±0.517
Tyr (M+4)	0.793±0.199^{b}	0.716±0.210^{ab}	0.995±0.232^{b}	0.470±0.098^{a}	0.704±0.180^{ab}
Tyr (M+2)	4.13±0.306	4.08±0.530	3.99±0.246	4.23±0.279	4.25±0.249

Values are means ± SD; n=6 per group, except for Base (n=5), Met&GAA (n=5), and GAA (n=7).
Data were analyzed using a one-way ANOVA followed by a Tukey's multiple comparisons test; different letters indicate statistical differences ($P < 0.05$).

3.5.2 Amino acid flux

Whole body turnover or flux of the amino acids, GAA and creatine was calculated by using the steady state enrichment of the isotope in the plasma and infusate, as well as the rate of the isotope infusion. These fluxes were then used to measure the whole body rate of the conversion of the precursor to the product.

Table 3.2 The fluxes of amino acids by diet treatment

Amino Acid	Base	GAA	Creatine	Arg&Met	Met&GAA
			umol.kg $BW^{-1}.h^{-1}$		
Arg	320±43.3[ab]	303±19.1[a]	339±25.8[b]	319±13.5[ab]	295±11.2[a]
GAA	25.8±5.46	24.55±9.16	14.2±1.98	21.9±9.91	20.4±6.76
Creatine	51.4±10.2[ab]	58.0±12.1[b]	49.9±10.7[ab]	35.7±5.14[a]	52.1±10.2[ab]
Phe	234±14.6	235±21.3	230±11.8	240±19.7	236±16.8
Tyr	210±16.8	215±27.7	218±14.3	205±15.3	204±11.9

Values are means ± SD; n=6 per group, except for Met&GAA (n=5), and GAA (n=7).
Data were analyzed using a one-way ANOVA followed by a Tukey's multiple comparisons test.; different letters indicate statistical differences ($P < 0.05$).

3.5.3 Precursor to product conversion rate

The whole body conversion rate of arginine to GAA was greatest when piglets were provided with high arginine and methionine (Arg&Met) in the PN diet (Figure 3.12). The data followed a similar pattern to that observed for kidney AGAT activity (Figure 3.4). When compared to excess arginine and methionine, the supplementation of GAA, creatine, or GAA&Met resulted in less arginine being converted to GAA. The data in Figure 3.13 demonstrate the conversion of arginine to creatine; similar to the production of GAA, supplementation with GAA, creatine, or GAA&Met also resulted in less arginine conversion to creatine.

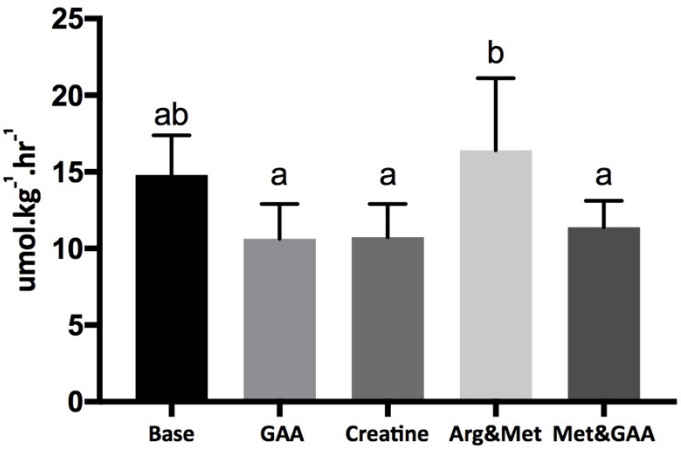

Figure 3.12 Arg (M+6) to GAA (M+1) whole body conversion rate across diet treatment.

Values are means ± SD; n=6 per group, except for GAA (n=7). Data were analyzed using a one-way ANOVA followed by a Tukey's multiple comparisons test; different letters indicate statistical differences ($P < 0.05$).

