Arginine Metabolism and Creatine Biosynthesis in Yucatan Miniature Piglets

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ABSTRACT

Arginine metabolism and creatine biosynthesis in Yucatan Miniature piglets

Creatine and creatine phosphate are amino acid-derived compounds that are necessary to meet short-term energy requirements. Creatine is a potent neuromodulator such that it is critical for neurological development in neonates. Neonates receive creatine in mother's milk. However, up to 77% of the daily creatine requirement must be synthesized endogenously. Creatine synthesis involves the conversion of arginine to guanidinoacetic acid (GAA) via L-arginine:glycine amidinotransferase (AGAT). The subsequent conversion of GAA to creatine requires methionine and guanidinoacetate *N*-methyltransferase (GAMT). Following preterm birth, total parenteral nutrition (PN) is often required as a means of nutritional support. However, creatine is not a component of pediatric PN. In this situation, the entire creatine requirement must be met by *de novo* synthesis which consequently must create considerable demand for the amino acid precursors. Poor arginine status is common in neonates during PN, and this may compromise optimal creatine accretion. In this thesis, the capacity of the neonatal piglet to synthesize creatine was addressed, particularly when dietary creatine was not supplied. The data from first experiment demonstrated that PN support with creatine led to greater creatine concentrations in plasma and tissues, suggesting that neonates receiving PN may not be able to sustain optimal creatine accretion. The second major objective of this thesis was to investigate whether the low creatine accretion in piglets fed creatine-free diets was due to limited enzyme capacity or limited substrate availability. Using a multiple isotope

tracer method, we determined that inadequate dietary arginine and methionine, not enzyme capacity, limits GAA and creatine biosynthesis. Lastly, because we measured high AGAT activity in kidney and pancreas, we investigated how the plasma concentrations of precursor amino acids (arginine/citrulline) and creatine affected the release of GAA and creatine from these organs as well as from the intestine. We determined both arginine and citrulline can provide precursors for renal GAA synthesis. However, in the pancreas and intestine, citrulline cannot provide arginine to serve as a precursor for GAA. Overall, the results of this thesis clearly demonstrate that dietary concentrations of arginine and methionine are important mediators of creatine synthesis; the absence of dietary creatine, such as during PN feeding, must be taken into consideration when determining dietary requirements of neonates.

Key words: Arginine, citrulline, methionine, creatine, AGAT, GAMT, tracer: tracee, enrichment, flux, organ balance

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List of Abbreviations

AAO	Amino acid oxidation
AGAT	L-arginine:glycine amidinotransferase
Ala	Alanine
APE	Atom percent excess
Arg	Arginine
Arg/Met	Arginine and methionine
ASL	Argininosuccinate lyase
ASS	Argininosuccinate synthetase
BBB	Blood brain barrier
BCA	Bicinchoninic acid
BHMT	Betaine:homocysteine methyltransferase
BCSFB	Blood cerebrospinal fluid barrier
BW	Body weight
CA	Carotid artery
Cit/Met	Citrulline and methionine
CK	Creatine kinase
CPS-I	Carbamoyl phosphate synthetase
+Cre	Creatine with Arg/Met
CT	Creatine transporter
DMG	Dimethylglycine

DNA	Deoxyribonucleic acid
DNMT	DNA methyltransferase
E	Enrichment
EN	Enteral Nutrition
FSR	Fractional synthesis rate
GAA	Guanidinoacetic acid
+GAA	Guanidineacetic acid in low arginine and low methionine diet
GAMT	Guanidinoacetate N-methyltransferase
GC-MS	Gas chromatography mass spectrometry
GI	Gastrointestinal
GNMT	Glycine N-methyltransferase
HA	Hepatic artery
HPLC	High performance liquid chromatography
HV	Hepatic vein
I	Infusion rate
IAAO	Indicator amino acid oxidation
IGF-1	Insulin-like growth factor-1
IV	Intravenous
LC-MS/MS	Liquid chromatography mass spectrometry/mass spectrometry
MAT	Methionine adenosyltransferase
Met	Methionine

MRI	Magnetic resonance imaging
MSyn	Methionine synthase
MTHFR	Methylenetetrahydrofolate reductase
mTOR	Mechanistic target of rapamycin
NAFLD	Non-alcoholic fatty liver disease
NEC	Necrotizing enterocolitis
NO	Nitric oxide
NRC	National Research Council
OAT	Ornithine aminotransferase
OTC	Ornithine transcarbamylase
PC	Phosphatidylcholine
PCA	Perchloric acid
P5C	Pyrroline-5-carboxylate
P5CDH	Pyrroline-5-carboxylate dehydrogenase.
P5CS	Pyrroline-5-carboxylate synthase
PE	Phosphatidylethanolamine
PEMT	Phosphatidylethanolamine methyltransferase
PFBBr	Pentafluorobenzyl bromide
PITC	Phenylisothiocyanate
PN	Parenteral nutrition
PPHN	Persistent pulmonary hypertension
PV	Portal vein
Q	Flux

RV	Renal vein
SAM	S-adenosyl-L-methionine
SAH	S-adenosyl-L-homocysteine
SD	Standard deviation
SF	Sow fed
SHMT	Serine hydroxymethyltransferase
TFA	Trifluoroacetic acid
TG	Triglyceride
THF	Tetrahydrofolate
VLDL	Very low-density lipoproteins

CHAPTER ONE

1.0 Introduction

1.1 Creatine

Creatine is an amino acid-derived nitrogenous compound which occurs naturally in food, particularly in meat products and fish. Creatine and phosphocreatine together serve as a short-term energy reservoir in tissues with high and variable energy demands such as cardiac and skeletal muscles. Creatine is critical for brain function and neurological development in neonates. Severe creatine deficiency secondary to inborn errors of creatine synthesis or transport results in profound neurological defects, demonstrating its importance in brain development (Leuzzi et al, 2000, Battini et al, 2002, deGrauw et al, 2002, Royes et al, 2006). Creatine can also be synthesized endogenously by a dual-organ process involving the kidneys and the liver. In terms of loss, creatine is subjected to continuous degradation and is excreted as creatinine at a daily rate of approximately 1.7% in adult humans (Wyss et al, 2000), which represents the maintenance cost of creatine in the body. In addition to fulfilling the maintenance requirement, rapidly growing neonates must accrue creatine as lean tissue pools expand. A factorial assessment in piglets suggested that the absolute creatine need in growing neonates is greater than that supplied by porcine milk (~12.7 versus 2.8 $mmol.wk^{-1}$, respectively); consequently, neonates rely on *de novo* creatine synthesis to supply approximately 75% of the daily requirement (Brosnan et al, 2009).

1.1.1 Creatine biosynthesis

Endogenous creatine synthesis is a simple two-step multi-organ reaction involving the kidney and the liver **(FIGURE 1.1)**. Creatine synthesis requires three amino acids (arginine, glycine and methionine) and two enzymes (Walker et al, 1979). The first enzyme, L-arginine: glycine amidinotransferase [AGAT, EC number 2.1.4.1] is expressed in the kidney and pancreas and transfers the amidino compound from arginine to glycine in order to synthesize ornithine and guanidinoacetic acid (GAA). It has been postulated by Wyss et al (2000) that GAA is then transported to the liver where the methyl group of S-adenosyl-L-methionine (SAM), which was donated by methionine, is transferred to guanidinoacetate. The enzyme guanidinoacetate N-methyltransferase [GAMT, EC 2.1.1.2], the second enzyme in the creatine synthesis pathway, is responsible for this methylation reaction.

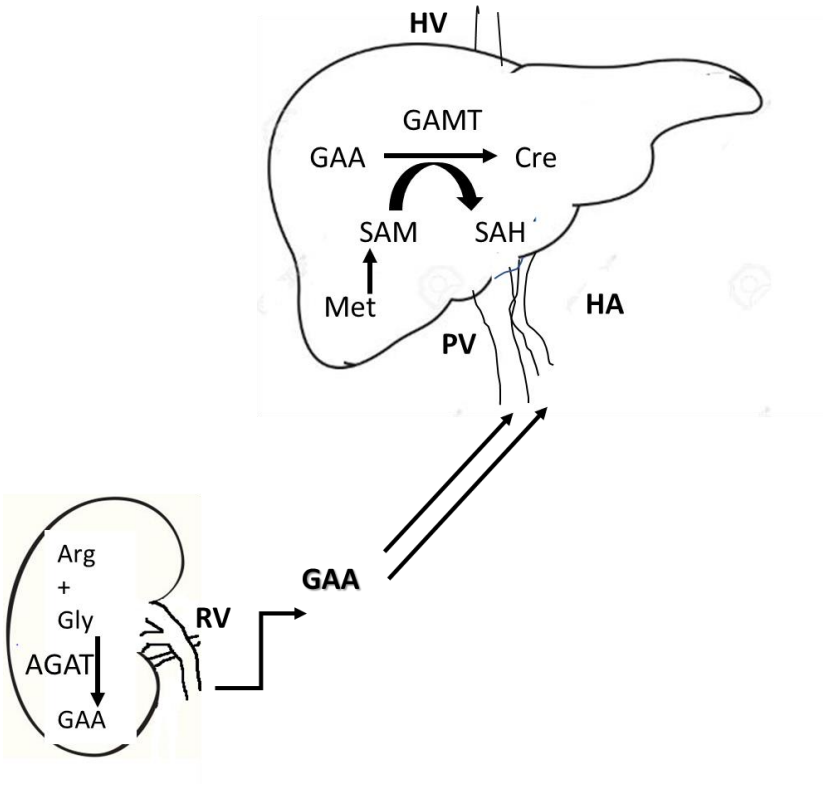

FIGURE 1.1 Creatine biosynthesis pathway

Abbreviations in the figure include, AGAT: L-arginine: glycine amidinotransferase, GAA: guanidinoacetic acid, GAMT: guanidinoacetate N-methyltransferase, HA: hepatic artery, HV: hepatic vein, PV: portal vein, SAM: S-adenosyl-L-methionine, SAH: S-adenosyl-L-homocysteine, Arg: arginine, Gly: Glycine.

The newly synthesized creatine is transported into the circulation via a sodium dependent creatine transporter (Fitch & Shields, 1966) for delivery to tissues and organs. Creatine is reversibly phosphorylated to phosphocreatine via creatine kinase. The larger pools of creatine and phosphocreatine are stored in tissues with high energy demands such as skeletal muscle, cardiac muscle and brain tissue (Wyss et al, 2000).

1.1.1.1 AGAT

The AGAT protein is highly expressed in the mammalian kidney and pancreas. A much lower level was reported in the brain, heart, lung, muscles, spleen and testes (Wyss et al, 2000, Braissant et al, 2005). According to early studies in the literature, AGAT is the rate-limiting enzyme for creatine biosynthesis and is subjected to end-product repression by ornithine and creatine (Sipila, 1980, McGuire et al, 1984).

1.1.1.2 Kidney and pancreatic AGAT activity

In the kidney, AGAT is predominantly bound to the external side of the mitochondrial inner membrane with a lesser amount located in the cytoplasm (Magri et al, 1975). According to immunochemical studies with monoclonal antibodies, the majority of transaminidase activity occurs in the proximal tubules of the kidney (McGuire 1984, Takeda et al, 1992). In the pancreas, immune-reactive transamidinase was detected only in acinar cells. The AGAT activity in pancreatic islet cells was below the detectable limit. Furthermore, the

whole pancreas AGAT activity was similar to that of the remaining pancreatic tissues after the islets had been separated from the acinar cells (Sorenson et al, 1995, da Silva, 2014).

1.1.1.3 Interorgan creatine biosynthesis in adults

In rats, the highest AGAT activity has been reported in kidney and pancreas; in contrast, a relatively high GAMT activity has been reported in the liver, and intermediate activity has been measured in the pancreas, compared to kidney and muscles (Walker et al, 1979). Furthermore, recent *in-vivo* studies have demonstrated the release of GAA by the kidneys in rats and humans (Edison et al, 2007). The conversion of GAA into creatine was further demonstrated in isolated rat hepatocytes (Edison et al, 2007, da Silva et al, 2009). Therefore, it is likely that a renal-hepatic axis for creatine biosynthesis is present in adults. A recent study by da Silva et al (2014) demonstrated that in pancreatic tissues from rats, GAMT activity was only 30% that of the specific activity of AGAT. This suggests that the capacity for GAA synthesis by the pancreas is greater than the capacity for creatine synthesis in this organ, and that the pancreas releases GAA into the portal blood (da Silva et al, 2014). However, no in vivo evidence for the role of the pancreas in whole body GAA and creatine supply currently exists to our knowledge.

1.1.1.4 Creatine biosynthesis in neonates

Neonates acquire only 23% of their creatine requirement via mother's milk (Brosnan et al, 2009). This means that the remaining 77% of the creatine requirement must be synthesized

by the neonate. In 7 – 8 d old sow-reared neonatal piglets, Brosnan et al (2009) studied the liver, kidney, pancreas, intestine, brain and muscle specific AGAT and GAMT activities. Pancreas had the highest AGAT specific activity among the organs they measured, with the kidney having the second highest. The AGAT activity in the pancreas was five times of that measured in the kidney on a per g of tissue basis. Liver had the highest specific GAMT activity among the tissues studied followed by pancreas and then kidney. The GAMT activity in liver tissue was four times higher than pancreas GAMT activity, and the pancreas GAMT activity was three times higher than the kidney. Based on carcass analysis, piglets between the ages of 4 and 11 days accrued 12.5 mmol creatine, and spontaneous loss was estimated as 2.3 mmol per week. Therefore, neonates must experience a high rate of de novo creatine synthesis, supported by high rates of activity by creatine synthesizing enzymes (Brosnan et al, 2009). Similar to adults, the highest AGAT activity appears to exist in the kidney and pancreas compared to liver, intestine, brain and muscle, whereas the highest GAMT activity occurs in the liver in neonatal piglets (Brosnan et al, 2009). However, in neonatal piglets, the existence of a renal-hepatic axis for the creatine biosynthesis has yet to be identified. The role of the pancreas in creatine synthesis also remains unknown in neonatal piglets. A direct measurement of GAA and creatine inter-organ fluxes in piglets is required to confirm the importance of the kidney, pancreas and liver for creatine bio-synthesis.

1.1.1.5 Interactions between creatine supplementation and AGAT and GAMT activity

In adult humans and rodents, creatine synthesis appears to be regulated by the activity of AGAT. Studies in rats that were fed diets supplemented with creatine reported lower AGAT activity and lower expression of AGAT mRNA compared to controls (McGuire et al., 1984, da Silva et al, 2014), demonstrating that creatine synthesis appears to be regulated via lower GAA production. In healthy adults, dietary creatine lowers the circulating GAA levels, suggesting dietary creatine inhibits endogenous GAA synthesis (Derave et al, 2004). In recent studies conducted in healthy rodents, kidney AGAT activity was lowered by more than 80% when creatine was provided in the diet at a concentration that was four-fold higher than the daily requirement (da Silva et al, 2009, 2014 & Deminice et al, 2011). However, the GAMT activity was not altered with creatine supplements. Therefore, AGAT is considered to be the rate limiting step in the creatine biosynthesis pathway in adult humans and rodents (Walker et al, 1979, McGuire et al, 1984). The capacity for creatine biosynthesis has not be been studied in neonates, nor has the AGAT and GAMT activities in response to creatine supplementation. In an acute study with neonatal piglets, GAA was infused intravenously to measure the effect of high circulating GAA on GAMT activity. It was demonstrated that the GAMT activity was driven by the GAA concentration, with no apparent feed back regulation via creatine (McBreairty et al, 2013). The effects of dietary creatine supplementation on the activities of AGAT and GAMT must be explored to elucidate the factors that affect the regulation of creatine biosynthesis in neonates.

1.1.1.6 Differential regulation of AGAT activity in the kidney and pancreas

The regulation of kidney and the pancreatic AGAT activity in response to dietary creatine may differ between tissues. A recent publication by da Silva et al (2014) supports the idea that the feeding of creatine induces a reduction of AGAT activity in both kidney and pancreas in adult rats. In addition to the reduction of AGAT activity, the kidney had lower AGAT mRNA expression and lower AGAT protein abundance in rats fed creatine compared to rats fed a creatine-free diet. In contrast to this, pancreatic AGAT mRNA levels and AGAT protein abundance were not different between creatine-fed and creatine-free groups. Thus, kidney AGAT was likely regulated by a pre-translational mechanism while this mechanism seemed to be absent in pancreas. Furthermore, the reduction of rat pancreatic AGAT activity was only 30% compared to 80% in the kidney (da Silva et al 2014). The different responses to creatine supplementation in kidney versus pancreas may be due to different roles for these organs in creatine homoeostasis. The kidney and pancreatic responses to creatine supplementation should be addressed in future studies of neonates.

1.1.2 Creatine transporter (CT)

In various tissues, cellular creatine uptake occurs via a sodium and chloride ion dependent creatine transporter protein (SLC6A8). The CT expresses in most tissues, with the highest levels found in skeletal muscle (Murphy et al, 2001), brain, kidneys and the intestine (Brosnan et al, 2007). Based on the intra-cellular to extra-cellular creatine gradient, the

same creatine transporter (SLC6A8) does not seems to be responsible for creatine release from the liver as is found in skeletal muscle (da Silva, 2009). This suggests the expression of different creatine transporters.

1.1.3 Creatine availability during fetal development

In a spiny mouse model, the concentration of creatine in the kidney, liver, heart and brain of the fetus gradually increases during gestation, especially during the second half of the pregnancy (Ireland et al, 2009). Placental creatine concentrations also increase similarly to the fetal pattern. The placenta expresses relatively high amounts of creatine transporters and the transporter mRNA expression level peaks during late gestation in full-term pregnancies (Ireland et al, 2009). mRNA and protein levels for kidney AGAT and liver GAMT peak at 37 d or immediately after birth of a full-term pregnancy (Ireland et al, 2009) suggesting that the fetus relies on a supply of creatine from the placenta during fetal development. Growing neonates must accumulate substantial amounts of creatine; however, in a study using rats, only 12% of the total creatine accrued originated from rat milk (Lamarre et al, 2010). This suggests that the capacity for creatine synthesis gradually increases during fetal development and reaches a mature level sometime after birth to facilitate the shift from maternal dependency to endogenous synthesis of the creatine required. It is yet to be explored whether a premature birth interrupts the maturing of enzymes involved in creatine biosynthesis compared to that in a full-term birth.

1.1.4 Brain creatine homeostasis

The source of creatine for the brain was thought, for many years, to come from the periphery (Wyss et al, 2000). However, it was more recently determined that AGAT and GAMT expresses within the brain, and within the brain, AGAT and GAMT express in a dissociated manner (Braissant et al, 2016). This suggests that within the central nervous system, GAA must be transported to GAMT expressing cells. Isotopic kinetic experiments demonstrated that GAA transported from AGAT expressing brain cells to GAMT expressing cells via the same transporter as creatine, SLC6A8 (Braissant et al, 2016). The transporter SLC6A8, is expressed by microcapillary endothelial cells at the blood-brain-barrier (BBB) but is absent from surrounding astrocytes (Braissant et al, 2016). This suggests that the BBB may have a limited permeability for peripheral creatine. The literature suggests that no transport of GAA occurs at the BBB under normal physiological conditions. However, a net exit of GAA appears to occur at the blood-cerebrospinal-fluid-barrier (BCSFB) most probably though the taurine transporter TauT (Braissant et al, 2016).

1.1.5 Creatine deficiency and neurological disorders

The brain creatine pool is relatively insignificant compared to the whole-body creatine pool, representing less than 2% of the entire body pool in neonatal piglets (Brosnan et al, 2009). However, adequate brain creatine is of physiological importance and is critical to neurological developmental processes in neonates. Profound negative neuro-developmental effects have been reported secondary to inborn errors of the enzymes

involved in creatine synthesis or defects in the creatine transporter. Infants with these genetic abnormalities exhibit neurological symptoms, including epilepsy, speech delay and mental retardation, if left untreated. Studies using magnetic resonance imaging (MRI) has revealed a profound depletion of brain creatine in infants with these genetic abnormalities (Clark et al, 2015). Case studies of infants suffering from creatine deficiency syndromes (ie. GAMT deficiency), have reported slow and variable recovery of cerebral creatine concentrations with creatine supplementation (Leuzzi et al, 2000; Stockler et al, 1996). Low permeability of the blood-brain-barrier to creatine has been suggested as the reason for the slow recovery of brain creatine levels (Hanna-El-Daher & Braissant, 2016). Creatine supplementation does not entirely reverse the pathology; however, very early provision of creatine appears to be beneficial for preventing negative neurological effects (Item et al, 2001). Evidence from rodent models and humans supports the capacity for brain de novo creatine synthesis. However, in neonatal piglets, brain AGAT activity was not detected and GAMT activity was very low (Brosnan et al, 2009). The precise mechanisms by which GAA and creatine are made available to the brain are not clear. The recovery of brain creatine levels in infants who suffer creatine deficiency syndromes suggests that the healthy developing brain has the capacity to capture creatine from the periphery (Stockler-Ipsiroglu et al, 1996). However, the complete recovery of brain creatine concentrations may take one to two years following the initiation of supplementation (Stockler-Ipsiroglu et al, 2015). Therefore, the accrual of brain creatine seems to be a very slow process, even in a pathophysiological state of compromised creatine synthesis. Whether human neonates are capable of de novo creatine synthesis in the brain is unclear.

Accretion of creatine in the fetal brain likely depends on both the maternal supply and endogenous de novo synthesis in the fetus (Hanna-El-Daher & Braissant, 2016). In the first weeks after birth, piglets grow remarkably fast, demanding a high rate of creatine accretion for the expanding muscle mass. In the study on creatine accretion in early life, the piglets' body weight increased by 50% between 4 and 11 days of age but there was no change in the brain weight (Brosnan et al, 2009). The total body creatine pool increased by 71% over 7 days and the circulating creatine pool increased by 171%. In contrast, the brain creatine pool did not change. Therefore, unlike muscle, the brain does not appear to have the same need for rapid creatine accretion in the neonatal period. The rate of creatine degradation in the neonatal brain is also unknown but is likely very slow. In infants with AGAT deficiency, neurological symptoms do not become apparent until the second year of life (Mercimek-Mahmutoglu et al, 2009). Thus, brain creatine likely degrades too slowly to create a deficit until the second year of life. Based on current knowledge, it is unclear whether the brain synthesizes its own creatine locally or takes it up from the periphery, or whether both synthesis and uptake contribute to maintain the brain creatine equilibrium in neonatal piglets. Creatine deficiency disorders secondary to inborn errors in creatine metabolism have been shown to result in profound neurological defects, demonstrating the importance of creatine in brain metabolism. Under normal health conditions, neonates receive a dietary supply of creatine. However, certain dietary treatments, such as the use of soy-based infant formulas or pediatric parenteral nutrition (PN), are devoid of creatine. In these circumstances, the requirement for creatine must be met entirely by *de novo* synthesis from the amino acids arginine, glycine and methionine.

1.2 Arginine

Arginine is an aliphatic straight chain amino acid with a guanidine group at the distal end of the chain. As the guanidine group is positively charged in neutral, acidic and even somewhat basic environments, arginine shows basic chemical properties.

1.2.1 Biological importance of arginine

Arginine is an important and abundant amino acid. It constitutes approximately 6.9% of total body proteins in fetal and neonatal piglets (Williams et al, 1954) and 7.7% in the human fetus (Widdowson et al, 1979). Arginine is also involved in the synthesis of other biologically important molecules such as nitric oxide (NO), polyamines and creatine. Arginine plays a critical role as a urea cycle metabolite and is therefore important for ammonia detoxification. The fate of arginine catabolism via the urea cycle or through NO or creatine synthesis depends on the cellular activities of the enzymes involved in the different synthetic pathways, as well as the availability of substrates.

Nitric oxide is an end product of arginine metabolism whereby arginine is converted into citrulline and nitric oxide. NO acts as a vasodilator which modulates blood pressure and thus blood flow. NO also acts as a neurotransmitter and mediator of the immune response (Homer & Wanstall, 2000). The amount of arginine utilized for NO synthesis accounts for a small amount in adult man, such that the fraction of whole-body arginine flux associated with NO synthesis in healthy humans was 1.2% (Castillo et al, 1996).

Furthermore, whole-body arginine flux associated with urea synthesis is 15% in healthy humans (Castillo et al, 1996). Currently, there are no data available for neonates to describe the proportion of arginine flux dedicated to urea and NO synthesis.

Arginine is also a precursor of ornithine, which is the amino acid precursor for polyamine synthesis. Polyamines (putrescine, spermidine and spermine) regulate gene expression, DNA and protein syntheses, apoptosis, cell proliferation and differentiation, and thus are essential for growth and differentiation of cells in many tissues including intestinal epithelial cells (Johnson, 1988; Blachier et al, 1995).

The necessity for de novo creatine synthesis may place a significant demand on arginine, potentially limiting the partitioning of arginine towards growth or other metabolic pathways. However, the proportion of arginine flux required to meet the demands for creatine synthesis have not yet been empirically determined in human infants or neonatal piglets.

1.2.2 Arginine de novo synthesis

1.2.2.1 Arginine de novo synthesis in adults

In an *in-vitro* experiment, it was determined that the small intestine of adults converts proline, glutamate and glutamine effectively into citrulline (Jones, 1985), a precursor for arginine. This is due to high activities of pyrroline-5-carboxylate synthase (P5CS) (Jones, 1985), ornithine aminotransferase (OAT), carbamoyl phosphate synthetase (CPS-1) and

ornithine transcarbamylase (OTC) activities in adult intestinal mucosa (Wu, 1998) **(FIGURE 1.2).** However, a study by Marini et al (2010) demonstrated that the dietary glutamine and proline made only a minor contribution to the synthesis of citrulline in mice, even when the mice were fed an arginine-free diet. The extraction of plasma arginine and ornithine by the small intestine was adequate to support citrulline synthesis even with the arginine-free diet. In another study conducted in mice, using multiple isotope tracers, Marini et al (2012) showed that dietary and plasma arginine were the main precursors for citrulline synthesis in the gut during feeding and food deprivation, respectively (Marini et al, 2012).

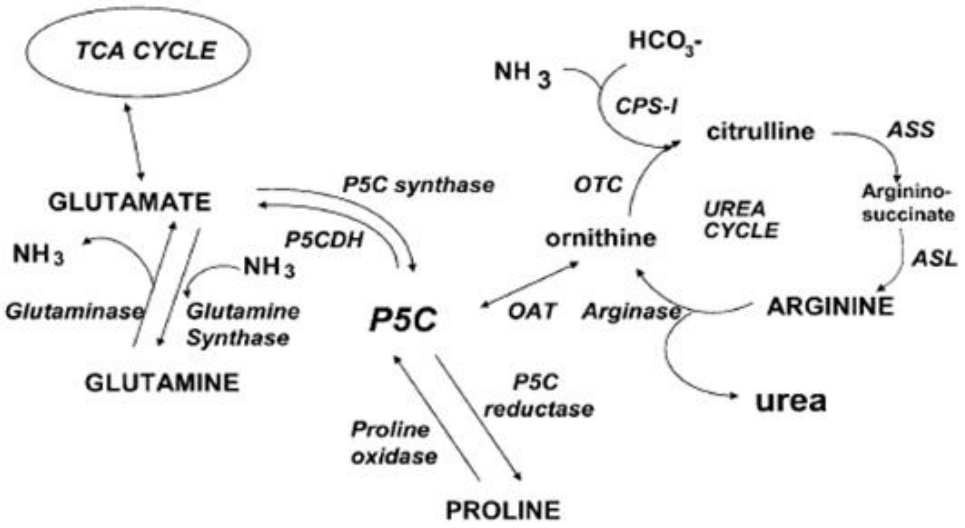

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FIGURE 1.2: Metabolic pathway of arginine

This figure represents arginine metabolism in adult human (small intestinal-renal axis). Abbreviations ASL: argininosuccinate lyase; ASS: argininosuccinate synthetase; CPS-I: carbamoyl phosphate synthetase; OAT: ornithine aminotransferase; OTC: ornithine transcarbamylase; P5C: pyrroline-5-carboxylate; P5CDH: pyrroline-5-carboxylate dehydrogenase.

Low intestinal activities of argininosuccinate synthetase (ASS) and argininosuccinate lyase (ASL), and high activity of arginase results in the net release of citrulline into the portal vein (Cynober et al, 2002). In contrast, appreciable ASS and ASL enzyme activities in the kidney in conjunction with very low renal arginase activity facilitate net arginine release into the peripheral circulation (Brosnan et al, 2003; Dhanakoti et al, 1990). Therefore, small intestinally-derived citrulline is the major precursor for renal de novo arginine synthesis; thus, the small intestinal–renal axis is considered to be the major pathway for arginine de novo synthesis in adult humans. Using an *in-vivo* amino acid tracer technique, Ligthart-Melis et al (2007; 2008) reported that arginine was synthesized from glutamate and glutamine in adult humans. However, those results were obtained using nitrogen-labelled tracers, which have been criticized because the movement of the tracer could potentially reflect transamination. Recently, a study by Tomlinson et al (2011a) used multiple carbon labelled tracers (arginine and proline) to determine whether dietary proline is a precursor for de novo arginine synthesis in adult humans. They demonstrated that 40% of newly synthesized arginine was derived from dietary proline. Thus, proline is considered an important dietary precursor for arginine synthesis in the adult human. A subsequent study by the same research group also clearly identified glutamine as a precursor for arginine, by using multiple glutamine tracers (^{13}C and ^{15}N). Based on the ^{13}C tracer enrichments and flux, 50% of newly synthesized arginine was derived from glutamine (Tomlinson et al, 2011b).

1.2.2.2 Arginine de novo synthesis in neonatal piglets

Arginine is a metabolically important amino acid for neonates during the rapid growth period, and the consequences of limited arginine availability have captured the attention of clinicians and researchers.

Arginine concentration in sow milk is relatively low (4.4% of total amino acids at d 21 of lactation) compared to the concentration of some other indispensable and conditionally indispensable amino acids such as proline (11.7%), leucine (8.9%), lysine (7.9%) or glutamate and glutamine (20.8%) (Davis et al, 1994). Mature sow milk provides approximately 0.47 $g.kg^{-1}.d^{-1}$ of arginine to the suckling neonatal piglet (Moughan et al, 1992). According to the NRC recommendations (1998), the dietary requirement for arginine for a healthy neonatal piglet is 0.38 $g.kg^{-1}.\ d^{-1}$; as such, it is met by the arginine concentration in sow milk. However, Brunton et al (2003) measured protein synthesis rates as a functional determinant of the adequacy of arginine intake in parenterally-fed piglets; the intravenous arginine supply necessary to maximize muscle protein synthesis was greater than 1.2 $g.kg^{-1}.d^{-1}$. Sow milk is a relatively poor source of arginine as sow milk provides only 40% of the arginine required by the neonate. However, sow milk contains substantial amounts of the arginine precursors glutamine, glutamate and proline and trace amounts of citrulline and ornithine (Wu et al, 1994). Thus, sow-reared, neonatal piglets must synthesize a significant portion of their daily requirement of arginine to maximize growth.

De novo arginine synthesis in neonates does not follow the same intestinal-renal axis as has been described in adults. A series of studies in neonatal piglets have clearly shown that arginine synthesis occurs in the small intestine (Brunton et al, 1999; Bertolo et al, 2003; Wilkinson et al, 2004). Brunton et al (1999) were the first to demonstrate in neonatal piglets that feeding an arginine-free diet intravenously resulted in the rapid onset of severe hyperammonemia, despite the presence of proline in the diet. The same diet delivered intragastrically resulted in only moderate hyperammonemia after eight hours of feeding, indicating that de novo arginine synthesis occurred from dietary proline only when delivered into the gut. Bertolo et al (2003) used isotope kinetic analyses to isolate the metabolic roles of the small intestine in the inter-conversions of arginine, proline and ornithine in neonatal piglets. This study isolated intestinal metabolism from hepatic and peripheral metabolism by infusing isotopes into the portal vein. They observed an absence of proline to ornithine conversion and very low ornithine tracer conversion into arginine during intraportal compared to intragastric tracer infusions. Furthermore, there was no difference in arginine to ornithine or ornithine to proline conversion between the two routes of feeding. Only 10% of proline requirement was synthesized in the small intestine compared to 50% of arginine requirement (Bertolo et al, 2003). Accordingly, near absence of small intestinal proline de novo synthesis facilitated a net positive release of arginine into the portal blood. Bertolo et al (2003) were the first to use multiple tracers to estimate the arginine de novo synthesis in the small intestine. Wilkinson et al (2004) also found 42–63% of whole-body arginine synthesis occurred during the first pass intestinal metabolism in neonatal piglets. Later, Urschel et al (2005) demonstrated that the hepatic contribution to net arginine synthesis is negligible. Indeed, they found that net arginine de novo

synthesis is dominated by the small intestinal mucosa of neonatal piglets by isolating the first pass hepatic metabolism through the infusion of intraportal and intravenous tracers. Based on the enzyme activities (*in-vitro*) measured in mucosal tissues in humans and piglets (Kohler et al, 2008; Wu, 1998), and *in-vivo* multi-tracer studies using various routes of administration in neonatal piglets (Bertolo et al, 2003; Wilkinson et al, 2004), it is clear that arginine de novo synthesis occurs in the small intestinal enterocytes in neonates.

1.2.2.3 Intestinal-renal axis for arginine synthesis in neonates

In the early stages of neonatal life, the functionality of the intestinal-renal axis for the arginine *de novo* synthesis is doubted. Urschel et al (2007) conducted a study in piglets which provided citrulline in the diet in an amount that was equimolar to a high arginine intake (1.8 arginine $g.kg^{-1}d^{-1}$) along with an arginine deficient diet (0.2 g $arginine.kg^{-1}d^{-1}$) fed intragastrically. Normal plasma ammonia and urea concentrations were sustained; however, compared to piglets fed the arginine deficient diet, the whole-body arginine flux was more than two-fold higher in piglets supplemented with citrulline. Interestingly, arginine fluxes did not differ between piglets fed high arginine and those supplemented with citrulline. Further studies are warranted to explore whether citrulline is a potent precursor for arginine synthesis in neonatal piglets and whether the conversion of citrulline to arginine occurs in the renal tissues of neonatal piglets.

1.2.3 Arginine as a semi-indispensable amino acid for sow-reared neonatal piglets

In contrast to healthy adults, healthy neonatal piglets have been shown to require at least part of their whole-body arginine requirement from diet. Apparently, the capacity for de novo synthesis is not sufficient to meet the whole-body needs during rapid growth and development (Wilkinson et al, 2004; Kim & Wu, 2004). A study by Kim & Wu (2004) demonstrated that even with relatively normal feeding circumstances, dietary arginine intake at a concentration delivered in sow milk might be inadequate. In that study, a sow milk replacer was used to feed piglets. The formulas contained arginine at either the sow milk level or was supplemented with arginine at 0.2% and 0.4%. The piglets fed arginine at the sow-milk level had significantly lower plasma arginine, citrulline and ornithine concentrations by days 14 and 21 of age; this was accompanied by significantly higher plasma ammonia (Kim & Wu, 2004). Arginine supplementation enhanced daily weight gain by 28 and 66% in the two groups, compared to control piglets who received arginine at the sow milk level. The authors suggested that even normal, healthy neonatal piglets cannot synthesize adequate amounts of arginine to meet the requirement for rapid growth during early life. However, it is important to note that colostrum and sow milk are also important sources of polyamines and other growth factors (Kelly et al, 1991; Cheng et al, 2006) as well as providing exogenous creatine (Brosnan et al, 2009). Thus, a formula diet containing arginine at the concentration found in sow milk may not produce a rate of growth that is truly reflective of the sow-reared piglet.

1.2.4 Arginine partitioning into NO and urea

Arginine is the precursor for NO synthesis. The fraction of whole-body arginine flux associated with NO synthesis in healthy adult humans is small (1.2%) (Castillo et al, 1996). In healthy adults, the first-pass splanchnic use of dietary arginine was about 38% (Castillo et al, 1993). Interestingly, the amount of dietary arginine intake did not have an effect on NO synthesis, and a similar proportion of arginine flux was used for NO synthesis in both fed and fasted situations (Castillo et al, 1996). In contrast, dietary L-arginine supplementation stimulated endothelial NO synthesis in adult rats (Kohli et al, 2004). Currently, there are no such data available for healthy human neonates. In sick neonates recovering from persistent pulmonary hypertension (PPHN), NO synthesis accounted for less than 0.5% of plasma arginine flux, which was less than the proportion of arginine converted into NO in healthy adults. In the same study of infants with PPHN, Castillo et al (1995) reported that the rate of plasma arginine conversion to NO synthesis was higher (45.6 versus 10.3 μmol. d^{-1}) when dietary arginine intake was 36 compared to 16 μmol.kg^{-1}.h^{-1}. Urschel et al (2007) studied the synthesis of NO from dietary arginine in healthy neonatal piglets. In contrast to sick human neonates (0.5%), Urschel et al (2007) demonstrated that NO synthesis accounted for 13% of whole-body arginine flux in enterally fed neonatal piglets, irrespective of the amount of arginine in the diet. In the Urschel study, the isotope tracers were infused into a gastric catheter, and as such included small intestinal NO synthesis, as opposed to Castillo's study which used IV infusion. The difference between the two studies in the amount of arginine flux directed to NO synthesis is quite large, which may be due to the route of infusion, or the model used. Urschel et al (2007)

fed either generous (1.8 $g.kg^{-1}.d^{-1}$) or deficient (0.2 $g.kg^{-1}.d^{-1}$) arginine diets intragastrically to piglets and found significantly higher whole-body arginine conversion into NO with the higher intake of arginine. Accordingly, piglets fed the generous arginine diet had significantly higher NO synthesis compared to piglets fed the deficient arginine diet, but the proportion of whole-body arginine converted into NO was not different. Thus, greater arginine intake led to greater NO production in both sick human neonates and neonatal piglets; however, arginine deficient piglets could not up-regulate NO synthesis.

1.2.5 Importance of arginine in creatine synthesis in neonatal piglets

Despite the importance of arginine and creatine in neonates, there have been no reports of the impact of dietary arginine availability on GAA and creatine concentrations in an *in-vivo* neonatal model. A piglet study from our lab produced preliminary data on the tissue and plasma GAA and creatine concentrations when piglets were provided with very low to very high concentrations of dietary arginine in an intravenous diet for a duration of less than 20 hours. We found that the liver and plasma GAA and creatine concentrations reflected the short-term manipulation of dietary arginine in PN, suggesting the importance of adequate arginine intake to maintain creatine concentrations in neonatal piglets. Understanding the relationship between dietary arginine intake and creatine status in neonates is also important as the concentrations of arginine and creatine in the commercial infant formulas are quite variable, and at the present time all commercial parenteral products are devoid of creatine.

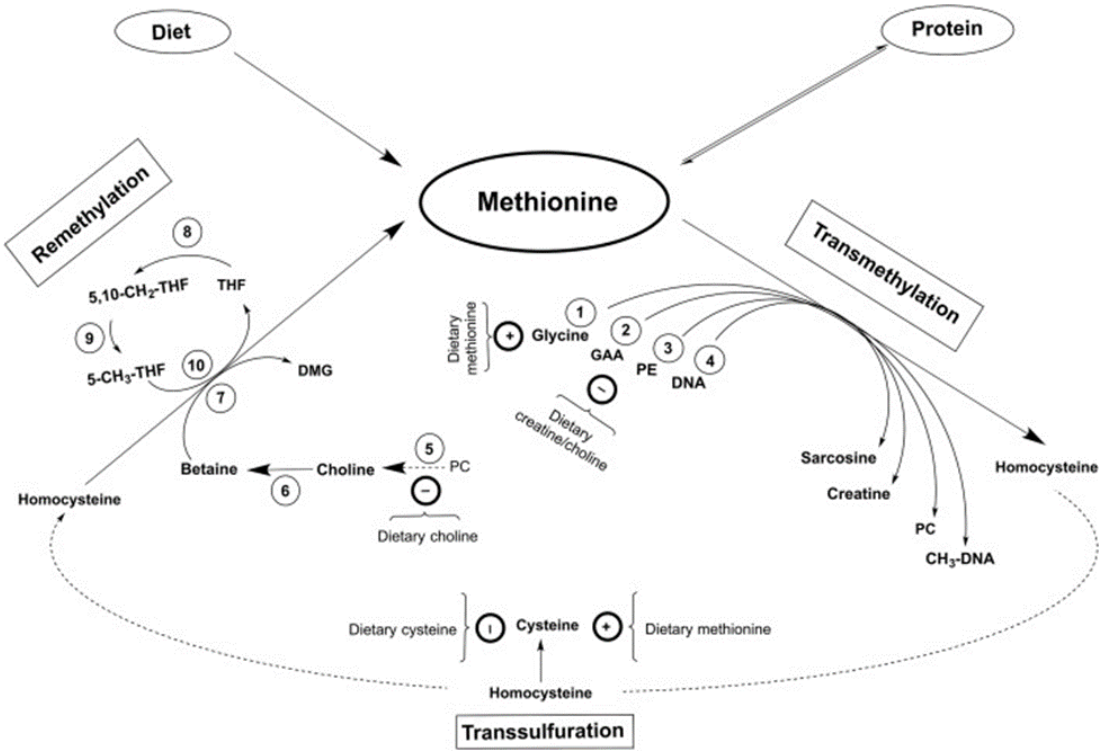
1.3 Methionine

Methionine is a sulfur amino acid that is involved in multiple important metabolic pathways. Methionine, similar to most other amino acids, is primarily involved as a substrate for protein synthesis. Methionine is also a key precursor for the synthesis of non-protein metabolites such as cysteine, taurine, glutathione, and as the major methyl donor, it is involved in the synthesis of number of methylated products including creatine, phosphatidylcholine (PC) and DNA.

1.3.1 Methionine cycle

In the methionine cycle, methionine is adenylated to form S-adenosylmethionine (SAM) via methionine adenosyltransferase (MAT). SAM is a universal methyl donor for many transmethylation reactions. SAM transfers terminal methyl groups (labile methyl groups) to various substrates for the synthesis of transmethylated products and S-adenosylhomocysteine (SAH) via various methyltransferases. Creatine and PC are quantitatively the major methylated products, but SAM is involved in numerous other methylation reactions including DNA methylation, and it is also involved in polyamines synthesis. SAH conversion into homocysteine is a reversible reaction. Homocysteine has two pathways and can transfer its sulfur atom irreversibly to cysteine through a "transsulfuration" reaction. Alternatively, homocysteine may be remethylated to methionine by methyl donors through one of two distinct pathways. In one reaction, methionine synthase (MSyn) transfers the labile methyl group from 5-

methyltretrahydrofolate (folate) to homocysteine. Cyanocobalamin (B12) act as a cofactor for this reaction. A second route involves betaine: homocysteine methyltransferase (BHMT) which transfers a methyl group from betaine, a nutrient derived from choline, to homocysteine (Robinson et al, 2016a).



Used permission from the Oxford University Press (Robinson et al, 2016a)

FIGURE 1.3 Schematic diagram of methionine cycle

Circled numbers indicate the enzymes: 1) glycine N-methyltransferase (GNMT, (EC 2.1.1.20); 2) guanidinoacetate methyltransferase (GAMT, EC 2.1.1.2); 3) phosphatidylethanolamine methyltransferase (PEMT EC 2.1.1.17); 4) DNA methyltransferase (DNMT, EC 2.1.1.204/37); 5) various phospholipases (mostly phospholipase D; EC 3.1.4.4); 6) both choline dehydrogenase (EC 1.2.1.8) and betaine aldehyde dehydrogenase (EC 1.1.3.17); 7) betaine:homocysteine methyltransferase (BHMT, EC 2.1.1.5); 8) serine hydroxymethyltransferase (SHMT, EC 2.1.2.1); 9) methylenetetrahydrofolate reductase (MTHFR, EC 1.5.1.15); and 10) methionine synthase (MSyn, EC 2.1.1.13). DMG, dimethylglycine; GAA, guanidinoacetic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; THF, tetrahydrofolate.