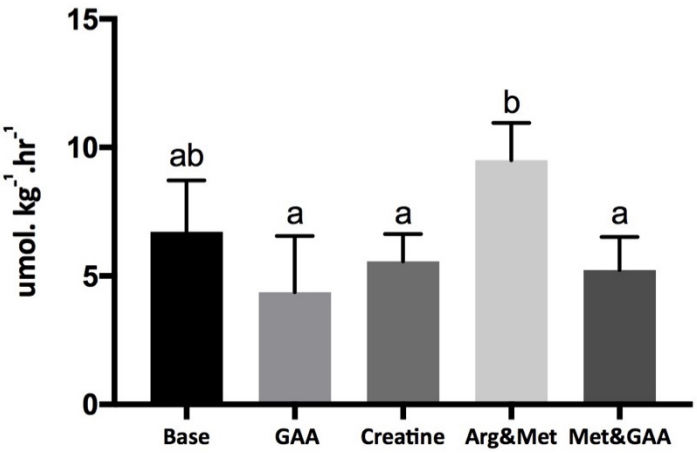

Figure 3.13 Arg (M+6) to creatine (M+1) whole body conversion rate across diet treatments.

Values are means ± SD; n=6 per group, except for Base (n=5), Met&GAA (n=5), and GAA (n=7). Data were analyzed using a one-way ANOVA followed by a Tukey's multiple comparisons test; different letters indicate statistical differences ($P < 0.05$).

There were no differences in the conversion rate of GAA to creatine regardless of the label. The amount of GAA converted to creatine that was derived from the arginine tracer did not differ among groups (Figure 3.14). In addition, there were no differences observed in the rate of creatine production from the infused GAA tracer (Figure 3.15).

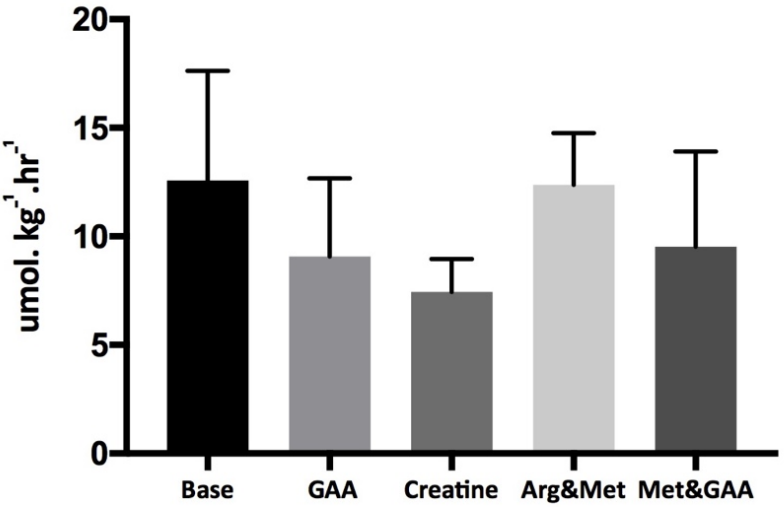

Figure 3.14 GAA (M+1) to creatine (M+1) whole body conversion rate across diet treatments.

Values are means ± SD; n=6 per group, except for Base (n=5), Met&GAA (n=5), and GAA (n=7). Data were analyzed using a one-way ANOVA followed by a Tukey's multiple comparisons test; different letters indicate statistical differences ($P < 0.05$).

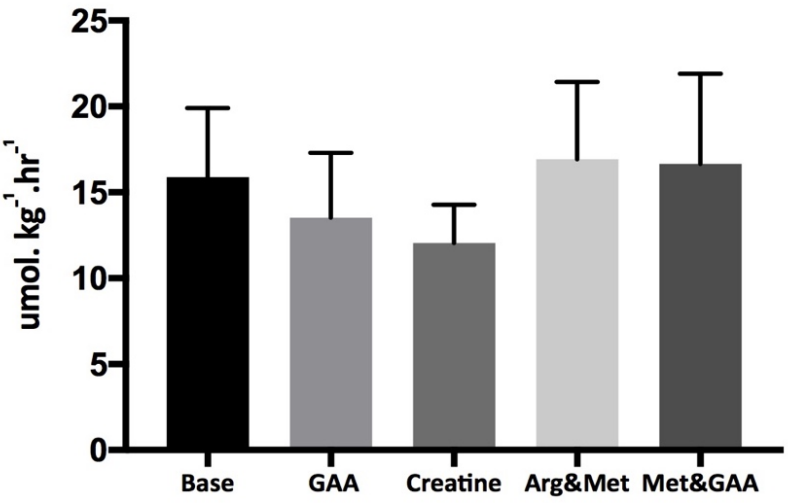

Figure 3.15 GAA (M+2) to creatine (M+2) whole body conversion rate across diet treatments.