1.3.2 Methionine synthesis

Methionine is an indispensable amino acid, but its metabolism is partially cyclic; when methionine participates in transmethylation reactions, it can be reformed (remethylated) from methyl donors such as folate (MSyn pathway) or betaine/choline (BHMT pathways). According to current knowledge on methionine metabolism, methionine becomes available to the body in three ways, dietary methionine intake, remethylation of homocysteine by dietary methyl donors such as folate and choline (via betaine) and methionine release via body protein break down. In a study by Robinson et al (2016b), piglets were fed either a methyl donor deficient diet or a methyl donor sufficient diet. These piglets were fed methionine at the requirement concentration for 5 days. On day six, dietary methionine was reduced to 80% of the requirement in both groups. Transmethylation and remethylation were affected by deficient methionine, however, neither transsulfuration or protein synthesis were affected. This suggests the deficiency of methionine sustained protein synthesis by sacrificing transmethylation and remethylation reactions (Robinson et al, 2016b). According to Robinson et al (2016b) and Riedijk et al (2007), the remethylation accounts for only 7 - 10% of the whole-body methionine flux, however, a major portion of the rate of appearance of methionine was directed towards protein synthesis and transmethylation reactions. Transmethylation reactions are critical during neonatal development and because they are sacrificed during methionine deficiency, the methionine requirement must include the demands for all the fates of methionine, including protein synthesis, transsulfuration, and transmethylation.

1.3.3 Methionine requirement

The methionine requirement of piglets was determined using the indicator oxidation technique (Shoveller et al, 2003a). This method measures the methionine intake necessary to optimize whole-body protein synthesis. Piglets supported on parenteral nutrition have a lower methionine requirement (0.26 $g.kg^{-1}.d^{-1}$) compared to those fed enterally (0.44 $g.kg^{-1}.d^{-1}$), suggesting that first pass splanchnic metabolism consumes approximately 30% of the dietary methionine in healthy, enterally-fed piglets. This requirement was determined using a cysteine-free diet. Parenteral and enteral methionine requirements were lower (0.18 $g.kg^{-1}.d^{-1}$ and 0.25 $g.kg^{-1}.d^{-1}$, respectively) when excess cysteine was included in the diet, indicating that ~40% of the methionine requirement was spared by adequate dietary cysteine in growing piglets (Shoveller et al, 2003b).

1.4 The metabolic burden of creatine synthesis on precursor amino acids

Creatine biosynthesis requires three amino acids: glycine, arginine and methionine. Piglets can synthesize glycine from serine, choline or threonine. Glycine is also available from endogenous protein breakdown. Other than de novo synthesis and protein breakdown, sow milk also provides a source of dietary glycine during the neonatal period (Davis et al, 1994). The estimated amount of glycine necessary for creatine synthesis in neonatal piglets is 10% of the amount provided in sow milk. However, the glycine deposited as body proteins in the neonatal period is greater than four times the intake of glycine from sow milk (Brosnan et al, 2009). Accordingly, glycine appears to be a dispensable amino acid for healthy

neonatal piglets. In contrast, a study was conducted with preterm infants fed human donor breast milk in which ^{15}N glycine was infused as a tracer. None of the tracer appeared in urea, and the authors speculated that the glycine provided in the milk was insufficient to meet the demand for growth, and that glycine might be a conditionally indispensable amino acid for sick neonates (Jackson et al, 1981). However, further experiments are warranted to estimate the capacity for glycine synthesis in healthy neonates.

Arginine is available to neonates via endogenous synthesis and both arginine and methionine are available through the release from protein breakdown and intake from sow milk. The absolute amount of arginine needed for creatine biosynthesis is equivalent to 20% of arginine available from sow milk (Brosnan et al, 2009). The net arginine deposition during neonatal growth was estimated as 6.7% of total body proteins (Wu et al, 2004). Based on this calculation, the arginine deposition into proteins was equivalent to twice the amount of arginine provided by sow milk. Therefore, the arginine requirement for protein synthesis without considering the arginine requirement for urea, NO, creatine and other non-protein metabolite synthesis, exceeds the arginine intake from sow milk. This demonstrates that substantial endogenous arginine synthesis must occur in growing piglets.

Similar to the calculation for arginine, Brosnan et al (2009) also estimated the net methionine deposition in neonates (Brosnan et al, 2009). Subsequently, Robinson et al (2016b) reported an estimate of methionine accretion that was similar Brosnan et al (2009), despite the fact that Robinson et al (2016b) restricted the methionine intake to 80% of requirement. In the Robinson et al (2016b) study, piglets were fed either a methyl deficient

(methionine at 80% of the requirement) or methyl sufficient (restricted methionine with added folate, choline or betaine) diet. The methionine deposition in proteins was not different between treatments, and an equivalent amount of methionine was directed towards transsulfuration; however, the remethylation and transmethylation reactions were affected by the availability of the methyl donors (Robinson et al, 2016b). A methyl deficit would likely place a metabolic burden on the availability of methionine for the methylation reactions. This should be addressed during the formulation of neonatal diets as methylation reactions such as creatine and phosphatidylcholine synthesis are of paramount importance.

1.5 Parenteral Nutrition (PN)

Parenteral nutrition (PN) refers to the provision of a nutritionally complete, elemental formula via an intravenous route; the provision of nutrients into the gastrointestinal tract is known as enteral nutrition (EN). PN is frequently necessary for the nutritional management of preterm or premature neonates who cannot tolerate complete enteral feeding. It is common for newborn preterm infants to require short term PN for nutritional support, often due to severe respiratory distress syndrome that precludes oral feeding (Hay, 2008). Long term PN support is sometimes required by infants born with congenital gastrointestinal (GI) malformations, or those who develop necrotizing enterocolitis (NEC) which can lead to short bowl syndrome (Quiros-Tejeira et al, 2004). In neonatal intensive care units, providing small amounts of enteral feeding, known as partial enteral nutrition, in parallel with PN feeding is a common practice to avoid complications that are known to develop with prolonged PN feeding.

1.5.1 PN and amino acids

One of the main aims of PN support is to provide adequate but not excessive amounts of indispensable amino acids to fulfill the requirement for whole-body protein synthesis and growth. Excess amounts of indispensable amino acids or their by-products could stress immature metabolic systems in neonates, and lead to the accumulation of toxic end products such as ammonia. For human neonates, the amino acid profiles of the various commercial parenteral products currently available were devised based on the amino acid profiles in human milk, infant plasma or cord blood (Brunton et al, 2000).

Ideally, the specific enteral or parenteral requirement for each indispensable amino acid should be determined in neonates; currently, they are not well defined. What is currently known about the requirements for the indispensable amino acids has been determined by a number of different methods in adults and to a lesser extent in children. In the past, nitrogen balance was used to determine individual amino acid requirements of adult humans (Zello et al, 1995). However, more precise metabolic approaches such as plasma amino acid concentrations, amino acid oxidation (AAO) and the indicator amino acid oxidation (IAAO) techniques were subsequently introduced to estimate amino acid requirements (Zello et al, 1995). The AAO technique is based on the concept that amino acids in excess of the amounts needed for protein synthesis are preferentially oxidized. An important methodological issue with AAO is that all of the excess amino acids are not always oxidized completely but catabolized to other metabolic products (Zello et al, 1995). The IAAO technique was developed to address such issues. The IAAO technique is based

on the partitioning of a single indispensable amino acid between oxidation and protein synthesis, and the balance is sensitive to the most limiting indispensable amino acids in the diet. The provision of excess amounts of the previously limiting amino acid will reduce the oxidation of the indicator amino acid to a low and constant level. Lately, this method has been adapted to be minimally invasive and has been employed to measure the requirement for some amino acids in parenterally fed neonates (Bertolo et al, 1998; House et al, 1998).

The amino acid requirements of infants are not the same for parenteral versus enteral feeding for many amino acids. Wykes et al (1992) demonstrated that plasma amino acid concentrations were very different in human neonates fed the same elemental formula via the two different routes, suggesting that a significant role is played by the splanchnic organs in amino acid metabolism. A series of studies in neonatal piglets demonstrated that the requirements for lysine, threonine, branched-chain amino acids and methionine are substantially lower during PN feeding compared to enteral feeding (House et al, 1998; Bertolo et al, 1998; Elango et al, 2002; Shoveller et al, 2003ab). The methionine, threonine and lysine requirements for post-surgical intravenously fed human neonates estimated by IAAO technique were also lower than the amounts provided in commercially available pediatric PN products (Courtney-Martin et al, 2008, Chapman et al, 2009; Chapman et al, 2010). These studies have demonstrated the importance of considering the concentration of individual amino acids in the diet when the route of feeding by-passes the splanchnic tissues.

1.6 Arginine metabolism in pathological situations

1.6.1 Arginine de novo synthesis in human neonates during pathological situation

PN feeding reduces plasma arginine in human neonates: Arginine is considered to be a conditionally indispensable amino acid for human neonates. It is unlikely, based on current evidence, that the healthy neonate can synthesize adequate arginine in the small intestinal mucosa from abundantly available precursors, suggesting that arginine is semi-indispensable. Early in the history of PN use for neonates, it was clear that there was a requirement for arginine in PN solutions. Hypoargininemia in parallel with hyperammonemia was observed in three infants receiving total parenteral nutrition with a synthetic amino acid mixture (Heird et al, 1972). Interestingly, the hypoargininemia and accompanying hyperammonemia resolved with the administration of arginine intravenously, clearly demonstrating the inadequacy of arginine in neonatal PN. However, the absolute requirement for arginine was not known; nor was it known that bypassing small intestinal metabolism would impair de novo synthesis of arginine. Later studies by Batshaw et al (1984) reported that premature neonates had elevated plasma ammonia and significantly lower plasma arginine and ornithine concentrations within the first two months of life, compared to normal birth weight neonates. Again, elevated plasma ammonia was corrected by the provision of additional arginine in PN. These findings suggest that the concentration of arginine in the intravenous diets used for premature human neonates was not sufficient to maintain ammonia detoxification, even with full PN feeding.

1.6.2 Arginine metabolism and PN feeding in neonatal piglets

The recommended intake of arginine has not been established during PN feeding for neonatal piglets. Therefore, commercial pediatric PN formulas vary greatly in arginine content. Brunton et al (2000) reported the amino acid compositions of different brands of pediatric PN solutions, and the arginine levels in those brands varied from 0.7 $g.kg^{-1}d^{-1}$ to 1.8 $g.kg^{-1}d^{-1}$. According to the NRC (1998), the dietary requirement of arginine for healthy neonatal piglets is 0.38 $g.kg^{-1}d^{-1}$. However, hypoargininemia and hyperammonemia occurred during a PN feeding trial in piglets provided arginine at the NRC requirement (Brunton et al, 2003). Even with slightly more than double the amount of arginine than the NRC recommendation, low plasma arginine and high ammonia concentrations were observed. This amount of arginine (1.0 g $arginine.kg^{-1}d^{-1}$) is mid-range among the concentrations delivered by the various commercial pediatric products. In the neonatal piglet, it is apparent that the concentration is not adequate to meet the metabolic demands for arginine during PN feeding. Brunton et al (2003) demonstrated that 1.0 $g.kg^{-1}d^{-1}$ of arginine in PN did not maximise muscle protein synthesis in PN-fed neonatal piglets, which implies that although ureagenesis might be maintained, the demand for arginine for creatine biosynthesis, NO synthesis and growth may not be fulfilled. In the same study, plasma arginine concentration increased with increasing dietary arginine, and reached a plateau at a concentration similar to sow-fed reference piglets only when the piglets received greater than 1.2 $g.kg^{-1}d^{-1}$ of arginine. The plasma ammonia concentration was low and stable at this arginine intake, although it was unclear whether muscle protein synthesis was maximised. Therefore, they suggested that metabolic arginine requirement for PN-fed,

neonatal piglets must be 1.2 $g.kg^{-1}d^{-1}$ or higher. Arginine de novo synthesis becomes compromised during PN feeding, and as such, the whole-body arginine requirement must be met by the dietary (IV) intake. In infant studies that provided arginine in PN at concentrations similar to human milk, hypoargininemia and hyperammonemia were reported among premature neonates (Batshaw et al, 1984). Therefore, de novo arginine synthesis in the neonatal intestine seems to be impaired in both humans and piglets during PN feeding.

1.7 PN, arginine, creatine and protein synthesis

The growing fetus relies at least partially on the maternal supply of creatine via the placenta, but after birth the newborn must rely on endogenous synthesis (Ireland et al, 2009, Lamarre et al, 2010). It remains to be determined whether the prematurely born infant has the capacity to synthesize creatine efficiently, particularly if a dietary creatine source is not supplied. Following preterm birth, PN is often required as a means of nutritional support for infants who are medically unstable, or with gastrointestinal disorders or prolonged intolerance of enteral feeding. However, creatine is not a component of paediatric parenteral nutrition products. In this situation, the entire creatine requirement must be met by *de novo* synthesis. This must necessitate considerable demand for the amino acid precursors, particularly arginine and methionine. Parenteral nutrition interferes with normal arginine synthesis, but whether this affects optimal creatine accretion in the growing neonate has not yet been investigated. The neonatal period is characterized by rapid growth and with very high rates of protein synthesis to support the growth. Compromised *de novo*

arginine synthesis during PN feeding, as well as an increased demand for arginine to support creatine synthesis may limit the arginine available for protein synthesis. Alternatively, the sparing of arginine through the provision of pre-formed creatine in PN may lead to enhanced protein synthesis in growing neonates.

1.8 PN feeding and associated liver damage

The use of parenteral nutrition (PN) alone or in combination with enteral nutrition in neonates is effective in providing sufficient nutrients to maintain growth in the ill newborn infant. However, PN can result in hepatic dysfunction, complicated by fat infiltration in the liver. Abnormal lipid deposition in the liver is exacerbated by prematurity and in piglets, can be detected within few days of PN initiation (Hyde et al, 2008). In many cases, a mild fat infiltration progresses to chronic lipid accumulation and then to fibrotic changes and cirrhosis. Cholestasis is also a common complication of PN which presents in one-fifth of neonates receiving PN for more than two weeks' duration (Jolin-Dahel et al, 2013). The exact etiology of PN liver disease is unknown and likely multifactorial; suggested contributing factors include low birth weight, prematurity, duration of PN, sepsis, absence of enteral feeding, quality or quantity of amino acid intake and trace mineral toxicity (Wang et al, 2006). Liver transplantation is used to treat the most severe condition. However, the morbidity and mortality associated with this procedure is high. Therefore, the prevention of PN-induced liver damage has become an important part of managing infants requiring intravenous nutrition support.

1.8.1 Creatine and lipid deposition in the liver

Lipid accumulation in the liver has been associated with impaired methionine metabolism (Deminice et al, 2011). Both phosphatidylcholine (PC) and creatine synthesis require methylation reactions to occur in the liver and rely on an adequate methionine pool to serve as a methyl donor. Approximately 70% of PC synthesis occurs via the cytidine-diphosphate-choline pathway, also known as the Kennedy pathway, which is not SAM dependent. The remaining 30% of PC is synthesized via the PEMT pathway (Reo et al., 2002) which is SAM dependent. SAM provides three methyl groups to phosphatidylethanolamine to produce PC via the PEMT pathway, which demonstrates the quantitative importance of methyl consumption for PC synthesis. In contrast, creatine synthesis only consumes one methyl group (Stead et al., 2006). PC is important for the secretion of very low-density lipoproteins (VLDL) from the liver. Recently, it was demonstrated that creatine could protect the liver from non-alcoholic fatty liver disease (NAFLD) in a rodent model (Deminice et al, 2011). In that study, rats were fed a very high fat diet, with or without creatine supplementation. Creatine supplementation prevented lipid accumulation in the liver and maintained a more normal histology (Deminice et al, 2011). It is tempting to speculate that creatine may have a role in ameliorating PN-induced liver damage, but clear evidence is lacking. A dietary source of creatine may spare methyl groups for the synthesis of PC, allowing for more efficient transport of cholesterol and triglycerides out of the liver as PC-rich lipoproteins. In a situation of a creatine-free diet, the necessity for creatine synthesis may place a burden on other metabolic pathways that involve arginine or methionine. However, no studies have been done to assess the impact

of creatine added to PN, including the amelioration of liver damage in neonates. Whether the addition of creatine to PN could support total body creatine accretion in neonates and concomitantly be useful in ameliorating PN-induced liver damage should be investigated in rapidly growing neonates.

1.9 Porcine neonatal model

The piglet is an appropriate animal model to investigate questions related to nutritional metabolism in human neonates because of similar gastrointestinal tract morphology, physiology and metabolic changes during development (Pond et al, 1978). The gross body composition of the newborn piglet is more similar to the preterm infant than the term-born infant which has greater fat accretion at birth (Shulman et al, 1993). Piglets are also developmentally immature compared to term-born infants (Shulman et al, 1993). Therefore, the neonatal piglet is a clinically relevant model to study the complications associated with PN feeding in prematurely born infants. Furthermore, larger species such as piglets, in contrast other animal models such as rats, are suitable for investigating metabolic kinetics as when multiple blood samples have to be taken over time.

The neonatal miniature piglet has not been widely used for studies of nutrient metabolism compared to the domestic piglet. Shulman et al (1988) reported that the small intestinal mucosal enzyme activities of 6-wk-old miniature piglets were similar to those found in human infants. The mean growth rate of sow-fed Yucatan miniature piglets is less rapid during first month of life compared to domestic piglets, growing at a rate of ~45 $g.kg^{-}$

$^{1}d^{-1}$ (Myrie et al, 2012) compared 79 $g.kg^{-1}d^{-1}$ (Wykes et al, 1993). In contrast, low birth weight premature infants grew at ~20.5 $g.kg^{-1}d^{-1}$ during first month of their life when fed fortified human milk (Kashyap et al, 1990). Thus, studies using a piglet model may quickly identify nutrient deficiencies or metabolic changes because of the accelerated growth rate; however, the miniature pig model may represent a better model for nutrient metabolism in infants, growing at a rate that is half the growth rate of domestic piglet and more similar to infants.

For the study of amino acid metabolism and requirements, there are data to demonstrate that the piglet is an ideal model for the human infant. There have been some studies in piglets which determined specific amino acid requirements in both enteral (EN) and parenteral nutrition (PN) (House et al (1997a; 1997b), and subsequent studies in human infants (Roberts et al, 2001) that confirmed the piglet findings. The absolute amino acid requirement values for human infants were also predicted from the piglet data by dividing the piglet value by five, to accommodate the differing growth rates. Using this correction, the similarity between the predicted (from piglets) and measured requirements in humans have now been established for threonine (Bertolo et al, 1998; Chapman et al, 2009), methionine (Shoveller et al, 2003a,b; Courtney-Martin et al, 2008), and tyrosine (House et al, 1997a; Roberts et al, 2001). It is now clear that estimates of amino acid requirements derived from piglet data are transferable to human infants (Chapman et al, 2010). Unfortunately, the requirement for arginine, a metabolically important amino acid for the neonate, has not yet been determined for the human infant. An attempt was made to determine the arginine requirement for PN-fed neonatal piglets using the IAAO technique.

However, the researchers were not successful in determining a breakpoint estimate of requirement, likely because the capacity for de novo arginine synthesis changes with development, resulting in highly variable data.

CHAPTER TWO

2.0 Problem of investigation

2.1 Rationale, objectives and hypotheses

2.1.1 Rationale

Creatine is an important neuromodulator which is critical for neonatal brain development. Neonates receive creatine from their mother's milk; however, up to 77% of the daily creatine requirement must be synthesized in the neonatal body (Brosnan et al, 2009). De novo creatine synthesis is an inter-organ mechanism which requires three amino acids, arginine, glycine and methionine, and two simple step reactions. The first step involves the conversion of arginine to guanidinoacetic acid (GAA) via the enzyme L-arginine: glycine amidinotransferase (AGAT) predominantly in the kidney. The, subsequent step, the conversion of GAA to creatine, requires methionine and the enzyme guanidinoacetate *N*-methyltransferase (GAMT) (Walker et al, 1979, Wyss et al, 2000). In adult humans and rodents, creatine synthesis appeared to be regulated at the AGAT level. However, the effect of creatine supplementation on *in-vitro* enzyme activities of creatine synthesis has not been studied in a neonatal model.

Arginine is synthesized in the small intestinal enterocytes in healthy neonates, which is bypassed during PN feeding. Therefore, PN feeding interferes with normal arginine synthesis. However, whether the PN-induced gut atrophy in neonates affects

arginine supply and optimal creatine accretion in the growing neonate has yet to be investigated.

Unpublished data from our lab demonstrated that tissue and plasma creatine concentrations were proportionate to the parenteral arginine delivery which suggests that arginine availability limits de novo creatine synthesis. The lower *in-vitro* AGAT enzyme activity demonstrated by adult humans or rats may be a consequence of limited substrate availability rather than limited enzyme capacity. This has never been studied.

Methionine is also an essential amino acid for neonates which is involved in nearly 50 transmethylation reactions in the body, and both arginine and methionine are important for incorporation into body proteins. Therefore, guanidine and methyl group incorporation into creatine or proteins will depend on the amount of arginine and methionine available. Hepatic synthesis of creatine utilizes a significant proportion of methyl groups which originate from methionine. In an acute study with neonatal piglets, the intraportal infusion of GAA led to high creatine synthesis (McBreairty et al, 2013), and this limited the methionine available for liver protein synthesis. Brosnan et al (2009) conducted a factorial approximation to quantify the amount of arginine, glycine and methionine incorporated into newly synthesized proteins and creatine in neonatal piglets. However, direct measures of arginine and methionine incorporation into creatine and proteins have not been studied *in-vivo*, particularly when differing amounts of precursors are available or when GAA or creatine have been supplemented in the diet of piglets.

Tissue GAA and creatine concentrations and *in-vitro* enzyme activities involved in the creatine synthesis pathway are used to describe the inter-organ system of creatine synthesis in rats, adult humans and in piglets (Walker et al, 1979, Wyss et al, 2000, Brosnan et al, 2009). Based on the *in-vitro* enzyme activities or the tissue GAA and creatine concentrations, the kidney has been considered the major organ responsible for GAA synthesis and the liver has been considered the major organ responsible for creatine synthesis, using data from rats and piglets (Brosnan et al, 2009). In spite of the presence of both enzymes, the role of the pancreas in creatine synthesis remains unclear in neonatal piglets including the quantitative contribution of the pancreas to whole-body GAA supply. Furthermore, individual contributions of the kidney or pancreas in situations where differing amounts of precursors are available may be of importance. A direct measurement of inter-organ fluxes of GAA and creatine in piglets is required to confirm the importance of the kidney, pancreas and liver for creatine biosynthesis.

Citrulline is converted to arginine in the kidney, but only three quarters of citrulline entering the kidney appeared as arginine in a study of young pigs (Marini et al, 2012). The importance of citrulline as a precursor for GAA synthesis in the kidney, pancreas and gut also needs to be assessed, and compared to arginine as a precursor for GAA synthesis in neonatal piglets.

2.1.2 Objectives:

The objectives for the work in the entire thesis are separated by Chapter.

For the first experimental Chapter (Chapter Three, titled "Creatine supplementation to total parenteral nutrition improves creatine status and supports greater liver and kidney protein synthesis in neonatal piglets"), the objectives were to:

a) determine whether creatine in PN could normalize the tissue and plasma GAA and creatine concentrations; and,

b) to determine whether creatine supplemented PN fed to piglets with gut atrophy could spare arginine (and/or methionine) for protein synthesis.

The objectives for the second study (Chapter Four, "Low dietary arginine sacrifices creatine biosynthesis while maintaining whole-body protein synthesis in neonatal piglets") were to:

a) to determine whether the limited creatine accretion is due to limited enzyme capacity or limited substrate availability; and

b) to determine whether the availability of arginine and/or methionine in a situation of a high methyl demand diet, or a methyl sparing diet, will affect protein synthesis.

The objectives for the final experiment (Chapter Five, '"Kidneys are quantitatively more important than pancreas and gut as a source of GAA for hepatic creatine synthesis in sow-reared Yucatan miniature piglets") were to:

a) to measure the net flux (release or uptake) of amino acids involved in GAA and creatine synthesis across the kidney, pancreas and gut with differing amounts of substrates; and
b) to describe the inter-organ pathway of creatine synthesis and the capacity of the kidney, pancreas and gut for GAA and creatine synthesis in sow-reared piglets using an *in-vivo* model.

2.1.3 Hypotheses

The hypotheses for the work in the entire thesis are separated by Chapter.

For the first experimental Chapter (Chapter Three, "Creatine supplementation to total parenteral nutrition improves creatine status and supports greater liver and kidney protein synthesis in neonatal piglets"), the hypotheses were:

a) Piglets fed creatine-supplemented PN will have greater creatine concentrations but lower GAA concentrations in tissues and plasma, compared to littermates fed creatine-free PN. Tissue and plasma GAA and creatine concentrations in creatine-fed piglets will be closer to the reference values of sow-reared piglets.

b) The provision of creatine in PN will reduce the demand for arginine and/or methionine for creatine synthesis and consequently will spare arginine (and/or methionine) to enhance the tissue protein synthesis in neonatal piglets compared to littermates provided creatine-free PN.

The hypotheses for second study (Chapter Four, "Low dietary arginine sacrifices creatine biosynthesis while maintaining whole-body protein synthesis in neonatal piglets") were:

a) Limited substrate (arginine and methionine) availability will have a more pronounced effect on tissue creatine accretion and the partitioning of precursors of creatine than the capacity of enzymes involved in creatine synthesis.
b) Provision of dietary GAA with excess methionine will result in greater creatine accretion compared to littermates fed GAA with limited methionine.

The hypotheses for third study (Chapter Five, "Kidneys are quantitatively more important than pancreas and gut as a source of GAA for hepatic creatine synthesis in sow-reared Yucatan miniature piglets") were:

a) The majority of GAA entering the liver will originate from the kidney as opposed to pancreas and gut.
b) Infusion of citrulline will result in greater GAA flux across the kidney compared to the infusion of arginine or alanine.

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CHAPTER THREE

Creatine supplementation to total parenteral nutrition improves creatine status and supports greater liver and kidney protein synthesis in neonatal piglets

Pediatr Res. 2018 Jan; 83 (1-1):135-141

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3.0 Creatine supplementation to total parenteral nutrition improves creatine status and supports greater liver and kidney protein synthesis in neonatal piglets

Running title: Creatine-supplemented PN for neonates

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Category: Basic research

3.1 ABSTRACT

Background: Creatine is not included in commercial pediatric parenteral products; the entire creatine requirement must be met by *de novo* synthesis from arginine during parenteral nutrition (PN). Poor arginine status is common during PN in neonates which may compromise creatine accretion. We hypothesized that creatine supplementation will improve creatine status and spare arginine in PN-fed piglets. *Methods:* Piglets (3-5 d old) were provided PN with or without creatine for 14 d. Tissue concentrations of creatine metabolites and activities of creatine synthesizing enzymes were measured as well as tissue protein synthesis rates and liver lipid parameters. *Results:* Creatine provision lowered kidney and pancreas L-arginine: glycine amidinotransferase (AGAT, EC number 2.1.4.1) activities and plasma guanidinoacetic acid (GAA) concentration, suggesting down-regulation of *de novo* creatine synthesis. Creatine increased plasma creatine concentrations to sow-fed reference levels and increased the creatine concentrations in most tissues, but not brain. PN creatine resulted in greater protein synthesis in the liver and in the kidney, but not in the pancreas, skeletal muscle or gut. Creatine supplementation also reduced liver cholesterol concentrations, but not triglyceride or total fat. *Conclusions:* The addition of creatine to PN may optimize the accretion of creatine and reduce the metabolic burden of creatine synthesis in rapidly growing neonates.

3.2 INTRODUCTION

Creatine and creatine phosphate are amino acid-derived compounds that are necessary to meet short-term energy requirements in tissues that have high and variable rates of energy demand. Severe creatine deficiency because of inborn errors of creatine synthesis or transport results in profound neurological defects, demonstrating its importance in brain development [1-4]. Creatine can be acquired from the diet or synthesized endogenously as a multi-organ process. In terms of loss, creatine is subjected to continuous degradation and in adults, is excreted as creatinine at a rate of approximately 1.7 $g.d^{-1}$ [5], which represents the maintenance cost of creatine. In addition, rapidly growing neonates must accrue creatine as lean tissues expand. Factorial assessment in piglets suggests that the absolute creatine needs in growing neonates is greater than that supplied by porcine milk (~12.7 versus 2.8 $mmol.wk^{-1}$, respectively); as such, neonates rely on *de novo* creatine synthesis [6]. Creatine synthesis requires three amino acids (arginine, glycine and methionine) and two enzymes [7]. The first enzyme, L-arginine: glycine amidinotransferase (AGAT; EC number 2.1.4.1), is expressed in the kidney and pancreas, and transfers the amidino compound from arginine to glycine in order to synthesize ornithine and guanidinoacetic acid (GAA). The second enzyme, guanidinoacetate *N*-methyltransferase (GAMT; EC 2.1.1.2), methylates GAA to synthesize creatine, primarily in the liver [6]. Methionine donates a methyl group via *S*-adenosylmethionine (SAM).

The growing fetus relies at least partially on the maternal supply of creatine via the placenta, but after birth the newborn must rely on endogenous synthesis [6, 8, 9]. It remains

to be determined whether the prematurely born infant has the capacity to synthesize creatine effectively, particularly if a dietary creatine source is not supplied. Following preterm birth, total parenteral nutrition (PN) is often required as a means of nutritional support for infants with gastrointestinal disorders or prolonged intolerance of enteral feeding. However, creatine is not a component of paediatric PN products. In this situation, the entire creatine requirement must be met by *de novo* synthesis which must create considerable demand for the amino acid precursors, arginine and methionine. Arginine is a conditionally essential amino acid for neonates, although *de novo* arginine synthesis does occur in the small intestinal mucosa during first-pass metabolism, predominantly from dietary proline [10, 11, 12]. PN feeding by passes the gut metabolism and thus causes gut atrophy, therefore the PN feeding interferes with normal arginine synthesis; whether this affects optimal creatine accretion in the growing neonate has not yet been investigated.

The neonatal period is characterized by rapid growth and very high rates of protein synthesis to support the growth. Compromised *de novo* arginine synthesis during PN feeding, as well as an increased demand for arginine to support creatine synthesis, may limit arginine availability for protein synthesis. Alternatively, the sparing of arginine through the provision of pre-formed creatine in PN may lead to enhanced protein synthesis in growing neonates. We hypothesized that the addition of creatine to PN would reduce the need for *de novo* creatine synthesis and spare arginine for protein synthesis in a PN-fed piglet model.

3.3 METHODS

Animals, surgical procedure and daily care: All procedures were approved by the Institutional Animal Care Committee of Memorial University of Newfoundland (St. John's, Canada) and conformed to the guidelines of the Canadian Council on Animal Care. Fourteen Yucatan miniature piglets (3 – 5 days old) were obtained from the breeding herd (Animal Care Services, Memorial University of Newfoundland) as littermate pairs. Animals immediately underwent surgical implantation of two silastic venous catheters (jugular and femoral) under general anaesthesia. Detailed descriptions of surgical procedure and post-surgical care were published elsewhere [13]. Immediately following surgery, piglets were housed individually in metabolic cages with a single-port swivel and tether system (Lomir Biomedical, Notre-Dame-de-l'Île-Perrot, Canada) that facilitates continuous PN feeding while allowing piglets to move freely. Piglets were weighed each morning and diet infusion rates were adjusted daily according to the piglet body weight. Five additional piglets were identified in the herd but were left with the sow until the study end. These piglets were of the similar age to the catheterized piglets but were not littermates.

Diet Regimen*:* Piglets were randomized to either creatine-supplemented PN (Creatine) or creatine-free PN (Control). Intravenous diets were provided as continuous infusions; PN was initiated immediately following surgery at 50% of target rate, then increased to 75% on the following morning and to 100% of target rate (13.5 $mL.kg^{-1}.d^{-1}$) on the evening of day 1. Both diets provided similar amounts of total amino acids (Sigma Aldrich, Oakville,

Canada) **(SUPPLEMENTAL TABLE 3.1 (online)**. Vitamins, iron dextran, minerals and trace elements were added to each diet just prior to feeding **(SUPPLEMENTAL TABLE 3.2 (online))**, along with Intralipid 20% (52 $mL.kg^{-1}.d^{-1}$) as previously described [13]. Creatine-supplemented piglets received 0.12 $g.kg^{-1}.d^{-1}$ (0.79 $mmol.kg^{-1}.d^{-1}$) creatine as creatine monohydrate (Sigma Aldrich). This concentration satisfied the piglets' total whole-body creatine accretion and maintenance based on previous work in sow-fed neonatal piglets from 4 to 11 days old [6]. The diets provided 0.94 g $arginine.kg^{-1}.d^{-1}$, which is considered a moderate arginine intake [14]. Piglets were maintained on the same PN for 14 days.

***Necropsy procedure*:** After 14 days, a flooding dose of L-phenylalanine (1.5 mmol.kg of body $weight^{-1}$) (Sigma Aldrich) containing 0.15 mmol.kg of body $weight^{-1}$ L-[ring-$^{2}H_5$] phenylalanine (Cambridge Isotopes Inc., Tewksbury, MA) was administrated into the jugular vein to measure tissue-specific fractional rates of protein synthesis. The phenylalanine (labeled and unlabeled) was dissolved in pyrogen-free water (11 mL.kg of body $weight^{-1}$) and warmed before administering. Thirty min after dosing, piglets were anaesthetized with halothane and delivered with oxygen by mask. Tissues including the whole right kidney and the pancreas were frozen in liquid nitrogen and stored at -80°C for later analyses of AGAT activity, creatine, and GAA concentrations. Samples of brain, skeletal muscle and liver were also removed and frozen for the analysis of creatine and GAA concentrations. Small intestinal mucosa, liver and skeletal muscle samples were taken to analyze rates of protein synthesis. The five sow-fed (SF) piglets underwent the same necropsy protocol for establishing SF reference data.

GAA and creatine analyses: Tissue (kidney, pancreas, brain, and liver) and plasma GAA and creatine concentrations were assayed using an HPLC method modified from Buchberger and Ferdig [15] using a C18 reverse phase column (Hypersil ODS 5 U 150x4.6 mm column) with ninhydrin (Sigma Aldrich) derivatization and fluorescence detection (Ex/EM 390/470).

Skeletal muscle creatine concentrations*:* Creatine concentration of gastrocnemius muscle was determined using the simplified method of Lamarre et al [9]. Tissues were homogenized in a 50 mmol.L^{-1} Tris buffer (pH 7.4) and kept for 30 min at room temperature to convert phosphocreatine into free creatine. The homogenates were then deproteinized with trifluoroacetic acid (TFA) in methanol and micro-centrifuged at 13,500 x *g* for 10 min. Creatine was eluted with an isocratic mobile phase of 0.1% TFA and 3% methanol in an N/RP-HPLC Hypercarb 100 x 4.6 mm column with UV detection at 210 nm. Total creatine concentrations were determined by reference to a standard curve which was run with samples.

AGAT assay: AGAT activity was assayed using a modified method of Van et al [16]. The assay measured the amount of ornithine converted from arginine due to AGAT activity (transamidinase). Whole kidney was pulverized, and a representative sample was used to measure transamidinase activity. Supernatants prepared from homogenized frozen kidney or pancreas samples were incubated at 37^{o}C in a shaking water bath with different substrate buffers containing either arginine or glycine or arginine and glycine or phosphate buffer

with no substrates. The resulting supernatants were incubated in a 92ºC water bath with ninhydrin colour reagent dissolved in 1-propanol anhydrous (Sigma Aldrich). The absorbance of the final incubations was read at 505 nm in a spectrophotometer. The protein content of homogenates was assayed using the Pierce™ BCA protein assay kit (Thermo Fisher Scientific, Mississauga, Canada). The ornithine produced by AGAT was presented as nmol of ornithine per mg of protein per min.

GAMT assay: GAMT activity was assayed as described previously by da Silva et al [17] which was modified from Ogawa et al [18]. Reactions, stop reactions and blanks were prepared from fresh liver samples immediately following necropsy and frozen at -80ºC for later analysis. Creatine synthesized by GAMT activity was measured via HPLC and ninhydrin derivatization [15] and activity was expressed per mg of protein per min.

Tissue-specific protein synthesis: A 100 mg sample of frozen tissue was homogenized and prepared for extraction of phenylalanine as per the method of Lamarre et al [19]. Both the tissue free and hydrolyzed protein fractions were applied to a hydrophobic solid phase extraction cartridge (Bond Elute C18, 100 mg 1 mL; Agilent Technologies, Santa Clara, CA). The eluent was dried overnight and re-suspended in 80 µL of HPLC water for the derivatization. The isotopic enrichment of L-[ring-$^{2}H_{5}$] phenylalanine in tissue free and protein bound fractions was determined by GC-MS, pentafluorobenzyl bromide (PFBBr) derivative (Sigma Aldrich) with a model 6890 GC linked to a 5976N quadrupole MS (Agilent Technologies) operating in the electron ionization mode [19]. A mixed sample of L-[ring-$^{2}H_{5}$] phenylalanine and unlabeled phenylalanine was run in scan mode, in which

91 and 96 ions or 300 and 305 were identified as potential ions. A standard curve was run before analyzing samples to identify the linear ranges. Ions with mass-to-charge ratio of 91 and 96 ions were monitored via selected ion monitoring for liver, small intestinal mucosa and muscle tissues; for kidney and pancreas samples, 300 and 305 ions were monitored. Percent molar enrichment (mol%) was determined, and fractional synthesis rate (FSR, %/d) of protein was calculated as follows:

$$FSR = IE_{bound} / IE_{free} \cdot 1440/t \cdot 100$$

Where IE_{bound} and IE_{free} are the isotopic enrichments (mol%) of L-[ring-$^{2}H_{5}$] phenylalanine of the PCA-insoluble (protein bound) and PCA-soluble (tissue free) phenylalanine pool; t is time of labeling in min and 1440 is the number of minutes per day.

Plasma and tissue free amino acids: Tissues were homogenized as previously described [20]. Plasma and tissue free amino acid concentrations were measured by reverse-phase HPLC (C18 column) following derivatization with phenylisothiocyanate (PITC) (Waters, Woburn, MA) as per the method of Bidlingmeyer et al [21].

Liver lipids and plasma cholesterol and triglyceride (TG) analyses*:* Lipid was extracted using the method of Folch et al [22]. Extracted lipid from triplicate liver samples was dried under nitrogen gas and weighed to quantify the lipid content. Extracted lipid was also used to analyze hepatic cholesterol and triglyceride (TG) concentrations via spectrophotometer

assays (505 nm and 520 nm respectively) using commercially available kits (Genzyme DC-CAL, Triglyceride-SL and Cholesterol-SL) from Sekisui Diagnostic (Charlottetown, Canada). Plasma samples were also analyzed via spectrophotometer assays (505 nm and 520 nm respectively) using a commercially available glycerol standard (Sigma Aldrich) and cholesterol standard (Pointe Scientific, Canton, MI), and the same commercial kits as used for liver samples (Triglyceride-SL and Cholesterol-SL, Sekisui Diagnostic, Charlottetown, Canada).

***Statistical analysis**:* Data were analyzed using two tailed paired t-tests (GraphPad 5, Graph Pad Software Inc., La Jolla, CA), and were considered significantly different if $P < 0.05$. Data are expressed as mean ± SD. Data collected from the sow-fed (SF) reference group were used for reference only and were not included in the statistical analyses, as this group was not treated identically to the treatment groups (i.e., no surgical or flooding dose procedures). SF reference group means ± SD are presented for comparison purposes.

3.4 RESULTS

Growth rates of the piglets in the two treatment groups were not significantly different.

Enzyme activities: Creatine supplementation to PN down-regulated the kidney AGAT activity by 52% ($P = 0.05$) and pancreas AGAT activity by 39% ($P = 0.002$) compared to the Control group (**FIGURE 3.1**). Mean kidney AGAT activity in the Creatine animals fell within the mean ± SD measured in the age-matched SF piglets, while the mean for the

Control piglets was ~139% above the SF mean. Similarly, pancreatic AGAT activity in the Creatine group was within the SF reference mean ± SD, whereas the Control group pancreatic AGAT activity was 40% higher than the SF reference mean **(FIGURE 3.1)**. Creatine supplementation did not affect liver GAMT activity and both mean PN values were approximately 55% below the SF reference mean. The liver GAMT activity in the SF piglets was quite variable (**FIGURE 3.2**).

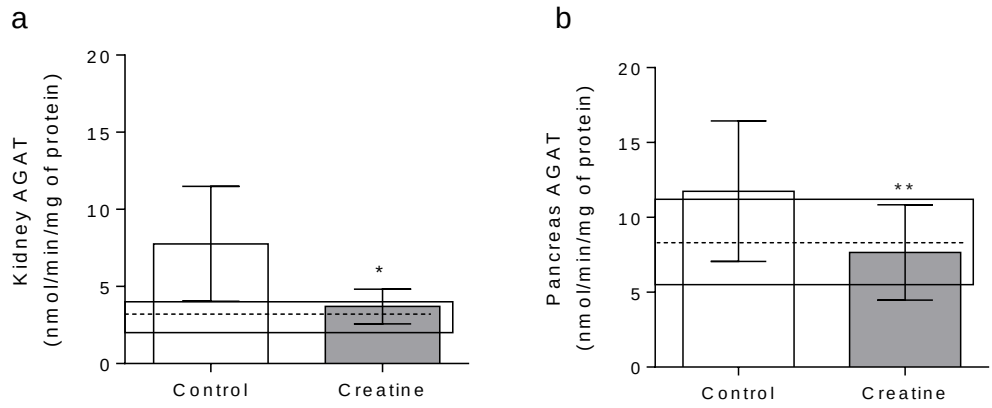

FIGURE 3.1 AGAT enzyme activity in a) kidney and b) pancreas. Open bars: Control group, n = 7; gray bars: Creatine group, n = 7. Values are expressed as means ± SD. **P < 0.01 and *P < 0.05 by paired t-test. The broken line and box represent the mean ± SD derived from a group of sow-fed reference piglets (n = 5) that were not littermates to the treatment groups.

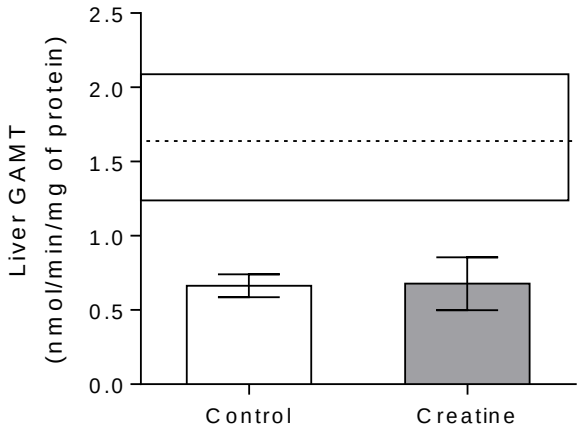

FIGURE 3.2 GAMT enzyme activity in liver. Open bars: Control group, n = 7; gray bars: Creatine group, n = 7. Values are expressed as means ± SD. The broken line and box represent the mean ± SD, derived from a group of sow-fed reference piglets (n = 5) that were not littermates to the treatment groups.

***Tissue GAA and creatine concentrations**:* GAA concentrations in the kidney, pancreas, liver and brain did not differ between treatment groups (**TABLE 3.1**). The kidney and pancreas GAA concentrations were highly variable in both treatment groups (**TABLE 3.1**). GAA concentrations were comparable to SF reference piglets for most tissues, except liver where the mean concentration in SF group was approximately twice that of the experimental piglets (**TABLE 3.1**). Creatine supplementation resulted in significantly higher creatine concentrations in kidney, pancreas and liver compared to the Control group (**TABLE 3.2**). Total creatine content (i.e., creatine and phosphocreatine) in the skeletal muscle was significantly higher in the Creatine compared to the Control group. SF pigs tended to have creatine concentrations between those of the treatment groups for most tissues. Lack of creatine in Control piglets did not alter GAA (**TABLE 3.1**) or creatine (**TABLE 3.2**) concentrations in the brain. Furthermore, the mean brain GAA and creatine concentrations measured in both Creatine and Control groups were within the SF reference range (**TABLE 3.1 & 3.2**).

TABLE 3.1 Tissue and plasma guanidinoacetic acid (GAA) concentrations in piglets given Control versus Creatine PN[a]

	Control	**Creatine**	**SF[b]**
		$nmol.g^{-1}$	
Kidney	435 ± 234	376 ± 206	480 ± 123
Pancreas	240 ± 185	271 ± 170	268 ± 107
Liver	60 ± 10	68 ± 29	97 ± 50
Brain	64 ± 14	62 ± 15	79 ± 33
Plasma ($\mu mol.L^{-1}$)	15.2 ± 5.0	8.3 ± 2.2**	6.0 ± 1.2

[a]Values are mean ± SD; n = 7 per group for PN treatments except for plasma, n = 6.

[b]SF (n = 5) data were derived from a group of sow-fed piglets that were not littermates to the PN groups; SF data were not included in the statistical analyses.

Asterisk indicates different from Control, **P = 0.01 by paired t-test.

PN, parenteral nutrition; SF, sow-fed.