Values are means ± SD; n=6 per group, except for Met&GAA (n=5), and GAA (n=7).
Data were analyzed using a one-way ANOVA followed by a Tukey's multiple comparisons test; different letters indicate statistical differences ($P < 0.05$).

The conversion of phenylalanine to tyrosine was measured to estimate the oxidation of phenylalanine, in order to calculate whole body protein synthesis (Figure 3.16). Greater hydroxylation of phenylalanine to tyrosine was measured in the creatine-supplemented group compared to Arg&Met group only. There was no effect of supplementing GAA or GAA and excess methionine on the rate of phenylalanine hydroxylation.

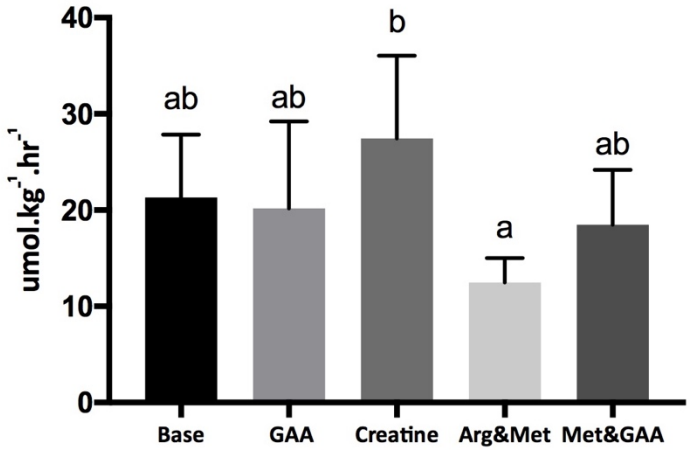

Figure 3.16 Phe (M+5) to Tyr (M+4) whole body conversion rate across diet treatments.

Values are means ± SD; n=6 per group, except for GAA (n=7). Data were analyzed using a one-way ANOVA followed by a Tukey's multiple comparisons test; different letters indicate statistical differences ($P < 0.05$).

3.5.4 Whole body protein dynamics

Whole body protein synthesis and breakdown did not differ by treatment group (Figures 3.17 and 3.18); however, protein accretion was significantly greater in the Arg&Met group compared to the creatine-supplemented group only (Figure 3.19).

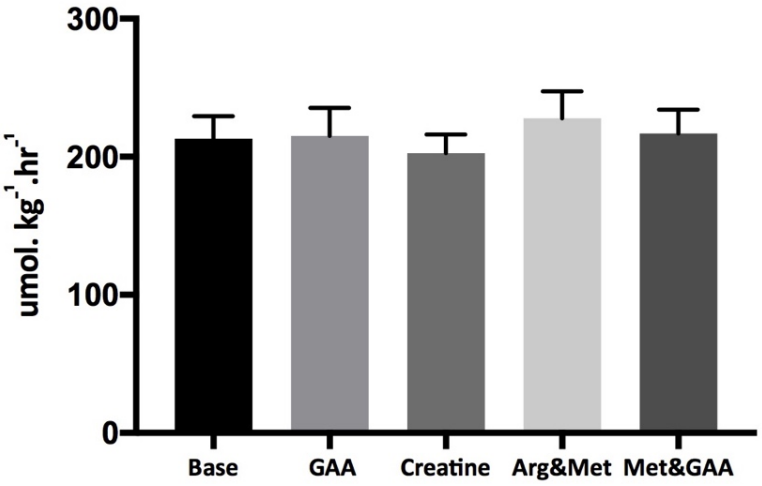

Figure 3.17 Whole body protein synthesis rate across diet treatments.

Values are means ± SD; n=6 per group, except for GAA (n=7). Data were analyzed using a one-way ANOVA followed by a Tukey's multiple comparisons test; different letters indicate statistical differences ($P < 0.05$).

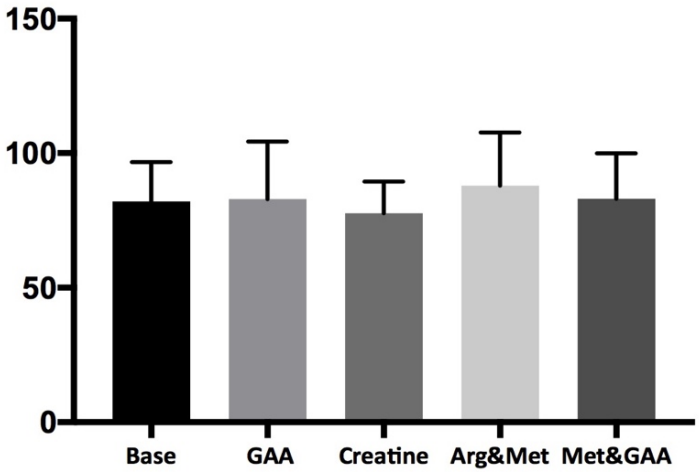

Figure 3.18 Whole body protein breakdown rate across diet treatments.

Values are means ± SD; n=6 per group, except for GAA (n=7). Data were analyzed using a one-way ANOVA followed by a Tukey's multiple comparisons test; different letters indicate statistical differences (P< 0.05).

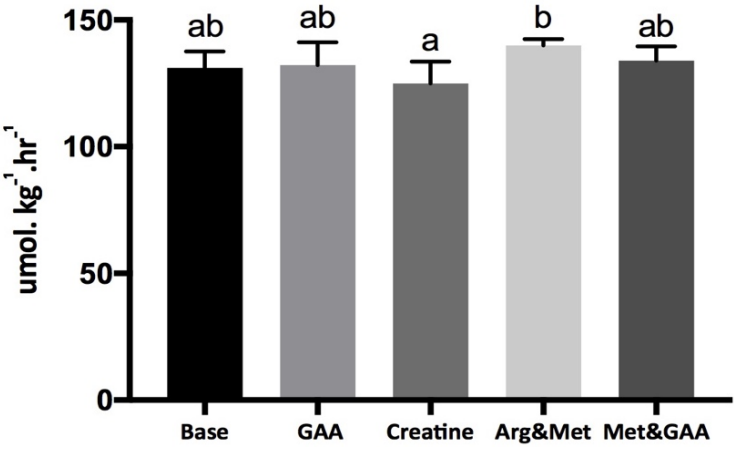

Figure 3.19 Whole body protein accretion rate across diet treatments.

Values are means ± SD; n=6 per group, except for GAA (n=7). Data were analyzed using a one-way ANOVA followed by a Tukey's multiple comparisons test; different letters indicate statistical differences (P< 0.05).

Chapter 4: Discussion

Creatine biosynthesis is an important metabolic pathway in the neonatal piglet (Brosnan et al., 2009). In spite of creatine intake from milk, the suckling neonatal piglet must still synthesize 75% of its creatine requirement from amino acid precursors (Brosnan et al., 2009). Creatine biosynthesis consumes appreciable amounts of the precursor amino acids, equivalent to approximately 20% and 35% of the dietary intake of arginine and methionine, respectively, even when considering the creatine provided in milk (Brosnan et al., 2009). Neonates can synthesize arginine and the main site of endogenous arginine synthesis is the enterocytes of the small intestine (Brunton et al., 1999). It was estimated that approximately 50% of the neonate's arginine requirement is synthesized in the small intestine from proline (Bertolo et al., 2003). Therefore, if intestinal metabolism is compromised, such as with atrophy resulting from TPN feeding, arginine synthesis will be limited and will place the animal at risk of deficiency (Dinesh et al., 2014). In this situation, greater dietary arginine is necessary to satisfy all of the metabolic roles for arginine, including creatine synthesis. The supplementation of GAA, or its product creatine, to spare the precursor amino acids has been reported in the literature. The supplementation of GAA was studied in broiler chicks (Dilger et al., 2013), and weaned pigs (McBreairty et al., 2015) and was found to increase tissue creatine concentrations compared to when creatine was given as the supplement. In a different study, creatine supplementation was tested in newly weaned pigs; however, it did not affect piglets' growth regardless of the amount added to the diet (Guzik et al., 2000). Another study tested creatine supplementation in growing finishing pigs and also found no positive effect on growth (O'Quinn et al., 2000). However, both of these studies provided sufficient amounts of the precursor amino acids, likely satisfying the requirement for creatine synthesis; as a consequence, no additional sparing benefit of