TABLE 3.2 Tissue and plasma creatine concentrations in piglets given Control versus Creatine PN[a]

	Control	**Creatine**	**SF[b]**
		$\mu mol.g^{-1}$	
Kidney	1.2 ± 0.6	2.5 ± 0.7**	1.8 ± 0.5
Pancreas	1.0 ± 0.4	2.3 ± 0.8**	1.4 ± 0.4
Liver	1.1 ± 0.7	2.2 ± 1.5*	1.1 ± 0.2
Skeletal Muscle[c]	22 ± 3.0	26 ± 2.6*	25 ± 5.0
Brain	9.8 ± 3.1	10.1 ± 1.5	8.2 ± 2.1
Plasma ($\mu mol.L^{-1}$)	54 ± 21	232 ± 46**	249 ± 40

[a]Values are mean ± SD; n = 7 per group for PN treatments except for plasma, n = 6.

[b]SF (n = 5) data were derived from a group of sow-fed piglets that were not littermates to the PN groups; SF data were not included in the statistical analyses.

[c]Total creatine.

Asterisks indicate different from Control, $^{**}P \leq 0.01$ and $^{*}P < 0.05$ by paired t-test.

PN, parenteral nutrition; SF, sow-fed.

Plasma GAA, creatine and amino acid concentrations: Creatine supplementation resulted in significantly lower plasma GAA concentration (**TABLE 3.1**), but higher creatine concentration (**TABLE 3.2**) compared to Control pigs. Creatine supplementation to PN resulted in plasma GAA and creatine concentrations that were like SF reference pigs, whereas omission of creatine for Control pigs increased plasma GAA by 60% and reduced plasma creatine by 80% compared to the mean reference values in SF pigs. Amino acid concentrations were similar between Creatine and Control groups, except for proline, which tended to be higher in the Control pigs (P = 0.06) (**SUPPLEMENTAL TABLE 3.3 (online))**.

Tissue specific fractional protein synthesis rates: Liver and kidney fractional protein synthesis rates were significantly higher in Creatine piglets compared to the Control group (**FIGURE 3.3**). There were no differences in protein synthesis rates in pancreas, small intestinal mucosa or skeletal muscle between experimental groups (**FIGURE 3.3**).

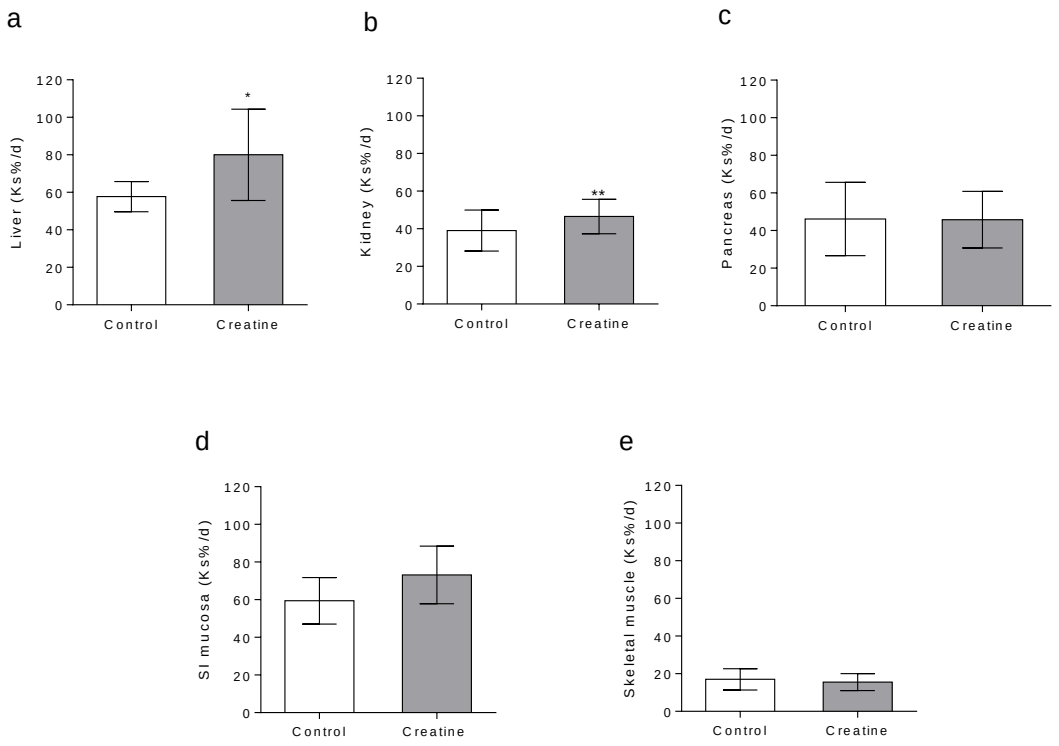

FIGURE 3.3 Tissue specific rate of protein synthesis in a) liver, b) kidney, c) pancreas d) small intestinal mucosa and e) skeletal muscle. Open bars: Control group; gray bars: Creatine group, n = 7 per group except for kidney, n = 6 and pancreas, n = 5. Values are expressed as means ± SD. **P = 0.01 and *P < 0.05 by paired t-test. SI, small intestine.

***Tissue free amino acid concentrations**:* No differences in free amino acid concentrations between treatments were found in liver or kidney tissues **(SUPPLEMENTAL TABLE 3.4 and 3.5 (online)).**

***Liver weight, liver lipid, triglyceride and cholesterol**:* Liver cholesterol in the Creatine piglets was significantly lower than in the Control group (**TABLE 3.3**). However, the triglyceride concentration, total lipid or liver weights were not different between groups. Regardless of the treatment, both PN-fed groups had liver weights, liver lipid concentrations and liver triglyceride concentrations that were 1.7 to 2.5 times higher than the SF reference ranges. Plasma cholesterol and TG were not different between treatment groups. However, the mean cholesterol SF value was approximately two times higher than that of the PN-treated piglets. The mean plasma triglyceride concentrations measured in the two PN-treated groups were within the range of values measured in the SF piglets **(TABLE 3.3).**

TABLE 3.3 Body and liver weights, liver and plasma lipid concentrations at necropsy in piglets given Control versus Creatine PN[a]

	Control	Creatine	SF[b]
Body weight (BW) (kg)	3.6 ± 0.4	3.6 ± 0.6	3.7 ± 0.2
Liver weight ($g.kg^{-1}$ BW)	52 ± 6.4	55 ± 8.4	32 ± 2.3
Whole liver:			
Lipid ($g.kg^{-1}$ BW)	2.0 ± 0.4	2.3 ± 0.6	1.0 ± 0.1
Triglyceride ($\mu mol.kg^{-1}$ BW)	407 ± 259	351 ± 164	166 ± 81
Cholesterol ($\mu mol.kg^{-1}$ BW)	372 ± 151	151 ± 73**	146 ± 56
Plasma:			
Triglyceride ($\mu mol.mL^{-1}$)	0.31 ± 0.01	0.29 ± 0.10	0.43 ± 0.13
Cholesterol ($\mu mol.mL^{-1}$)	2.3 ± 0.7	2.2 ± 0.7	4.4 ± 1.9

[a]Values are mean ± SD; n = 7 for PN treatments.

[b]SF (n = 5) data were derived from a group of sow-fed piglets that were not littermates to the PN groups; SF data were not included in the statistical analyses.

Asterisk indicates different from Control, $^{**}P < 0.01$ by paired t-test.

PN, parenteral nutrition; SF, sow-fed.

3.5 DISCUSSION

Previously, we demonstrated that suckling neonatal piglets cannot meet creatine requirements from milk alone and that 75% of creatine needs must come from endogenous synthesis from arginine and methionine [6]. Although methionine is required for creatine synthesis, it was provided in the PN at 20% over requirement, so likely was not limiting. In PN-fed piglets, arginine synthesis is diminished with gut atrophy [10] and we demonstrated that tissue creatine concentrations were proportionate to the i.v. arginine delivery (unpublished data), suggesting that arginine availability limited *de novo* creatine synthesis. In this study, we hypothesized that the addition of creatine to PN would spare arginine for protein synthesis. Indeed, an important finding is that liver and kidney protein synthesis were higher with creatine supplementation. However, there was no difference in muscle protein synthesis.

The concentration of arginine supplied in the PN diet was designed to be lower than that necessary to maximize protein synthesis in the muscle [14] to allow spared arginine to affect muscle protein synthesis; however, the amount of arginine spared by creatine was not conveyed to muscle. In the liver, the enhanced protein synthesis may be due to greater arginine availability, but this was not demonstrated by liver free arginine concentrations, which were not different between treatment groups. A similar situation occurred in kidney, with greater protein synthesis but no difference in free arginine concentrations. However, free concentrations are not necessarily reflective of arginine availability; it may be that arginine turnover was more rapid in the Creatine group, directing more arginine toward

protein synthesis without changing free concentrations. This possibility could be clarified with a tracer study to quantify arginine flux rates. Alternatively, creatine may increase tissue protein synthesis via mechanisms unrelated to arginine. *In-vitro* and *in-vivo* studies have identified several mechanisms by which creatine stimulates protein synthesis in muscle. Myogenic cells in culture exposed to oxidative stress have diminished proliferation and differentiation, and creatine attenuated these effects [23]. Creatine also upregulates the expression of a number of trophic factors in muscle, including IGF-1, which can stimulate protein synthesis through activation of mTOR pathway intermediates [24]. To our knowledge, none of these mechanisms related to creatine have been studied in liver or kidney. The lack of response to creatine in muscle may have been due to limited amino acid substrate in that tissue, or due to the relatively low dose of creatine used in this study compared to human supplementation trials.

Skeletal muscle contains over 95% of total body creatine, with one third as the free form and the rest as phosphorylated creatine [25]. Creatine kinase (CK) activity is found in the cytoplasm of several tissues including skeletal muscle, cardiac muscle and brain. We hypothesized that two weeks of creatine supplementation to PN would increase skeletal muscle and brain creatine concentrations. Interestingly, skeletal muscle total creatine concentration was higher in our creatine-fed piglets, but we found no difference in the brain creatine concentrations, and both groups were like SF reference piglets. The brain creatine pool is relatively minor compared to the creatine stored in muscle, representing less than 5% of the entire body pool. However, adequate brain creatine must be of physiological importance, as profound negative neuro-developmental effects have been reported

secondary to inborn errors of creatine synthesis or transport [26]; thus, creatine is critical to normal neurological developmental processes in neonates. Previously, we reported that brain AGAT activity was not detected and GAMT activity was very low in neonatal piglets [6]. In this study, there was a low ratio of GAA to creatine in brain tissues compared to major GAA and creatine synthesizing organs, which suggests that the brain's capacity to synthesize its own GAA seems unlikely, and accretion likely depends on peripheral de novo synthesis [27]. The mean plasma creatine concentration was 80% lower in piglets given the creatine-free PN compared to the Creatine and SF reference piglets; as such, perhaps the brain has priority for circulating creatine, even in the situation of low plasma creatine during PN. Alternatively, it may be that the brain creatine pool was not measurably affected during the short-term use of creatine-free PN. SF neonatal piglets studied at 4 and 11 days of age had 50% greater body weight at the older age, but no change in the brain weight or brain creatine concentration [6]; so unlike muscle, brain does not have the same need for rapid creatine accretion and might be less sensitive to dietary supply. The rate of creatine degradation in the neonatal brain is also unknown but is likely very slow. In infants with AGAT deficiency, neurological symptoms do not become apparent until the second year of life [26]. Thus, brain creatine likely degrades too slowly to create a deficit after only 14 days of creatine-free PN.

With the addition of dietary creatine, down-regulation of creatine synthesis was evident by lower AGAT activity in kidney and pancreas as well as by lower GAA concentrations in plasma. AGAT has been demonstrated as the rate limiting step in creatine bio-synthesis in humans [28] and in rodents [7, 17, 29, 30] as creatine feeding induced

lower AGAT activity with no change in liver GAMT activity. Similarly, in our piglets the AGAT activity was affected with a dietary supply of creatine, and liver GAMT activity did not change. Therefore, it appears that creatine synthesis is regulated at the level of AGAT in neonatal piglets as well.

The kidney has been identified as the major organ responsible for GAA synthesis in rodents [5]. Interestingly, in piglets, we measured pancreatic AGAT specific activity that was 53 to 159% higher (per gram of protein) than measured in the kidney. However, considering piglet kidneys are ~4 times the mass of the pancreas [6], the kidneys are likely still the organ responsible for the most of endogenous GAA synthesis in neonatal piglets. Although the pancreas has higher AGAT specific activity than kidney, its GAMT specific activity is relatively low (i.e., only ~25% that of the liver) [6], suggesting the pancreas has a net release of GAA with minimal net release of creatine into portal blood. Therefore, the high pancreatic AGAT specific activity may contribute some GAA directly to the liver for creatine synthesis, although this requires confirmation.

Because prolonged PN can lead to hepatic fat accumulation and PN-associated liver disease [31], we also measured lipid parameters in the liver as a secondary objective. As expected, the PN-fed groups had higher liver lipid content and heavier livers compared to SF piglets of the same age. Lipid accumulation in the liver has been associated with impaired methionine metabolism [32]. Both PC and creatine synthesis require hepatic methylation reactions, which rely on an adequate methionine pool to serve as a methyl donor. Moreover, adequate PC synthesis is required for VLDL assembly and secretion of

lipids from the liver. It is possible that creatine supplementation might spare methionine for transmethylation to PC, thereby reducing liver lipids in this PN-fed model. We found that creatine supplementation lowered the total liver cholesterol concentration, but no differences in liver weight or triglyceride concentration were detected. While creatine supplementation may have enhanced PC synthesis allowing for more efficient transport of cholesterol out of the liver, it is unclear why total lipids or triglycerides were also not measurably reduced. In rodents fed a high fat diet, creatine supplementation led to a profound reduction in lipid accumulation in the liver [33], but creatine was provided in the diet at almost 30 times the concentration delivered in our PN. It is tempting to speculate that creatine has a role in ameliorating PN-induced liver steatosis, but more research is needed on lipid metabolism outcomes.

To our knowledge, this is the first time that creatine has been studied as a supplement to PN for use in neonates. Our data suggest that the requirement for creatine synthesis might be a burden on other arginine metabolic pathways. The addition of creatine to PN appears necessary to support optimal creatine accretion and liver and kidney protein synthesis in rapidly growing neonates.

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DISCLOSURE

The authors declare no conflict of interest.

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3.7 ACKNOWLEDGEMENTS

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3.8 ONLINE SUPPLEMENTARY MATERIAL

SUPPLEMENTAL TABLE 3.1 Amino acid profile of Control and Creatine PN[a,b]

Amino acid	**g·L^{-1}**	**g·kg^{-1}·d^{-1}**	**Amino acid**	**g·L^{-1}**	**g·kg^{-1}·d^{-1}**
Amino acids related to arginine metabolism			*Indispensable amino Acids*		
Arginine	3.47	0.94	Histidine	1.76	0.48
Aspartate	3.47	0.94	Isoleucine	2.64	0.72
Glutamate	5.99	1.63	Leucine	5.94	1.62
Proline	4.73	1.29	Lysine	4.73	1.29
Dispensable Amino Acids			Methionine	1.10	0.30
Alanine[c]	5.34 (6.12)	1.45 (1.66)	Phenylalanine	3.03	0.82
Cysteine	0.83	0.22	Threonine	3.03	0.82
Glycine	1.76	0.48	Tryptophan	1.21	0.33
Serine	3.19	0.87	Valine	3.03	0.82
Taurine	0.28	0.08			
Tyrosine	0.43	0.12			

[a]PN, parenteral nutrition. [b]The creatine-supplemented PN contained 0.44 g·L^{-1} creatine monohydrate and 5.34 g·L^{-1} alanine while the Control PN (within the brackets in the table) contained 6.12 g·L^{-1} alanine and no creatine monohydrate. [c]Alanine was manipulated to make the diets isonitrogenous. With the exception of alanine, the amino acid composition of the diets was similar.

SUPPLEMENTAL TABLE 3.2 Composition of the Control and Creatine PN[a,b]

Composition of Elemental Diets	*g/L*
L-Amino acids	56.0
D-Glucose	90.3
Trihydrate K_2HPO_4	1.57
Monobasic KH_2PO_4	1.09
Potassium acetate	1.47
NaCl	2.17
$MgSO_4$	0.78
$ZnSO_4$	0.09
Calcium gluconate	6.41
Multivitamin solution	
Ascorbic acid, *mg*	17.4
Vitamin A, μg	150
Vitamin D, μg	2.2
Thiamine (as hydrochloride), *mg*	0.26
Riboflavin (as phosphate), *mg*	0.30
Pyridoxine hydrochloride, *mg*	0.22
Niacinamide, *mg*	3.70
d-Panthenol, *mg*	1.1
Vitamin E (*dl*-alpha tocopheryl acetate), *mg* IU)	
Vitamin $K_{1,}$ *mg*	1.4
Biotin, μg	0.04
Folic Acid, μg	4.35
Vitamin $B_{12,}$ μg	30.5
	0.22
Trace Element Mix[d]	
Zinc (as $ZnSO_4.7H_2O$), *mg*	10.07
Copper (as $CuSO_4.5H_2O$), *mg*	0.86
Manganese (as $MnSO_4.H_2O$), *mg*	0.66
Chromium (as $CrCl_3.6H_2O$), *mg*	0.01
Selenium (as SeO_2), *mg*	0.05
Iodide (as NaI), *mg*	0.02
Iron Dextran[e]	
Iron (as ferric hydroxide), *mg*	3.0

[a]Creatine-supplemented and Control treatments were identical, with the exception of the amino acid composition (**SUPPLEMENTAL TABLE 3.1**). The diets were comprised of amino acids, glucose and minerals mixture, which was infused at a rate of 272 $mL \cdot kg^{-1}$ $BW \cdot day^{-1}$.

[b]Intralipid® 20% (Baxter, Canada) was infused with the parenteral diets at a rate of 52 $mL \cdot kg^{-1}$ $BW \cdot day^{-1}$ (10.4 $g.kg^{-1}$ $BW.day^{-1}$).

[c]Multi-12/K_1 Pediatric® multivitamin solution for parenteral nutrition (Baxter, Canada).

[d]The the trace element mix (components from Sigma-Aldrich, Canada) was prepared in the laboratory, and added to the PN just prior to use.

[e]Iron dextran (Bimeda-MTC Animal Health, Canada).

SUPPLEMENTAL TABLE 3.3 Plasma amino acid concentrations in Control versus Creatine PN[a].

	Control	Creatine	SF[b]
	($\mu mol.L^{-1}$)		
Amino acids related to arginine metabolism			
Arginine	55 ± 15	68 ± 17	135 ± 54
Aspartate	20 ± 13	20 ± 9	86 ± 90
Citrulline	91 ± 32	68 ± 25	100 ± 51
Glutamine	218 ± 44	194 ± 39	242 ± 46
Glutamate	182 ± 41	186 ± 80	87 ± 38
Ornithine	51 ± 21	50 ± 18	89 ± 35
Proline	481 ± 130	329 ± 88	469 ± 202
Indispensable amino acids			
Histidine	30 ± 9	24 ± 10	46 ± 14
Isoleucine	120 ± 53	125 ± 46	139 ± 56
Leucine	204 ± 49	183 ± 92	220 ± 88
Lysine	250 ± 82	242 ± 81	306 ± 82
Methionine	30 ± 9	22 ± 7	20 ± 5
Phenylalanine	1237 ± 462	991 ± 554	111 ± 47
Threonine	201 ± 49	209 ± 98	153 ± 47
Tryptophan	130 ± 29	112 ± 33	80 ± 31
Valine	284 ± 50	249 ± 90	310 ± 137
Dispensable amino acids			
Alanine	419 ± 105	332 ± 108	408 ± 139
Glycine	841 ± 373	740 ± 208	558 ± 146
Hydroxyproline	47 ± 15	46 ± 30	94 ± 38
Serine	474 ± 169	357 ± 111	223 ± 74
Taurine	249 ± 68	233 ± 68	167 ± 68
Tyrosine	144 ± 96	118 ± 50	135 ± 68

[a]Values are mean ± SD by paired t-test; n = 7 (for PN treatments). [b]SF data were derived from a group of sow-fed piglets (n = 5) that were not littermates to the PN groups; SF data were not included in the statistical analyses. PN, total parenteral nutrition. SF, sow-fed.

SUPPLEMENTAL TABLE 3.4 Liver free amino acid concentrations in piglets fed Control versus Creatine PN[a].

	Control	Creatine	SF[b]
	($\mu mol.g^{-1}$)		
Amino acids related to arginine metabolism			
Arginine	0.21 ± 0.13	0.29 ± 0.19	0.22 ± 0.16
Aspartate	1.95 ± 1.37	2.29 ± 1.70	1.05 ± 0.32
Citrulline	1.45 ± 0.64	1.62 ± 0.54	0.35 ± 0.15
Glutamine	0.54 ± 0.20	0.36 ± 0.22	1.92 ± 0.26
Glutamate	0.33 ± 0.21	0.50 ± 0.21	2.01 ± 0.58
Ornithine	0.91 ± 0.51	0.83 ± 0.49	0.47 ± 0.12
Proline	3.65 ± 1.14	2.61 ± 1.53	0.90 ± 0.14
Indispensable amino acids			
Histidine	0.66 ± 0.25	1.17 ± 1.03	0.37 ± 0.15
Isoleucine	0.66 ± 0.17	0.76 ± 0.46	0.36 ± 0.14
Leucine	1.09 ± 0.52	0.88 ± 0.25	0.55 ± 0.19
Lysine	1.63 ± 0.98	1.08 ± 0.48	0.03 ± 0.02
Methionine	0.48 ± 0.37	0.91 ± 0.46	0.25 ± 0.18
Phenylalanine	3.32 ± 0.51	3.31 ± 0.48	1.56 ± 0.25
Threonine	0.67 ± 0.31	0.63 ± 0.27	0.37 ± 0.10
Tryptophan	0.07 ± 0.03	0.07 ± 0.03	0.07 ± 0.03
Valine	0.92 ± 0.35	0.93 ± 0.40	1.00 ± 0.60
Dispensable amino acids			
Alanine	6.72 ± 1.96	6.25 ± 2.86	1.63 ± 0.64
Glycine	10.11 ± 2.18	11.04 ± 2.56	3.64 ± 0.78
Hydroxyproline	0.26 ± 0.09	0.28 ± 0.21	0.22 ± 0.05
Serine	5.02 ± 2.33	4.66 ± 1.84	1.73 ± 0.32
Taurine	9.14 ± 3.03	9.78 ± 4.31	8.20 ± 1.25
Tyrosine	0.94 ± 0.42	0.92 ± 0.47	0.50 ± 0.20

[a]Values are mean ± SD by paired t-test; n = 7 (for PN treatments). [b]SF data were derived from a group of sow-fed (n = 5) piglets that were not littermates to the PN groups; SF data were not included in the statistical analyses. PN, total parenteral nutrition. SF, sow-fed.