supplementing creatine was observed. We measured the effects of GAA or creatine supplementation when arginine and methionine were marginal in the diet, as well as diets where both arginine and methionine were presumably in excess. We also aimed to determine the effect of GAA supplementation on the availability of methionine; therefore, we added an additional diet group that was supplemented with GAA with methionine in excess. The objectives of this study were to assess the piglet's response to supplementation of varying amounts of dietary arginine, methionine, creatine, or GAA during TPN. We did this by quantifying the molar conversions of arginine into GAA, creatine, and whole body protein dynamics, by measuring tissue AGAT activity, quantifying the distribution of creatine and GAA in various tissues, and measuring nitrogen retention in order to determine the efficiency of dietary amino acid utilization.

4.1 The effect of GAA or creatine supplementation on whole body arginine kinetics and whole body protein dynamics

Theoretically, if dietary arginine and methionine are limited, then sparing these amino acids by the supplementation of creatine or its precursor GAA would lead to increased protein synthesis and growth. In this study, we observed no beneficial growth effect with the supplementation of GAA (alone) or creatine (Figure 3.1). Similar to McBreairty et al. (2015), GAA or creatine supplementation to weaned pigs did not affect growth compared to controls. Only when methionine was supplied in excess with GAA did we observe an advantage in weight gain, which demonstrated that dietary methionine was limiting growth. Indeed, all of the groups on low methionine PN grew more slowly than the group given GAA and methionine in excess. Limited methionine availability will limit protein synthesis; however, weight gain is not necessarily indicative of lean mass accretion. Nitrogen balance is a better marker of whole body

protein gain. In this study, the pattern in the nitrogen balance data (Figure 3.2) tended to parallel the weight gain data (Figure 3.1), but the only significant difference occurred between the piglets provided with GAA and those given GAA with high methionine when nitrogen balance was expressed as nitrogen retention (Figure 3.3). Taken together, these data demonstrate that methionine was limiting and that supplemental methionine enhanced whole body protein retention and growth.

GAA requires methionine for its conversion to creatine via GAMT, which is responsible for the methylation of GAA via SAM, to produce SAH and creatine in the liver (Wyss & Kaddurah-Daouk, 2000). Our group recently showed that when methionine is limited, creatine synthesis is prioritized over other methylation reactions (McBreairty et al., 2013). In addition, GAMT activity is not regulated by creatine; hence, creatine synthesis in the liver is always proportional to the amount of GAA available, provided sufficient methionine is available (McBreairty et al., 2013). Indeed, methionine deficiency can be induced by experimentally depleting methyl groups through the IV infusion of GAA. McBreairty and colleagues (2013) measured the effect of infusing GAA into 15-18 day-old suckling piglets that were either of a normal weight or intrauterine growth-restricted. The infusion of GAA in both groups induced a very high rate of methylation of GAA to creatine, at the expense of PC and protein synthesis. In our GAA plus methionine supplemented piglets, excess methionine appeared to satisfy the GAA methylation needs for the conversion to creatine, as well as the need for protein synthesis as demonstrated by greater nitrogen retention (Figure 3.3).

Another method of assessing efficiency of amino acid utilization was the measurement of whole body protein synthesis using isotope tracers. We infused stable isotopes of phenylalanine and tyrosine to measure the oxidation of phenylalanine via its conversion to tyrosine (the first

step in phenylalanine oxidation), and the non-oxidative disposal of phenylalanine, which represents whole body protein synthesis. It should be noted that dietary tyrosine was supplied in excess to avoid the need for tyrosine synthesis from phenylalanine. In contrast to our hypothesis, we found significantly greater hydroxylation of phenylalanine to tyrosine in the creatine group (Figure 3.16), indicating less incorporation of phenylalanine into proteins. Theoretically, we expected that the supplementation of creatine or GAA to a diet marginal in arginine would alleviate the need for arginine use for creatine synthesis, and shift arginine towards protein synthesis. It is interesting that we did not find this, as it is in contrast to the evidence from the arginine isotope tracer results. The arginine tracer indicated that less arginine was partitioned toward GAA synthesis when creatine or GAA was included in the diet (Figure 3.12). So arginine sparing was occurring, but not to enhance whole protein synthesis. The spared arginine may have been directed towards other metabolic pathways, or it may have enhanced organ-specific protein synthesis, such as in the liver, but was not detectable at the level of whole body kinetics.

Only when arginine and methionine were supplied in excess did they enhance the rate of whole body protein accretion (Figure 3.19), but only compared to creatine which had the lowest protein accretion. However, neither protein accretion nor nitrogen balance was different between the deficient Base group and the adequate methionine and arginine group, which raises the question of whether the arginine provided in the adequate group was in fact high enough to satisfy all of the metabolic requirements for arginine. Brunton et al. (2003) determined that (creatine-free) PN-fed piglets require >1.2 g.kg BW^{-1}.day^{-1} of dietary arginine to satisfy its protein synthesis needs in the presence of an atrophied gut. Based on that, we determined that our adequate arginine group would be fed 1.8 g.kg BW^{-1}.day^{-1} to meet or perhaps exceed

arginine requirements. Therefore, we should expect that that amount of arginine in the diet would meet the requirements for both creatine and protein synthesis, and that a supply of 0.55 g.kg BW^{-1}.day^{-1} of arginine (the limiting groups) should impair protein synthesis, without adversely harming the piglets by the consequential hyperammonemia (Brunton et al., 2003). However, our limited arginine groups only negatively affected protein accretion when creatine was supplemented, which is contradictory to what we expected. It is also possible that the low arginine group was not deficient leading to similar protein accretion, although this is unlikely given that PN arginine levels of 0.45 g.kg BW^{-1}.day^{-1} are likely to lead to life-threatening hyperammonemia (Brunton et al., 2003). Because methionine was also deficient and supplemented with arginine, it is also notable that methionine deficiency did not lead to lower protein accretion. Using the same rationale, it is possible that the level of methionine used was not deficient in this Yucatan miniature pig model, perhaps because this strain of pigs is slower growing than domestic piglets used in previous PN methionine requirement studies (Shoveller, et al., 2003a; Shoveller, et al., 2003b).

4.2 The effect of GAA or creatine supplementation on AGAT activity and tissue distributions of GAA and creatine

In neonatal piglets, AGAT has been found to be highly expressed in the pancreas and kidney (Brosnan et al., 2009). The expression of renal AGAT is upregulated by growth and thyroxine hormones, and downregulated by creatine supplementation (Humm et al., 1997). Evidence in the literature is highly suggestive of the kidney being the major contributor to creatine synthesis through the production of renal GAA and subsequent methylation via hepatic GAMT (Brosnan et al., 2009). Dinesh and colleagues (2018) found renal AGAT downregulation

when neonatal piglets were supplemented with creatine in PN diets for two weeks compared to controls. We report the same result for creatine supplementation, and further, GAA in the PN also caused a down-regulation of AGAT activity in the kidney (Figure 3.4). Similarly, Edison et al. (2007) found a reduction of AGAT activity and GAA production in rat kidney when creatine was supplemented in the diet. It is interesting to note that the activity of AGAT in the pancreas (Figure 3.5) was also affected by the provision of creatine to the piglets, but GAA supplementation did not produce the same down-regulation as measured in the kidney. da Silva et al. (2014) also found a downregulation of AGAT activity of rat pancreas when supplemented with creatine. It is possible that the pancreas responds differently to GAA supplementation than the kidney. It has been identified that both human and pig AGAT share at least 94% of the amino acid sequence (Humm et al., 1994); in addition, Gross et al. (1986) found that humans express different isoforms of AGAT. Therefore, it is possible that the AGAT isoforms expressed in the pig kidney and pancreas are different, which may explain our reported differences in the AGAT responses to GAA in these tissues.