SUPPLEMENTAL TABLE 3.5 Kidney free amino acid concentrations in piglets fed Control versus Creatine PN[a].

	Control	Creatine	SF[b]
	($\mu mol.g^{-1}$)		
Amino acids related to arginine metabolism			
Arginine	0.09 ± 0.05	0.14 ± 0.01	0.14 ± 0.13
Aspartate	1.43 ± 0.63	1.40 ± 0.87	1.33 ± 0.38
Citrulline	0.49 ± 0.13	0.49 ± 0.07	0.35 ± 0.10
Glutamine	0.10 ± 0.05	0.10 ± 0.04	0.73 ± 0.15
Glutamate	0.34 ± 0.15	0.30 ± 0.12	2.70 ± 0.30
Ornithine	0.36 ± 0.12	0.36 ± 0.25	0.33 ± 0.16
Proline	0.94 ± 0.30	0.83 ± 0.19	0.90 ± 0.30
Indispensable amino acids			
Histidine	1.27 ± 0.37	1.82 ± 0.68	0.24 ± 0.08
Isoleucine	0.56 ± 0.14	0.46 ± 0.16	0.43 ± 0.08
Leucine	0.61 ± 0.16	0.57 ± 0.15	0.48 ± 0.12
Lysine	0.52 ± 0.19	0.71 ± 0.26	0.40 ± 0.20
Methionine	0.18 ± 0.02	0.22 ± 0.07	0.23 ± 0.06
Phenylalanine	2.58 ± 0.37	2.38 ± 0.65	1.85 ± 0.20
Threonine	0.43 ± 0.09	0.46± 0.21	0.35 ± 0.01
Tryptophan	0.18 ± 0.04	0.14 ± 0.07	0.07 ± 0.01
Valine	0.61 ± 0.21	0.51 ± 0.10	0.63 ± 0.09
Dispensable amino acids			
Alanine	3.69 ± 0.77	4.48 ± 2.57	1.80 ± 0.40
Glycine	4.26 ± 0.84	5.29 ± 3.66	4.75 ± 0.20
Hydroxyproline	0.22 ± 0.08	0.22 ± 0.12	0.09 ± 0.01
Serine	1.05 ± 0.25	1.57 ± 1.65	0.68 ± 0.16
Taurine	6.83 ± 1.38	8.23 ± 3.03	4.50 ± 0.90
Tyrosine	0.27 ± 0.13	0.35 ± 0.18	0.34 ± 0.10

[a]Values are mean ± SD by paired t-test; n = 7. PN, total parenteral nutrition. [b]SF data were derived from a group of sow-fed piglets (n = 5) that were not littermates to the PN groups; SF data were not included in the statistical analyses. PN, total parenteral nutrition. SF, sow-fed.

CHAPTER FOUR

Low dietary arginine sacrifices creatine biosynthesis while maintaining whole-body protein synthesis in neonatal piglets

The work presented in this Chapter was funded by a grant from the NSERC Collaborative Research and Development program, with Evonik Industries (Hanau, Germany) as the industry partner. It represents work that was presented at Canadian Nutrition Society Annual Conference (2015) and the conference abstract was published in the journal Applied Physiology Nutrition and Metabolism (Appl. Physiol. Nutr. Metab. Vol. 40, 2015). Part of this work was also presented at Experimental Biology 2016 in San Diego, CA, and the abstract was published in FASEB (Vol.30, Issue 1_supplement, April 2016). Included in this thesis are data from the same experimental piglets on liver GAMT activity and plasma cysteine and homocysteine; these data were generated and generously shared by Kankayaliyan Thillayampalam (in **TABLE 4.1** GAMT activity and **TABLE 4.2**: Cysteine and homocysteine). Dr. M. Rademacher is a scientist with the industry partner (Evonik). Dr. C. Tomlinson collaborated on this project because of his expertise in mass spec analysis of creatine-related metabolites. O. Chandani Dinesh performed all of the other laboratory and statistical analyses and data interpretation in this Chapter. The manuscript is formatted to follow the guidelines of the Journal of Nutrition, the journal of choice for this work.

4.0 Low dietary arginine sacrifices creatine biosynthesis while maintaining whole-body protein synthesis in neonatal piglets

4.1 ABSTRACT

Background: We previously demonstrated low tissue accretion of creatine in piglets fed creatine-free diets. However, it is unknown whether insufficient endogenous synthesis of creatine is due to limited enzyme capacity or inadequate substrate availability. Arginine (Arg) and methionine (Met) are indispensable amino acids for neonates as well as precursors for creatine biosynthesis. **Hypothesis:** The limited availability of dietary Arg and Met limits guanidinoacetic acid (GAA) and creatine syntheses, to conserve whole-body protein synthesis. **Methods:** Piglets (9 - 11 d old, n = 35) were fed one of five elemental diets for 5 days: 1) low Arg and low Met (Base), 2) Base plus GAA (+GAA), 3) Base plus GAA plus excess Met (+GAA/Met) 4) Base plus creatine (+Cre) 5) excess Arg and excess Met (+Arg/Met). Isotope tracers were infused to determine whole-body GAA, creatine and protein synthesis. **Results:** Piglets fed the Base diet had lower Arg conversion to GAA and creatine compared to the +Arg/Met group ($P < 0.0001$), demonstrating that creatine biosynthesis was limited by low dietary Arg and Met. Renal L-arginine:glycine amidinotransferase activity in piglets on the Base diet was higher than that in piglets fed +Arg/Met ($P < 0.0001$), suggesting that the lower Arg to GAA conversion was due to inadequate substrate availability, rather than lack of enzyme activity. One week manipulation of the dietary arginine, methionine, GAA or creatine did not affect the brain

creatine levels or whole-body protein synthesis. **Conclusions:** The substrate availability rather than the presence of creatine in the diet/tissues or in vitro enzyme capacities determined the rate of arginine conversion to GAA and creatine in our piglets. Whole-body protein synthesis is conserved even when the diet is low in arginine, by diverting less arginine to GAA and creatine synthesis. Supplemental GAA can be used to sustain required levels of creatine, but only if provided with excess methionine. Accommodation of all metabolic fates of Arg and Met need to be considered when determining dietary requirements for neonates.

Abbreviations: AGAT, L-Arginine:glycine amidinotransferase; APE, atom percent excess; arg, arginine; BCA, bicinchoninic acid; cre, creatine; GAA, guanidinoacetic acid; GAMT, guanidinoacetate *N*-methyltransferase; met, methionine; NO, nitric oxide; NRC, National Research Council.

4.2 INTRODUCTION

Creatine accretion in early life occurs at a rapid rate, to accommodate the expanding tissue pools during this period of swift growth (Brosnan et al, 2009); furthermore, creatine is critical for brain function and neurological development in neonates (Beard & Braissant, 2010). Neonates receive some creatine in mother's milk; however, up to 77% of the daily creatine requirement must be synthesized in the neonatal body (Brosnan et al, 2009), likely resulting in a high demand for the precursor amino acids. Arginine is a conditionally essential amino acid precursor of creatine, that is also involved in protein synthesis. Along with arginine, creatine biosynthesis utilizes glycine and methionine. L-Arginine:glycine amidinotransferase (AGAT) catalyzes the first step by the formation of guanidinoacetic acid (GAA), which is the intermediate metabolite in creatine biosynthesis. A second enzyme, guanidinoacetate *N*-methyltransferase (GAMT) methylates GAA to synthesize creatine in the liver. Methionine donates a methyl group for the final reaction to form creatine (Walker et al, 1979). As an essential amino acid, a dietary source of methionine is necessary for protein synthesis as well as to fulfill methyl donor functions for several important reactions including creatine synthesis. It may follow that the rate of creatine accretion depends on the amount of preformed creatine, or arginine and methionine that is available to the neonate; however, this has not been quantified. Nutritional management strategies used for term and preterm infants vary in the quantities of creatine and amino acids, and some are nearly devoid in creatine, including parenteral nutrition, and soy or lactose free formulas (Edison et al, 2013). Thus, it is of critical importance to understand

how the lack of dietary creatine or limiting precursor amino acids might affect overall creatine accretion, protein synthesis and growth. Similarly, an important question is whether the provision of preformed creatine or its precursor GAA could spare amino acids to be used for growth. Neither of these questions has been adequately addressed in neonates to date.

The use of GAA as a low-cost feed additive to enhance creatine accretion and spare amino acids has captured the interest of the agricultural industry. GAA supplementation to growing pigs enhanced muscle creatine accretion, but at the expense of liver protein synthesis (McBreairty et al, 2015). The effects on whole-body protein synthesis or the potential benefits of sparing arginine were not assessed in neonates but should be quantified. A further question is whether additional methionine supplemented with GAA would ameliorate the depletion of methionine and lead to better growth outcomes.

In our previous study, neonatal piglets that were fed creatine-free diets had lower plasma and tissue creatine concentrations compared to those fed a creatine-supplemented diet (Dinesh et al, 2018). However, AGAT activity was high in piglets fed the creatine-free diet. Whether low creatine accretion was due to limited enzyme capacity or limited substrate availability is not known. However, tissue and plasma creatine concentrations were proportionate to the concentration of arginine delivered to piglets as a parenteral diet

(Gagnon et al, 2010), suggesting that limited arginine availability will impact de novo creatine synthesis.

The addition of creatine to the diet has the potential to spare both arginine and methyl groups, through negative feedback regulation of AGAT activity (Dinesh et al, 2018). Therefore, in this study, we measured tissue creatine accretion and whole-body protein synthesis in piglets fed diets that were supplemented with creatine or GAA in combination with arginine and methionine intakes just slightly below requirements. This would facilitate detection of a change in protein synthesis when amino acids are spared. A diet with arginine and methionine concentrations slightly below requirement served as the negative control. As a positive control, we included a group with excess arginine and methionine, to meet the requirements for all synthesized products including protein as well as GAA/creatine. Lastly, because GAA has been shown to be proportionately converted to creatine with no regulatory feedback, we included a group that was fed GAA with low arginine plus excess methionine to ensure sufficient methionine was available for creatine synthesis.

In-vitro enzyme activities measure the maximum capacity of enzymes present in tissues without accounting for *in-vivo* substrate availability. In contrast, a multi-tracer approach allows for the measurement of whole-body *in-vivo* conversions of precursors to products and is a more sophisticated method to measure metabolic flux through the enzymes involved in creatine synthesis. Therefore, the present study used a multiple stable isotope tracer protocol to address how whole-body arginine and methionine were

quantitatively partitioned into GAA, creatine or protein synthesis when differing amounts of arginine and methionine were available or when pre-made GAA or creatine was supplied. Finally, the *in-vivo* flux through creatine biosynthesis enzymes was compared to *in-vitro* activities.

4.3 METHODS

Chemicals: All the amino acids, dipeptide, GAA, creatine and stable isotopes were pharmaceutical grade and were purchased from either Sigma, Evonik, Ajinomoto or CDN Isotopes. The other chemicals were analytical grade and purchased from either Sigma or Fisher Scientific.

Animal and surgical procedure: All procedures were approved by Institutional Animal Care Committee at Memorial University of Newfoundland (St. John's, Canada) and conformed to the guidelines of the Canadian Council on Animal Care. Thirty-five Yucatan miniature piglets (9 – 11 d old) were taken from the breeding herd at Memorial University of Newfoundland as littermate groups (sex-matched). The animals immediately underwent surgical implantation of two silastic central venous catheters and a gastric catheter under general anesthesia. Detailed descriptions of pre-anesthesia, surgical procedures, post-surgical care, and the diet feeding during recovery (until d 2) have been previously published (Dodge et al, 2012). On the morning of d 2, the piglets were randomized to

receive one of 5 experimental diets (n = 7 per group) for 5 days via the gastric catheter continuously (24 h) using pressure sensitive peristaltic pumps.

Experimental diets: Arginine and methionine were manipulated to provide excess arginine and methionine or low arginine and methionine with or without GAA and creatine in five different experimental diets: 1) Base diet was designed to marginally limit creatine and/or protein synthesis. Arginine was fed at 0.3 $g.kg^{-1}.d^{-1}$, which represents 80% of the arginine requirement (0.38 $g.kg^{-1}.d^{-1}$) according to NRC (1994). Methionine was added to provide 0.2 $g.kg^{-1}.d^{-1}$, which represents 80% of the methionine requirement for piglets at this age (Shoveller et al, 2003, NRC, 1994). These marginal levels of arginine and methionine allowed us to detect both decreases and increases in amino acid utilization due to treatments. 2) +GAA diet was designed to determine whether GAA can spare arginine; GAA was supplemented at a rate of 0.093 $g.kg^{-1}.d^{-1}$ which is the molar equivalent of the total creatine accretion rate in piglets (Brosnan et al, 2009). 3) +GAA/Met diet was designed to determine if methionine availability can limit creatine synthesis from supplemented GAA, so the Base-GAA diet was supplemented with excess methionine (0.5 $g.kg^{-1}.d^{-1}$). 4) +Cre diet was supplemented with creatine (as creatine monohydrate) at a rate equal to the total creatine accretion rate of piglets (0.12 $g.kg^{-1}.d^{-1}$) (Brosnan et al, 2009) to determine if creatine can spare arginine and/or methionine. 5) +Arg/Met diet was the positive control and included excess dietary arginine (1.8 $g.kg^{-1}.d^{-1}$) and methionine (0.5 $g.kg^{-1}.d^{-1}$) to enable protein and de novo creatine synthesis. For arginine, because we have some preliminary data that muscle protein synthesis is still increasing at 0.9 $g.kg^{-1}.d^{-1}$, we added arginine at twice that estimate. This level of arginine resulted in only one SD above

the upper limit of the sow fed plasma reference value in Wilkinson study (2004). Methionine was fed at twice the requirement.

The rest of the amino acid pattern of the diets was adapted from a commercially available paediatric parenteral nutrition solution (Vaminolact-Fresenius Kabi, Germany) with nitrogen provided as free amino acids with or without GAA or creatine. Glycine and tyrosine were provided as both the free form and as the soluble dipeptide glycine-L-tyrosine to ensure feeding adequate dietary tyrosine. All diets were designed to provide identical amounts of metabolizable energy and total amino acids. Alanine and aspartate concentrations were manipulated to accommodate the nitrogen provided by GAA, creatine, or higher arginine and/or methionine in the diets (**SUPPLEMENTAL TABLE 4.1)**. Minerals, iron dextran, trace elements (**SUPPLEMENTAL TABLE 4.2)** and Intralipid 20% (52 $mL.kg^{-1}.d^{-1}$) were added to the diet bags as previously published (Creatine paper), with the exception that the vitamin mix was prepared in our lab for this study **(SUPPLEMENTAL TABLE 4.2)**.

Tracer infusion and blood sampling: A continuous infusion of L-arg-$^{13}C_6$-HCl (28 $\mu mol.kg^{-1}.h^{-1}$; prime 16.8 $\mu mol.kg^{-1}$), guanidineacetic-2,2-D2 acid (8.6 $\mu mol.kg^{-1}.h^{-1}$; prime 5.16 $\mu mol.kg^{-1}$), creatine-D_3-H_2O (methyl-D_3) (9.9 $\mu mol.kg^{-1}.h^{-1}$; prime 9.9 $\mu mol.kg^{-1}$), L-[ring-$^2H_5$]-phenylalanine (20 $\mu mol.kg^{-1}.h^{-1}$; prime 6.39 $\mu mol.kg^{-1}$) and L-[ring-3,5-D2]-tyrosine (9 $\mu mol.kg^{-1}.h^{-1}$; prime 2.74 $\mu mol.kg^{-1}$) and was conducted for 6 hours. Only a bolus dose of L-[ring-D_4]-tyrosine (2.74 $\mu mol.kg^{-1}$) was given to prime the product pool.

Arginine, GAA and creatine tracers were used to measure the whole-body conversions of arginine to GAA and creatine whereas phenylalanine and tyrosine tracers were used to measure whole-body protein kinetics. The isotope infusion doses (both prime and constant) were based on a target atom percent excess (APE) of 5% for most tracers, and 7% for arginine, and considering pool size and the whole-body rate of appearance (Bruins et al, 2002). On d 6, the infusion was initiated as priming dose (1 $mL.kg^{-1}$), and the constant isotope infusion during the 6 h experiment was carried out as repeated half-hourly bolus doses of one eleventh of the total constant infusion volume (0.5 $mL.kg^{-1}.h^{-1}$) via the gastric catheter. An interim analysis using plasma from 10 piglets was conducted to ensure achieving a steady state of all the isotopes before recruiting the rest of the piglets. The prime and constant doses for creatine were changed from 1.16 $mg.kg^{-1}$ and 1.86 $mg.kg^{-1}.h^{-1}$ to 1.5 $mg.kg^{-1}$ and 1.5 $mg.kg^{-1}.h^{-1}$ after completing the analysis from the first 10 animals. Amino acid compositions of the experimental diets were adjusted to accommodate the arginine isotope molar contribution.

Blood was collected from the femoral vein every 30 min into a 4 mL vacutainer (K_2 EDTA 7.2 mg, BD, USA), and the plasma was separated by centrifugation and stored at -80^{o}C for later analyses of isotopic enrichments. Before each infusion experiment, three baseline blood samples were collected to determine the background enrichment of each compound of interest. At the end of the tracer infusion, the piglets were maintained on the experimental diets until necropsy the next day, when piglets were placed under general anesthesia (halothane/oxygen). The whole right kidney, pancreas and whole brain were

harvested and stored at -80°C for later analyses of AGAT activity, GAA and creatine concentrations.

Tissue enzyme activities: Renal and pancreatic AGAT activities were assayed using a modified method of Van et al (1970) as we described previously (Dinesh et al, 2018). Analysis of liver GAMT activity was conducted as per the method of Ogawa et al (1983) as modified by da Silva et al (2009) using fresh samples. The protein contents of both AGAT and GAMT homogenates were assayed using the Pierce^{TM} BCA protein assay kit (ThermoFisher Scientific). The final values were expressed as per mg of protein.

Tissue and plasma measurements: Tissue (kidney, liver and brain (cerebellum) and plasma ***GAA and creatine concentrations*** were assayed using an HPLC method modified from Buchberger and Ferdig (2004) using a C18 reverse phase column (Hypersil ODS 5 U 150 x 4.6 mm column) with ninhydrin (Sigma Aldrich) derivatization and fluorescence detection (Ex/EM 390/470). ***Plasma amino acid concentrations*** were measured via HPLC following derivatization with phenylisothiocyanate (Bidlingmeyer et al, 1984). Plasma homocysteine and cysteine concentrations were determined as per the method of Vester & Rasmussen (1991).

Mass spectrometric analysis: 25 µL of plasma were deproteinized with 200 µL of 100% methanol. The mixture was vortexed for 30 seconds and centrifuged at 18,000 x *g* for 10 minutes. Supernatants were frozen at -80^{o}C for 20 minutes and subsequently freeze-dried for one hour. Dried samples were reconstituted with mobile phase and analyzed for tracer

to tracee ratio of each isotope via LC-MS/MS as per the method of Tomlinson et al (2011) in the Analytical Facility for Bioactive Molecules at the Hospital for Sick Children, Toronto, Canada. Isotopic enrichments of L-arg-$^{13}C_6$-HCl, guanidineacetic-2,2-D_2, creatine-D_3-H_2O (Methyl-D_3), L-[ring-$^2H_5$] phenylalanine, L-[ring D_4]-tyrosine, L-[ring-3,5-D_2]-tyrosine and their products were determined by monitoring daughter ions (m/z) at 70 (M+0) and 76 (M+6) for arginine, 76 (M+0), 77 (M+1) and 78 (M+2) for GAA, 90 (M+0), 91 (M+1), 92 (M+2) and 93 (M+3) for creatine, 120 (M+0) and 125 (M+5) for phenylalanine and 136 (M+0), 138 (M+2) and 140 (M+4) for tyrosine.

Calculations: Isotope enrichment of precursor tracers and their products and flux (turnover) and precursor conversions into products (arginine to GAA and creatine, phenylalanine to tyrosine) were calculated as described by Tomlinson et al (2011). The equations are summarized in **SUPPLEMENTAL TABLE 4.3**. The whole-body phenylalanine kinetics were determined as described by Thivierge et al (2005) and were then used as a measure of whole-body protein kinetics. The detailed formulas can be found in **SUPPLEMENTAL TABLE 4.3**.

Statistical analyses: Isotopic steady state was demonstrated by a visual inspection of the data and the absence of a significant slope as determined by regression analysis. For all isotopes infused, steady state was achieved by ~3.5 h of constant infusion. Data were analyzed using one-way ANOVA and groups were compared by Newman-Keuls post hoc test (GraphPad5, Graph Pad Software Inc. CA, USA). All data were expressed as mean ± SD. When the group variances were significantly different (not normally distributed),

ANOVA testing was conducted using log-transformed variables, although values presented are mean ± SD of non-transformed data.

4.3 RESULTS

The dietary treatments did not significantly affect body weight gain of the piglets during the study.

Enzyme activities: Kidney AGAT activity in piglets fed the Base diet was higher than that in piglets fed +GAA/Met, +Cre and +Arg/Met diets, while piglets fed +GAA was higher only to +Cre diet (P = 0.0005) (**TABLE 4.1**). In the pancreas, the +Cre diet also had lower AGAT activity compared to the Base and +Arg/Met groups (**TABLE 4.1**). No other differences in AGAT were determined among groups in both kidney and pancreas. Liver GAMT activity was highest in piglets fed +GAA/Met diet compared to all other treatment groups (P = 0.01) (**TABLE 4.1**).