The use of isotope tracers of amino acids is one of the popular methods to quantify the conversion or oxidation of each amino acid; the technique also allows for *in vivo* measurement of protein dynamics (Matthews, 2007). Although costly to analyze, the use of stable isotopes allows multiple tracers to be infused simultaneously to measure the fate of different metabolites at the same time. In this study, the method was very valuable, as it facilitated whole body kinetics measurements without the need to kill the piglets. The piglets underwent the stable isotope study on one day, and then underwent an additional radio-isotope tracer study the following day (data reported in the thesis of S.B.). From the stable isotope tracer data, we found that plasma arginine to GAA conversion rates (Figure 3.12) were supported by the enzyme activity data (Figure 3.4),

sharing a similar pattern. As predicted by the AGAT activity, the inclusion of creatine or GAA led to a lower conversion of arginine to GAA (Figure 3.12). Therefore, the addition of GAA or creatine spared arginine from GAA synthesis. The conversion of plasma arginine to creatine (Figure 3.13) includes the methylation step via GAMT. The provision of creatine or GAA also led to a lower conversion of arginine to creatine. However, the presence of GAA or creatine in the diet did not affect how much GAA was being converted to creatine (Figures 3.14 & 3.15), which was not different among treatments. While the provision of creatine or GAA down-regulated AGAT, no apparent changes in GAMT activity occurred, supporting the concept that AGAT is the rate limiting step in creatine synthesis.

Furthermore, the supplementation of excess arginine and methionine caused greater GAA retention in the kidney (Figure 3.7) which suggests that more arginine was utilized for the synthesis of GAA via renal AGAT. These data also suggest that supplementing creatine alone or GAA plus methionine may have spared arginine from GAA synthesis in the kidney. The supplementation of GAA appeared to enhance the tissue concentration of creatine in the kidney, but only when methionine was provided in excess to facilitate GAA methylation (Figure 3.10).

Interestingly, the brain concentrations of both GAA (Figure 3.6) and creatine (Figure 3.9) were not affected by the addition of either in the treatment diets. This might suggest that the brain has priority for the amino acids needed for creatine synthesis when arginine and methionine are limiting in the diet (Brosnan et al., 2009). Creatine is critical for the normal neurological function, as evidenced by the consequences of creatine deficiency symptoms (Leuzzi, 2002). However, it is unlikely that the piglet's brain satisfies its creatine needs by de novo synthesis alone, because it expresses a very low activity of GAMT with no detectable levels of AGAT; hence, it is more likely that the brain depends on the periphery for a supply of

creatine (Brosnan et al., 2009; Dinesh et al., 2018). While rat studies have suggested that the brain satisfies its own creatine supply (El‐Daher & Braissant, 2016), this seems unlikely for the piglet. Another possible reason for the lack of differences among our treatment groups in brain creatine concentration is that the turnover rate for brain creatine is very slow; therefore, we were unlikely to observe any differences in brain creatine concentration within one week of diet manipulation. Brosnan et al. (2009) found that neonatal piglets' brain mass and creatine concentration did not change significantly over one week, between 4 and 11 days of age (Brosnan et al., 2009). Hence, it is not surprising that we did not measure any differences in creatine concentration within the duration of our experiment, which only lasted for one week.

In the pancreas, supplementation of excess arginine and methionine appeared to enhance GAA retention (Figure 3.8). These data are in agreement with the pancreas AGAT activity data (Figure 3.5) which demonstrated an upregulation of AGAT in the adequate group, suggesting that more arginine was used for the synthesis of GAA in the pancreas. However, pancreas creatine concentrations (Figure 3.11) were not different regardless of treatment. Unlike the kidney, it appears that the pancreas is not affected by the provision of either creatine or GAA.

4.3 Conclusion and future directions

We hypothesized that a parenteral diet supplemented with creatine or its precursor GAA could spare arginine for growth and protein synthesis in neonatal piglets. We concluded that GAA supplementation with adequate methionine supports growth and nitrogen retention in the body. GAA may be a useful supplement in PN for neonates to satisfy creatine accretion and spare arginine, particularly because PN-fed neonates are at a high risk for arginine deficiency

(Becker et al., 2000); however, the requirement for methyl groups from methionine should be taken into account to avoid depletion of the methionine pool.

One finding from this study that requires further investigation is the lack of difference in protein accretion rates between the piglets provided adequate arginine and methionine versus those fed diets known to be deficient. This may be because the requirement for arginine is actually higher than that supplied in the adequate group (1.8 g.kg $BW.^{-1}day^{-1}$).

GAA and creatine supplementation spared arginine, as evidenced by the arginine tracer kinetics; however, it was not clear that the spared arginine was diverted towards protein synthesis when assessed at the whole body level. It would also be of interest to assess whether creatine enhances the cognitive development of these piglets compared to no creatine supplementation. It remains to be determined whether arginine was directed towards other important metabolic functions, or used to support organ-specific protein synthesis. A better method of assessing the protein status would be to measure the fractional protein synthesis rates of specific organs such as the liver, small intestine, and the skeletal muscle using a flooding dose of labeled phenylalanine. It would also be of interest to explore other metabolic pathways of arginine, such as the nitric oxide or polyamines pathways, to identify which pathways took priority when dietary arginine was limited but GAA or creatine was added to the diet.

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Appendices

Appendix I: The composition of the parenteral adaptation diet

L-Amino Acid	$g.L^{-1}$	$g.kg\ BW^{-1}.d^{-1}$
Alanine	4.50	1.22
Arginine	3.68	1.00
Aspartic acid	3.36	0.91
Cysteine	0.80	0.22
Glutamate	5.81	1.58
Glycine	1.51	0.41
Histidine	1.71	0.47
Isoleucine	2.55	0.69
Leucine	5.76	1.57
Lysine	4.58	1.25
Methionine	1.07	0.29
Phenylalanine	3.03	0.82
Proline	4.58	1.25
Serine	5.33	1.45
Taurine	0.25	0.07
Tryptophan	1.17	0.32
Tyrosine	0.43	0.12
Valine	2.93	0.80
Threonine	2.24	0.61
D-glucose and major minerals		
Dextrose	90.30	
Trihydrate K_2HPO_4	1.57	
Monobasic KH_2PO_4	1.09	
K acetate	1.47	
NaCl	2.17	
$MgSO_4$	0.78	
$ZnSO_4$	0.09	
Calcium gluconate	6.41	

* modified from the work of Wykes et al. (1993)

Appendix II: The composition of the trace elements used in TPN diet

Element	g/L
Zinc, $ZnSO_4.7H_2O$	40.7
Copper, $CuSO_4.5H_2O$	3.12
Manganese, $MnSO_4.H_2O$	1.86
Chromium, $CrCl_3.6H_2O$	0.0512
Selenium, SeO_2	0.0592
Iodide, NaI	0.0220

Appendix III: The compositions of the multivitamin solutions

Vitamin	Unit/ 4 mL of vial 1
Ascorbic acid (Vitamin C), *mg*	80
Vitamin A (as palmitate), *IU*	2300
Vitamin D_3 (cholecalciferol), *IU*	400
Thiamine (Vitamin B_1) as hydrochloride, *mg*	1.2
Riboflavin (Vitamin B_2) as riboflavin-5-phosphate sodium, *mg*	1.4
Pyridoxine HCl (Vitamin B_6), *mg*	1
Niacinamide, *mg*	17
Dexpanthenol (as d-pantothenyl alcohol), *mg*	5
Vitamin E (d1- α-tocopheryl acetate), *IU*	7
Vitamin K_1, *mg*	0.2
	Unit/ 1 mL of vial 2
Folic acid, *mcg*	140
Biotin, *mcg*	20
Vitamin B_{12} (cyanocobalamin), *mcg*	1

Inactive ingredients in vial 1: 50 mg polysorbate 80, sodium hydroxide and/or hydrochloric acid for pH adjustment.
Inactive ingredient in vial 2: 75 mg mannitol, citric acid and/or sodium citrate for pH adjustment