TABLE 4.1 Activities of enzymes of creatine synthesis and tissue GAA and creatine concentrations in piglets fed either Base, +GAA, +GAA/Met, +Cre or +Arg/Met diets[1]

	Base	**+GAA**	**+GAA/Met**	**+ Cre**	**+Arg/Met**
	AGAT activity (nmol.min^{-1}.mg protein^{-1})				
Kidney	9.70 ± 3.10^{a}	7.40 ± 2.90^{ab}	5.00 ± 1.80^{bc}	4.00 ± 1.20^{c}	6.50 ± 1.80^{bc}
Pancreas	19.9 ± 4.70^{a}	16.9 ± 3.40^{ab}	17.8 ± 3.10^{ab}	13.7 ± 4.20^{b}	21.4 ± 2.10^{a}
	GAMT activity (nmol.min^{-1}.mg protein^{-1})				
Liver	0.60 ± 0.2^{a}	0.67 ± 0.30 ^{a}	1.12 ± 0.50^{b}	0.54 ± 0.20^{a}	0.79 ± 0.20^{a}
	GAA concentration (µmol.g tissue^{-1})				
Kidney	1.28 ± 0.45^{a}	1.41 ± 0.78^{a}	1.25 ± 0.25^{a}	0.89 ± 0.31^{a}	2.58 ± 1.31^{b}
Liver	0.07 ± 0.01^{a}	0.06 ± 0.03^{a}	0.10 ± 0.02^{b}	0.06 ± 0.02^{a}	0.11 ± 0.03^{b}
Brain	0.04 ± 0.01	0.05 ± 0.02	0.06 ± 0.04	0.06 ± 0.03	0.06 ± 0.03
	Creatine concentration (µmol.g tissue^{-1})				
Kidney	1.50 ± 0.03^{a}	1.80 ± 1.20^{a}	4.50 ± 1.80^{b}	4.00 ± 1.70^{b}	3.80 ± 1.80^{b}
Liver	1.70 ± 0.60^{a}	1.40 ± 0.80^{a}	10.50 ± 3.80^{c}	4.40 ± 1.50^{b}	9.40 ± 5.40^{bc}
Brain	14 ± 5	17 ± 7	18 ± 5	17 ± 6	16 ± 7

[1]Values are mean ± SD of non-transformed data; statistics were determined using log values when variances were different; n = 7. Means with differing superscripts are significantly different within rows (≤ 0.01) (produced by one-way ANOVA) and were compared by Newman-Keuls multiple comparisons test. AGAT, L-arginine-glycine amidinotransferase;

Arg, arginine; Cre, creatine; GAA, guanidinoacetic acid; GAMT, Guanidinoacetate methyltransferase; Met, methionine. Base, low Arg and low Met; +GAA, Base plus GAA; +GAA/Met, Base plus GAA plus excess Met; +Cre, Base plus creatine; +Arg/Met, excess Arg and excess Met.

Tissue GAA and creatine concentrations: The groups fed the excess methionine concentration (+Arg/Met and +GAA/Met) had significantly higher liver GAA concentrations compared to the other three treatments. However, in the kidney, the GAA concentration was higher in the +Arg/Met group compared to the +GAA/Met treatment (**TABLE 4.1**). Significantly higher kidney and liver creatine concentrations were measured in +GAA/Met, +Cre and +Arg/Met groups compared to the Base and +GAA groups (**TABLE 4.1)**, while +GAA/Met diet resulted in significantly higher creatine concentrations in the liver tissues than the +Cre diet. Regardless of the amount of arginine and methionine in the diet, or the provision of dietary GAA or creatine, the brain GAA and creatine concentrations were unaffected by treatments (**TABLE 4.1**).

Plasma metabolite concentrations: In spite of the moderately low arginine concentration in most of our treatments, plasma ammonia and urea concentrations were not significantly different among the treatment groups (**TABLE 4.2**).

Provision of dietary GAA did not affect plasma GAA concentrations, in that the +GAA and +GAA/Met treated piglets were similar to all other treatments. The only difference in plasma GAA was between +Arg/Met and Base groups (**TABLE 4.2**). However, plasma GAA concentrations in both groups were not different from the other diet groups that were given low arginine (**TABLE 4.2**). Similar to the patterns of creatine concentrations in liver and kidney, plasma creatine concentration was significantly higher in +GAA/Met, +Cre and +Arg/Met compared to Base and +GAA groups (**TABLE 4.2**). The excess dietary arginine and methionine provided in +Arg/Met piglets led to

significantly higher plasma arginine and methionine concentrations (**TABLE 4.2**). Plasma glycine concentration was significantly lower in +Arg/Met group compared to +GAA/Met and +Cre diet groups (**TABLE 4.2**) suggesting greater glycine utilization for GAA synthesis in +Arg/Met piglets. Excess methionine diets also led to higher plasma taurine concentrations, although there was no difference in plasma cysteine concentrations across diet treatments. Plasma homocysteine concentrations in Base diet-fed piglets was higher compared to +Cre and +Arg/Met treatments, with +GAA piglets also significantly higher than +Arg/Met piglets ($P = 0.002$) (**TABLE 4.2**).

TABLE 4.2 Selected plasma amino acid and other plasma metabolite concentrations in piglets fed either Base, +GAA, +GAA/Met, +Cre or +Arg/Met diets[1]

Amino acid	Base	+GAA	+GAA/Met	+ Cre	+Arg/Met
		P5C amino acid (μmol.L^{-1})			
Alanine	1400 ± 403^{a}	1013 ± 289^{ab}	1132 ± 520^{a}	1004 ± 301^{ab}	573 ± 133^{b}
Arginine	52 ± 19^{a}	67 ± 17^{a}	66 ± 28^{a}	77 ± 20^{a}	436 ± 89^{b}
Asparagine	48 ± 7^{ab}	50 ± 7^{ab}	55 ± 6^{b}	54 ± 19^{b}	38 ± 4^{a}
Glutamine	357 ± 62^{a}	273 ± 105^{a}	292 ± 64^{a}	358 ± 138^{a}	122 ± 11^{b}
Glutamate	101 ± 27^{a}	96 ± 21^{ab}	106 ± 23^{a}	92 ± 27^{ab}	67 ± 8^{b}
Glycine	1493 ± 138^{ab}	1475 ± 207^{ab}	1655 ± 315^{a}	1613 ± 280^{a}	1204 ± 208^{b}
Citrulline	154 ± 43	135 ± 33	153 ± 27	133 ± 40	107 ± 27
Methionine	54 ± 15^{a}	47 ± 9^{a}	90 ± 27^{b}	57 ± 12^{a}	99 ± 27^{b}
Ornithine	115 ± 19^{a}	112 ± 16^{a}	97 ± 18^{a}	100 ± 31^{a}	334 ± 66^{b}
Taurine	146 ± 17^{a}	153 ± 18^{a}	229 ± 43^{b}	139 ± 23^{a}	209 ± 210^{b}
		Other plasma metabolites (μmol.L^{-1})			
Cysteine	168 ± 20	178 ± 45	158 ± 32	154 ± 24	130 ± 37
GAA	5.9 ± 1.8^{a}	6.1 ± 2.1^{ab}	6.0 ± 1.8^{ab}	8.0 ± 4.6^{ab}	12.0 ± 4.0^{b}
Creatine	58 ± 17^{a}	55 ± 28^{a}	292 ± 117^{b}	259 ± 112^{b}	201 ± 100^{b}
Homocysteine	20 ± 8^{a}	18 ±7^{ab}	14 ± 4^{ac}	11 ± 2^{bc}	10 ± 3^{c}
Ammonia	84 ± 70	53 ± 25	109 ± 115	159 ± 116	65 ± 49
Urea (mmol/L)	6.9 ± 3.6	7.6 ± 3.4	5.8 ± 1.1	6.3 ± 2.2	7.8 ± 1.7

[1]Values are mean ± SD of non-transformed data; statistics were determined using log values when variances were different; n = 7 per group, except for the ammonia concentration (n = 4 per group). Means with differing superscripts are significantly different within the row ($P \leq 0.05$) (produced by one-way ANOVA) and were compared by Newman-Keuls multiple comparison test. Arg, arginine; Cre, creatine; GAA, guanidinoacetic acid; Met, methionine; P5C, Pyrroline-5-carboxylic. Base, low Arg and low Met; +GAA, Base plus GAA; +GAA/Met, Base plus GAA plus excess Met; +Cre, Base plus creatine; +Arg/Met, excess Arg and excess Met.

Arginine, GAA and creatine kinetics: There was significantly lower plasma enrichment of the arginine (M+6) and GAA (M+2) tracers in the +Arg/Met compared to the Base group, which is likely due to dilution in the larger plasma pools due to the provision of high dietary arginine and high endogenous GAA synthesis, respectively (**TABLE 4.3**). In correspondence to this, significantly higher arginine and GAA fluxes were observed with the +Arg/Met treatment. The enrichment of the creatine (M+3) tracer that was infused was unaffected by the diet treatments, as was the whole-body creatine flux.

TABLE 4.3 Plasma isotopic enrichments of arginine, GAA, creatine and their derived products, flux of arginine, GAA and creatine and fractional conversion of arginine into GAA and creatine during steady-state on study d 6 in piglets fed either Base, +GAA, +GAA/Met, +Cre or +Arg/Met diets[1]

	Base	**+GAA**	**+GAA/Met**	**+ Cre**	**+Arg/Met**
			Enrichment (APE)		
L-Arginine -$^{13}C_6$-HCL (M+6)	6.7 ± 1.1^{a}	8.3 ± 1.9^{a}	7.4 ± 1.8^{a}	6.3 ± 1.0^{a}	4.5 ± 0 .9^{b}
Guanidinoacetic acid $^{13}C_1$ (M+1)	3.7 ± 0.6^{a}	2.8 ± 0.8^{ab}	2.7 ± 0.5^{b}	3.1 ± 0.7^{ab}	3.6 ± 0.2^{ab}
Creatine $^{13}C_1$ (M+1)	1.8 ± 0.3^{a}	1.3 ± 0.4^{a}	0.5 ± 0.1^{b}	0.7 ± 0.1^{b}	1.5 ± 0.7^{a}
Guanidinoacetic-2,2-d_2 acid (M+2)	6.9 ± 1.3^{ab}	9.7 ± 2.9^{a}	8.3 ± 2.1^{a}	9.5 ± 4.5^{a}	4.9 ± 1.0^{b}
Creatine-(methyl d_2) monohydrate (M+2)	10.7 ± 1.3^{a}	8.6 ± 2.2^{ab}	5.5 ± 1.2^{c}	6.1 ± 1.7^{bc}	7.2 ± 2.9^{bc}
Creatine-(methyl d_3) monohydrate (M+3)	9.9 ± 3.9	10.0 ± 4.1	6.7 ± 1.4	6.6 ± 1.7	8.6 ± 1.6
			Flux (μmol.kg^{-1}.h^{-1})		
Arginine	398 ± 76^{a}	329 ± 98^{a}	368 ± 94^{a}	425 ± 79^{a}	610 ± 133^{b}
GAA	119 ± 24^{a}	84 ± 20^{a}	101 ± 35^{a}	101 ± 55^{a}	173 ± 50^{b}
Creatine	111 ± 47	111 ± 50	152 ± 31	157 ± 43	116 ± 29
			Fractional conversion		
Arginine to GAA	0.57 ± 0.12^{a}	0.34 ± 0.06^{a}	0.38 ± 0.14^{a}	0.50 ± 0.17^{a}	0.79 ± 0.14^{b}
Arginine to creatine	0.28 ± 0.08^{a}	0.16 ± 0.04^{b}	0.08 ± 0.03^{c}	0.10 ± 0.02^{c}	0.30 ± 0.17^{a}
GAA to creatine (M+1)	0.48 ± 0.06^{a}	0.47 ± 0.07^{a}	0.20 ± 0.07^{b}	0.23 ± 0.05^{b}	0.42 ± 0.20^{a}

[1]Values are mean ± SD of non-transformed data; statistics were determined using log values when variances were different; n = 6. Means with differing superscripts are significantly different within the row ($P \leq 0.05$) (produced by one-way ANOVA) and were compared by Newman-Keuls multiple comparisons test. APE, Atom percent excess; Arg, arginine; Cre, creatine; GAA, guanidinoacetic acid; Met, methionine. Base, low Arg and low Met; +GAA, Base plus GAA; +GAA/Met, Base plus GAA plus excess Met; +Cre, Base plus creatine; +Arg/Met, excess Arg and excess Met.

Whole-body arginine conversion to GAA and creatine: The rate of whole-body arginine conversion to GAA was significantly lower in all the diets with low arginine concentrations compared to the diet with additional arginine (+Arg/Met) (**TABLE 4.4**). Furthermore, in the +GAA group, the rate of GAA production from arginine was significantly lower than in piglets fed the Base diet (**TABLE 4.4**). Rate of arginine conversion to creatine followed a similar pattern as GAA production, with the highest rate of conversion in the +Arg/Met group (**TABLE 4.4**). The conversion rate of endogenously synthesized GAA (M+1) to creatine was not different among all groups (**TABLE 4.4**). Because mixed body proteins of neonates contain 3.60%, 6.70% and 1.96% phenylalanine, arginine and methionine, respectively (Wu et al, 1999), phenylalanine incorporation into whole-body proteins was used to estimate the arginine and methionine incorporation into whole-body proteins in our piglets. Irrespective of the amount of arginine or methionine in the diets, the incorporation of arginine and methionine into proteins was unaffected in our piglets (**TABLE 4.4**).

TABLE 4.4 Whole-body arginine, methionine, GAA and creatine kinetics at steady-state on study d 6 in piglets fed either Base, +GAA, +GAA/Met, +Cre or +Arg/Met diets[1]

	Base	**+GAA**	**+GAA/Met**	**+ Cre**	**+Arg/Met**
Fractional conversion rate (μmol.kg^{-1}.h^{-1})					
Arginine into GAA	66 ± 15^{a}	29 ± 9^{b}	41 ± 25^{ab}	46 ± 15^{ab}	143 ± 47^{c}
Arginine into creatine	29 ± 12^{a}	18 ± 9^{b}	12 ± 5^{ab}	17 ± 3^{ab}	38 ± 19^{c}
Arginine to proteins	241 ± 48	251 ± 7	262 ± 28	244 ± 25	249 ± 16
GAA to creatine (M+1)	52 ± 22	42 ± 16	30 ± 6	37 ± 17	46 ± 16
Methionine to proteins	82 ± 16	85 ± 6	72 ± 8	83 ± 9	85 ± 5
Glycine to proteins	945 ± 186	982 ± 66	1026 ± 108	954 ± 98	975 ± 63

[1]Values are mean ± SD of non-transformed data; statistics were determined using log values when variances were different; n = 6. Means with differing superscripts are significantly different within the row ($P \leq 0.05$) (produced by one-way ANOVA) and were compared by Newman-Keuls multiple comparisons test. Arg, arginine; Cre, creatine; GAA, guanidinoacetic acid; Met, methionine. Base, low Arg and low Met; +GAA, Base plus GAA; +GAA/Met, Base plus GAA plus excess Met; +Cre, Base plus creatine; +Arg/Met, excess Arg and excess Met.

Partitioning of arginine between GAA and protein synthesis: A higher proportion of arginine flux was directed towards GAA synthesis in our +Arg/Met diet compared to the diets with low arginine ($P < 0.0001$) (**TABLE 4.5**). Among the four low arginine diet treatments, the proportion of arginine flux directed towards GAA was highest in Base diet (17%) compared to piglets fed +GAA (9%), +Cre (11%) or +GAA/Met (11%). The proportion of arginine flux directed towards whole-body protein synthesis was 51 - 90% ($P < 0.01$) and was different between piglets fed +GAA or +GAA and +Arg/Met. Only the +Arg/Met piglets showed no difference to other diet groups ($P = 0.01$) **(TABLE 4.5**).

TABLE 4.5 Whole-body arginine flux directed to the product as a % of precursor amino acid flux during steady-state on study d 6 in piglets fed either Base, +GAA, +GAA/Met, +Cre or +Arg/Met diets[1]

	Base	**+GAA**	**+GAA/Met**	**+ Cre**	**+Arg/Met**
			%		
Arginine to GAA	17 ± 3^{a}	9 ± 4^{b}	11 ± 4^{b}	11 ± 3^{b}	23 ± 6^{c}
Arginine to protein	63 ± 21^{ab}	81 ± 21^{a}	76 ± 23^{a}	59 ± 14^{ab}	44 ± 9^{b}

[1]Values are mean ± SD of non-transformed data; statistics were determined using log values when variances were different; n = 6. Means with differing superscripts are significantly different within the row ($P \leq 0.01$) (produced by one-way ANOVA) and were compared by Newman-Keuls multiple comparisons test. Arg, arginine; Cre, creatine; GAA, guanidinoacetic acid; Met, methionine. Base, low Arg and low Met; +GAA, Base plus GAA; +GAA/Met, Base plus GAA plus excess Met; +Cre, Base plus creatine; +Arg/Met, excess Arg and excess Met.

Utilization of dietary arginine, glycine and methionine for GAA, creatine and protein synthesis: The fraction of dietary arginine utilized to synthesize both GAA and creatine was significantly higher in the Base diet group (91%) compared to all other treatments (32 - 63%) (**TABLE 4.6**). The proportion of dietary glycine used for the synthesis of GAA was significantly higher in +Arg/Met piglets compared to the piglets fed diets with low arginine (**TABLE 4.6**). The fraction of dietary methionine utilized to synthesize creatine was significantly lower only in the +Arg/Met group compared to all other diets (**TABLE 4.6**). The fractions of dietary arginine and methionine partitioned to whole-body proteins was only affected by the amount of arginine in the diets ($P < 0.0001$) (**TABLE 4.6**).

TABLE 4.6 Percentage of dietary amino acid utilization (arginine, glycine or methionine) to synthesize GAA & creatine (%) determined at steady-state on study d 6 in piglets fed either Base, +GAA, +GAA/Met, +Cre or +Arg/Met diets[1]

	Base	**+GAA**	**+GAA/Met**	**+ Cre**	**+Arg/Met**
		%			
Arginine to GAA	91 ± 21^{a}	40 ± 12^{bc}	44 ± 19^{bc}	63 ± 21^{b}	32 ± 10^{c}
Arginine to creatine	40 ± 16^{a}	24 ± 13^{b}	16 ± 7^{b}	23 ± 5^{b}	9 ± 4^{b}
Arginine to proteins	335 ± 66^{a}	348 ± 23^{a}	364 ± 39^{a}	338 ± 34^{a}	58 ± 4^{b}
(GAA M+1 to Cre M+1)	94 ± 40^{a}	97 ± 58^{a}	53 ± 11^{a}	65 ± 30^{a}	33 ± 11^{b}
Methionine to proteins	146 ± 29^{a}	152 ± 11^{a}	129 ± 14^{a}	148 ± 15^{a}	61 ± 4^{b}
Glycine to GAA	33 ± 8^{a}	15 ± 4^{b}	21 ± 13^{b}	23 ± 8^{ab}	72 ± 24^{c}
Glycine to creatine	14 ± 5^{ab}	9 ± 5^{ac}	6 ± 3^{c}	8 ± 2^{ac}	19 ± 8^{b}
Glycine to proteins	476 ± 94	495 ± 33	518 ± 54	481 ± 49	492 ± 31

[1]Values are mean ± SD of non-transformed data; statistics were determined using log values when variances were different; n = 6. Means with differing superscripts are significantly different within the row ($P \leq 0.01$) (produced by one-way ANOVA) and were compared by Newman-Keuls multiple comparisons test. Arg, arginine; Cre, creatine; GAA, guanidinoacetic acid; Met, methionine. Base, low Arg and low Met; +GAA, Base plus GAA; +GAA/Met, Base plus GAA plus excess Met; +Cre, Base plus creatine; +Arg/Met, excess Arg and excess Met.

TABLE 4.7 Fate of metabolites in the whole-body pool in piglets fed either Base, +GAA, +GAA/Met, +Cre or +Arg/Met diets[1]

	Base	**+GAA**	**+GAA/Met**	**+ Cre**	**+Arg/Met**
			$\mu mol.kg^{-1}.h^{-1}$		
Dietary intake					
Arginine	72	72	72	72	432
Methionine	56	56	140	56	140
Creatine	0	0	0	33	0
De novo synthesis					
Arginine	223 ± 72^{a}	155 ± 80^{ab}	192 ± 96^{a}	237 ± 87^{a}	56 ± 100^{b}
Creatine[2]	111 ± 47	111 ± 50	152 ± 31	124 ± 43	116 ± 29
Release from protein break-down					
Arginine	132 ± 60	110 ± 64	133 ± 38	150 ± 32	124 ± 22
Methionine	45 ± 21	38 ± 22	45 ± 13	51 ± 11	42 ± 8

[1]Values are mean ± SD of non-transformed data; statistics were determined using log values when variances were different; n = 7. Means with differing superscripts are significantly different within the row ($P \leq 0.05$) (produced by one-way ANOVA) and were compared by Newman-Keuls multiple comparisons test. [2]Sum of the creatine synthesis and stored creatine release from muscles. Arg, arginine; Cre, creatine; GAA, guanidinoacetic acid; Met, methionine. Base, low Arg and low Met; +GAA, Base plus GAA; +GAA/Met, Base plus GAA plus excess Met; +Cre, Base plus creatine; +Arg/Met, excess Arg and excess Met.

Whole-body phenylalanine, tyrosine kinetics: There was no diet effect on phenylalanine incorporated into whole-body proteins, or phenylalanine released from whole-body protein breakdown in our piglets (**TABLE 4.8**).

TABLE 4.8 Phenylalanine (Phe) enrichment, flux and rate of incorporation into whole-body proteins at steady-state on study d 6 in piglets fed either Base, +GAA, +GAA/Met, +Cre or +Arg/Met diets[1]

	Base	**+GAA**	**+GAA/Met**	**+ Cre**	**+Arg/Met**
			Enrichment (APE)		
L-Phenyl-[ring-$^2H_5$] alanine (M+5)	8.1 ± 0.5	8.5 ± 1.2	7.8 ± 0.5	7.8 ± 0.7	8.2 ± 0.4
L-Tyrosine-(phenyl-d_4) (M+4)	0.7 ± 0.2	0.7 ± 0.3	0.5 ± 0.1	0.6 ± 0.2	0.6 ± 0.3
L-[ring 3,5 d_2]-tyrosine (M+2)	4.3 ± 0.5	4.4 ± 0.6	4.2 ± 0.4	4.3 ± 0.8	4.1 ± 1.0
			Flux (μmol.kg^{-1}.h^{-1})		
Phe	254 ± 50	245 ± 63	264 ± 56	264 ± 41	250 ± 42
Tyr	201 ± 25	199 ± 24	208 ± 20	209 ± 37	199 ± 22
			(μmol.kg^{-1}.h^{-1})		
Phe into Tyr conversion rate	17 ± 5	14 ± 4	12 ± 5	16 ± 9	16 ± 4
Phe incorporation into proteins	211 ± 13	205 ± 37	224 ± 16	223 ± 21	211 ± 9
Phe released from protein break-down	75 ± 34	63 ± 36	75 ± 22	85 ± 18	70 ± 12
Net phe incorporation into whole-body proteins	137 ± 27	142 ± 10	149 ± 16	138 ± 14	141 ± 9

[1]Values are mean ± SD of non-transformed data; statistics were determined using log values when variances were different; n = 6. Means with differing superscripts are significantly different within the row (produced by one-way ANOVA) and were compared by Newman-Keuls multiple comparisons test. Arg, arginine; APE, Atom percent excess; Cre, creatine; GAA, guanidinoacetic acid; Met, methionine. Phe, phenylalanine; Tyr, tyrosine. Base, low Arg and low Met; +GAA, Base plus GAA; +GAA/Met, Base plus GAA plus excess Met; +Cre, Base plus creatine; +Arg/Met, excess Arg and excess Met.

4.5 DISCUSSION

This is the first report of the partitioning of arginine into GAA, creatine, and protein *in-vivo*, with the corresponding plasma and tissue GAA and creatine concentrations. As GAA and creatine synthesis depend on arginine availability (Gagnon et al, 2010), we quantified the partitioning of arginine into GAA/creatine and towards protein synthesis when low versus excess amounts of arginine and methionine were provided in the diet.

Determinants of GAA and creatine synthesis: substrate, dietary creatine and endogenously synthesized creatine availability or enzyme activity An important question from our previous study (Dinesh et al, 2018) was whether the low creatine accretion in our piglets fed a creatine-free diet was due to limited enzyme capacity or limited substrate availability. It is clear from this study, that arginine/methionine availability, rather than enzyme activity, affects GAA and creatine biosynthesis. The highest GAA/creatine synthesis occurred in piglets fed excess arginine and methionine, but tissues from these same piglets exhibited lower in-vitro AGAT activity with no differences in GAMT activity. Similarly, AGAT activity was highest in the piglets fed the lowest arginine concentration, but these piglets demonstrated lower GAA synthesis, clearly demonstrating that substrate availability rather than the enzyme activity was the primary determinant of GAA synthesis.

In our previous study (Dinesh et al, 2018) and in the present study, we have demonstrated that a dietary supply of creatine results in reduced *in-vitro* AGAT specific

activity in piglets. Creatine supplementation has been reported to down regulate creatine synthesis by lowering kidney AGAT activity, AGAT protein and AGAT mRNA levels in rats (McGuire et al, 1984, Edison et al, 2007, da Silva et al, 2009, 2014). Based on these findings, we predicted a lower rate of conversion of arginine to GAA and creatine in piglets fed creatine; however, this did not happen. We observed no difference in the rate of GAA or creatine synthesis between creatine supplemented and un-supplemented piglets when arginine and methionine were low, despite lower *in-vitro* AGAT activity with creatine in the diet. It should be noted that we provided low arginine in both diets, so again, it may have been substrate availability rather than the presence of creatine in the diet that determined the rate of arginine conversion to GAA and creatine.

The interpretation of data from the *in-vitro* enzyme capacity and the *in-vivo* isotope kinetics were inconsistent and perhaps the *in-vitro* enzyme capacity is misleading. It is clear that the information derived from *in-vitro* enzyme studies (enzyme capacity) does not necessarily reflect the *in-vivo* flux through the enzymes and strongly suggests that enzyme activity data should not be used in isolation to interpret metabolite conversions.

Similarly, we expected that providing dietary GAA with excess methionine would lower arginine conversion to GAA and creatine, as this condition led to plasma and tissue creatine concentrations which were similar to piglets fed creatine; but again, this was not the case, suggesting that it may be excess arginine availability, rather than endogenously synthesized creatine, that alters the rate of GAA synthesis.

Capacity of piglet to synthesize creatine Low dietary arginine in the present study and moderate arginine in our previous study (Dinesh et al, 2018) both led to deficits in plasma GAA and creatine even with high AGAT capacity. In the present study, as expected, excess arginine and methionine sustained de novo GAA and creatine synthesis and a concomitant increase in the plasma and tissue creatine concentrations similar to piglets fed their entire creatine requirement in the diet. Therefore, the amount of arginine and methionine provided in the excess arginine and methionine diet appeared to be sufficient to meet the whole-body creatine requirement in piglets of this age, at least in the short-term.

We calculated de novo arginine synthesis from the proportion of arginine released from protein break down, the dietary arginine intake and whole-body arginine flux. The calculated de novo arginine synthesis in piglets fed the low arginine diets was higher than in piglets fed excess arginine. However, this higher de novo arginine synthesis did not translate into higher peripheral plasma arginine concentrations. None of the low arginine treatments, even with de novo arginine synthetic capacity, could overcome arginine deficiency and the concomitant reduction in GAA and creatine synthesis. Arginine levels in different brands of pediatric PN solutions varied from 0.7 $g.kg^{-1}.d^{-1}$ to 1.8 $g.kg^{-1}.d^{-1}$ (Brunton et al, 2000). We included 0.3 $g.kg^{-1}.d^{-1}$ arginine in the low arginine treatments, while arginine was 1.8 $g.kg^{-1}.d^{-1}$ in the excess arginine treatment. Therefore, the provision of excess amounts of arginine and methionine, is required to meet the neonatal whole-body creatine requirement when pre-made creatine is not provided in the diet.

GAA, an effective precursor for creatine biosynthesis Because GAA is proportionately converted to creatine with no feedback regulation (da Silva et al, 2009, McBreairty et al, 2015), we hypothesized that supplemental GAA would be maximally converted to creatine only if sufficient methionine was available to provide methyl groups for GAMT. This strategy produced plasma and tissue creatine concentrations that were similar to those resulting from the direct provision of dietary creatine, which demonstrates that under such conditions, the piglets had the capacity to synthesize enough creatine. Moreover, liver GAMT activity was higher only in piglets fed dietary GAA with excess methionine, demonstrating that GAMT was induced to accommodate creatine synthesis, but only when methionine was abundant. Therefore, dietary GAA is an effective intermediate precursor for creatine biosynthesis, but only when provided with excess methionine.

Low methionine in the GAA diet hampered creatine synthesis, as plasma and tissue creatine concentrations were low compared to piglets fed creatine. We predicted, therefore, higher plasma and liver GAA concentrations in these same piglets. However, we observed no differences in plasma GAA concentrations between piglets fed dietary GAA with low methionine and other low arginine groups suggesting that dietary GAA did not appear in the peripheral circulation. Liver is considered to be the main organ that takes up GAA to synthesize creatine in the body (Brosnan et al, 2009). When dietary GAA was provided with excess methionine, it was clearly evident that GAA was absorbed via the small intestine, since we observed high plasma and liver creatine concentrations in these piglets, similar to piglets fed creatine. Further to this, it was evident that dietary GAA was channeled to the liver, as piglets fed excess methionine with dietary GAA exhibited high

liver GAA concentrations which were similar to piglets fed excess arginine and methionine. When methionine was low in the diet, it is possible that dietary GAA was still absorbed, but not channeled into the liver for the transmethylation given low methyl group availability. However, the fate of this absorbed, but unmethylated GAA is unclear because GAA did not accumulate in the liver or kidney or in the plasma for catabolism and excretion. We did not measure urinary GAA, but we would have expected plasma GAA to increase concomitant with high renal excretion, so it seems unlikely that urinary excretion was the fate of the dietary GAA.

Maintenance of whole-body protein synthesis versus whole-body GAA/creatine synthesis In the current study, surprisingly, short-term dietary manipulation of arginine, methionine, GAA or creatine and concomitant acute changes in plasma arginine and methionine did not affect the whole-body protein synthesis in neonatal piglets.

The proportion of arginine flux directed towards GAA synthesis was only 17% in low arginine and methionine treated piglets; however, the proportion of flux was 23% in piglets fed excess arginine and methionine, suggesting that GAA and creatine synthesis were disproportionately affected by the acute changes in arginine in the diet. This reduction in the partitioning towards GAA and creatine synthesis may have allowed the piglets to accommodate a lower arginine intake without sacrificing whole-body protein synthesis. A more severe arginine deficiency might be necessary to observe a sparing effect of arginine for protein synthesis. Alternatively, the measurement of whole-body protein synthesis might not capture small increases in organ specific protein synthesis.

Short-term diet manipulations on brain GAA/creatine concentrations In our previous study we supplemented creatine to parenterally-fed piglets for two weeks and found no differences in brain GAA or creatine levels (Dinesh et al 2018). In the current study, we wanted to test whether the short-term provision of dietary arginine and methionine or the supplementation of GAA or creatine would affect brain GAA and creatine concentrations. The brain GAA and creatine levels were unaffected, suggesting that brain creatine accretion may be conserved and not readily affected by changes in whole-body creatine metabolism. Whether more chronic changes in arginine and methionine intake affect brain creatine accretion needs to be examined.

Conclusion The data from this study demonstrated that the short-term changes in arginine, methionine, GAA and creatine synthesis did not translate into differences in the brain GAA and creatine levels in neonatal piglets. The novel finding in this study was that GAA/creatine synthesis have been driven by the arginine and methionine availability rather than enzyme capacity or creatine concentration in the tissues. Since *in-vitro* enzyme activities do not reflect *in-vivo* arginine conversion into GAA/creatine, therefore, they cannot solely be utilized to speculate on the regulation of creatine biosynthesis. We demonstrated that the piglet is capable of synthesizing its requirement for creatine provided sufficient arginine and methionine are fed. The noteworthy finding was that supplemental GAA can be used to satisfy whole-body creatine needs, but only if accompanied by adequate methionine in the diet, perhaps to facilitate intestinal absorption of the GAA, and certainly to transmethylate GAA to creatine in the liver. Overall, we demonstrated that

creatine biosynthesis was disproportionately sacrificed to maintain whole-body protein synthesis when arginine and methionine were limiting in the diets of neonatal piglets. Therefore, adequate amounts of precursors of creatine or pre-made creatine supplementation must be considered relevant to the amino acid requirements of the neonatal diet to accommodate creatine requirement.

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4.7 ACKNOWLEDGEMENTS

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4.8 ONLINE SUPPORTING MATERIAL

SUPLEMENTAL TABLE 4.1 Amino acid, GAA and creatine compositions of Base, +GAA, +GAA/Met, +Cre or +Arg/Met diets[1]

Amino acid	Base	+GAA	+GAA/Met	+Cre	+Arg/Met
		g.L^{-1} (g.kg^{-1}d^{-1})			
Alanine	11.16 (3.04)	10.39 (2.8[2])	**9.61(2.61)**	10.39 (2.82)	**0.00 (0.00)**
Arginine	1.10 (0.30)	1.10 (0.30)	1.10 (0.30)	1.10 (0.30)	**6.62 (1.80)**
Aspartate	3.47 (0.94)	3.47 (0.94)	3.47 (0.94)	3.47 (0.94)	**2.31 (0.63)**
Cysteine	0.83 (0.22)	0.83 (0.22)	0.83 (0.22)	0.83 (0.22)	0.83 (0.22)
Glutamate	6.00 (1.63)	6.00 (1.63)	6.00 (1.63)	6.00 (1.63)	6.00 (1.63)
Glycine	1.31 (0.36)	1.31 (0.36)	1.31 (0.36)	1.31 (0.36)	1.31 (0.36)
Histidine	1.76 (0.48)	1.76 (0.48)	1.76 (0.48)	1.76 (0.48)	1.76 (0.48)
Isoleucine	2.64 (0.72)	2.64 (0.72)	2.64 (0.72)	2.64 (0.72)	2.64 (0.72)
Leucine	5.94 (1.62)	5.94 (1.62)	5.94 (1.62)	5.94 (1.62)	5.94 (1.62)
Lysine	4.73 (1.29)	4.73 (1.29)	4.73 (1.29)	4.73 (1.29)	4.73 (1.29)
Methionine	0.74 (0.20)	0.74 (0.20)	**1.84 (0.50)**	0.74 (0.20)	**1.84 (0.50)**
Phenylalanine	2.24 (0.61)	2.24 (0.61)	2.24 (0.61)	2.24 (0.61)	2.24 (0.61)
Proline	4.73 (1.29)	4.73 (1.29)	4.73 (1.29)	4.73 (1.29)	4.73 (1.29)
Serine	3.19 (0.87)	3.19 (0.87)	3.19 (0.87)	3.19 (0.87)	3.19 (0.87)
Taurine	0.28 (0.07)	0.28 (0.07)	0.28 (0.07)	0.28 (0.07)	0.28 (0.07)
Tryptophan	1.21 (0.33)	1.21 (0.33)	1.21 (0.33)	1.21 (0.33)	1.21(0.33)
Tyrosine	0.43 (0.12)	0.43 (0.12)	0.43 (0.12)	0.43 (0.12)	0.43 (0.12)
Valine	3.03 (0.82)	3.03 (0.82)	3.03 (0.82)	3.03 (0.82)	3.03 (0.82)
Threonine	3.03 (0.82)	3.03 (0.82)	3.03 (0.82)	3.03 (0.82)	3.03 (0.82)
Creatine monohydrate	**0.00 (0.00)**	**0.00 (0.00)**	**0.00 (0.00)**	**0.44 (0.12)**	**0.00 (0.00)**
GAA	**0.00 (0.00)**	**0.34 (0.09)**	**0.34 (0.09)**	**0.00 (0.00)**	**0.00 (0.00)**
Glycine-L-tyrosine	1.42 (0.39)	1.42 (0.39)	1.42 (0.39)	1.42 (0.39)	1.42 (0.39)

[1]Alanine and aspartate were used to make the diets isonitrogenous. With the exceptions of arginine, methionine, alanine and aspartate, the amino acid compositions of the diets were similar. Arg, arginine; Cre, creatine; GAA, guanidinoacetic acid; Met, methionine. Base, low Arg and low Met; +GAA, Base plus GAA; +GAA/Met, Base plus GAA plus excess Met; +Cre, Base plus creatine; +Arg/Met, excess Arg and excess Met.

SUPPLEMETAL TABLE 4.2 Composition of either Base, +GAA, +GAA/Met, +Cre or +Arg/Met diets[1,2]

Composition of Elemental Diets	$g.L^{-1}$
L-Amino acids	56.0
D-Glucose	90.3
Trihydrate K_2HPO_4	1.57
Monobasic KH_2PO_4	1.09
Potassium acetate	1.47
NaCl	2.17
MgSO4	0.78
ZnSO4	0.09
Calcium gluconate	6.41
Composition of Admixtures	
Vitamin solutions[3]	
Fat soluble vitamins	$Dose \cdot kg^{-1}BW \cdot d^{-1}$
Vitamin A, μg	273
Vitamin D, μg	2.20
Vitamin E (*dl*-alpha tocopheryl acetate), *mg*	1.50
Vial 1	
Ascorbic acid, *mg*	17.4
Thiamine (as hydrochloride), *mg*	0.26
Riboflavin (as phosphate), *mg*	0.30
Pyridoxine hydrochloride, *mg*	0.22
Niacinamide, *mg*	3.70
d-Panthenol, *mg*	1.10
Vitamin K_1, *mg*	0.04
Vial 2	
Biotin, μg	4.35
Folic Acid, μg	30.46
Vitamin B_{12}, μg	0.22
Trace ElementMix[4]	$mg \cdot kg^{-1}BW \cdot d^{-1}$
Zinc (as $ZnSO_4.7H_2O$)	10.07
Copper (as $CuSO_4.5H_2O$)	0.86
Manganese (as $MnSO_4.H_2O$)	0.66
Chromium (as $CrCl_3.6H_2O$)	0.01
Selenium (as SeO_2)	0.05
Iodide (as NaI)	0.02
Iron Dextran[5]	
Iron (as ferric hydroxide)	3.0

[1]All five experimental diets were identical, with the exception of the amino acid composition **(SUPPLEMENTAL TABLE 1**). The diets were comprised of an amino acid, glucose mineral mixture, which was infused at a rate of 272 $mL \cdot kg^{-1}$ $BW \cdot d^{-1}$.

[2]Intralipid® 20% (Baxter, Canada) infused with the diets at a rate of 52 $mL \cdot kg^{-1}$ $BW \cdot d^{-1}$. Choline presents as free form (in the form of lecithin) and as bound to phosphatidylcholine in the Intralipid® 20% (Baxter, Canada). The bound form is not converted to choline to any significant degree. The free choline in Intralipid® 20% emulsion is 24 nmol/mL (Buchman AL, 2009).

[3]Vitamin solutions (components are from Sigma-Aldrich, Canada or Biochemica BDH or Fluka, China) during treatment diets were prepared in the laboratory. The compositions of all three solutions were as in the Multi-12/K_1 Pediatric® multivitamin solution for parenteral nutrition (Baxter). The PH was adjusted using citric acid and/or sodium citrate. Doses from Vial 1 and 2 were added to the amino acid mixture just prior to use. Fat soluble vitamins were dissolved in vegetable oil (Crisco) and dosed as 0.5 $mLkg^{-1}d^{-1}$ directly into gastric catheter in every morning. During intravenous feeding, commercial Multi-12/K_1 Pediatric® multivitamin solution was infused into diet bag.

[4]The the trace element mix (components from Sigma-Aldrich, Canada) was prepared in the laboratory, and added to the amino acid mixture just prior to use.

[5]Iron dextran (Bimeda-MTC Animal Health, Canada).

Base, low Arg and low Met; +GAA, Base plus GAA; +GAA/Met, Base plus GAA plus excess Met; +Cre, Base plus creatine; +Arg/Met, excess Arg and excess Met.

SUPPLEMENTAL TABLE 4.3 Equations for *in-vivo* kinetics analysis

Variable	Equation
• Enrichment (APE)(E)	$[(R_s-R_b)/(1+R_s)-R_b]*100$
• Whole-body turnover (Flux) ($\mu mol.kg^{-1}.hr^{-1}$) (q)	$q = I *[(E_i/E_p)-1]$
Conversion rates:	
○ Whole-body conversion rate of arginine into GAA	$Q_{Arg > GAA} = (E_{GAA}/E_{Arg})*q_{GAA}$
○ Whole-body conversion rate of arginine into creatine	$Q_{Arg > Cre} = (E_{Cre}/E_{Arg})*q_{Cre}$
○ Whole-body conversion rate of GAA into creatine	$Q_{GAA > Cre} = (E_{Cre}/E_{GAA})*q_{Cre}$
○ Fractional conversion	E_{pdc}/E_{pre}
Arginine de novo synthesis	$Flux_{Arg}$ - (Dietary Arg intake + Arg released from Protein-break down)
Creatine release from muscle (creatine synthesis in muscle + creatine release from muscle creatine pool)	$Flux_{Cre}$ - (Dietary Cre intake + *Creatine de novo synthesis*
Estimation of whole-body protein kinetics:	
○ Whole-body protein synthesis ($\mu mol.kg^{-1}.hr^{-1}$)	Flux - (Phe → Tyr conversion rate)
○ Whole-body protein break-down ($\mu mol.kg^{-1}.hr^{-1}$)	Flux - Phe infusion rate
○ Whole-body net protein synthesis ($\mu mol.kg^{-1}.hr^{-1}$)	Protein synthesis - Protein break-down
• Fractional conversion rate as a % of precursor amino acid flux	(Fractional conversion rate/$Flux_{precursor}$)*100
• Fraction of dietary precursor amino acid (arginine, glycine or methionine) used to synthesize creatine/proteins (%)	(Conversion rate/Dietary amino acid infusion rate)*100

APE, Atom percent excess, Arg, arginine; Cre, creatine; $E_{i,}$ enrichment in the infusate; $E_{p,}$ enrichment in the plasma; E_{pre}, enrichment of the precursor; E_{pro}, enrichment of the product; I, isotope infusion rate; $R_{s,}$ tracer:tracee in enriched sample; $R_{b,}$ tracer:trace in natural abundance; Phe, Phenylalanine; Tyr, tyrosine.

CHAPTER FIVE

Kidneys are quantitatively more important than pancreas and gut as a source of GAA for hepatic creatine synthesis in sow-reared Yucatan miniature piglets

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5.0 Kidneys are quantitatively more important than pancreas and gut as a source of GAA for hepatic creatine synthesis in sow-reared Yucatan miniature piglets

5.1 ABSTRACT

Background: Arginine:glycine amidinotransferase, necessary for the conversion of arginine to guanidinoacetic acid (GAA), is expressed mainly in the kidney and pancreas. The subsequent methylation of GAA to creatine primarily occurs in the liver. The quantitative importance of the kidney, pancreas and gut as sources of GAA for hepatic creatine synthesis has not been studied in neonates. **Methods:** In sow-fed and fasted neonatal piglets, the net balance of GAA and creatine across the kidney, pancreas and intestine was measured during a constant intravenous infusion of one of the following: 1) arginine + methionine (Arg/Met), 2) creatine + arginine + methionine (+Cre), 3) citrulline + methionine (Cit/Met) or 4) alanine (Ala). **Results:** The kidneys of sow-fed piglets contributed 88% of the measured GAA produced, with the remainder released by the pancreas. Infusion of arginine (Arg/Met and +Cre) or citrulline (Cit/Met) produced much greater net release of GAA from the kidneys compared to that released from the pancreas and intestine. However, renal GAA release was greatest with citrulline infusion, suggesting that citrulline is a better precursor than arginine for GAA synthesis in the kidneys. In the

intestine, arginine during creatine infusion, but not citrulline, resulted in a net release of GAA, suggesting that these organs do not have the capacity to convert citrulline into arginine. Creatine infusion resulted in a much higher net GAA release from the intestine and pancreas compared to other treatments, perhaps because GAA was not required for creatine synthesis in these tissues and was subsequently released. **Conclusions:** The kidneys, not the pancreas or intestine, are the major source of GAA for hepatic creatine synthesis in neonatal piglets, but the neonatal intestine has the capacity to synthesize GAA and creatine when arginine and methionine are available with creatine.

Abbreviations: AGAT, L-Arginine:glycine amidinotransferase; Ala, alanine; Arg, arginine; CA, carotid artery; Cit, citrulline; Cre, creatine; GAA, guanidinoacetic acid; GAMT, guanidinoacetate *N*-methyltransferase; Met, methionine; PITC, phenylisothiocyanate; PV, portal vein; RV, renal vein; SF, sow fed; SV, splenic vein.

5.2 INTRODUCTION

De novo creatine synthesis involves the conversion of arginine to guanidinoacetic acid (GAA) via the enzyme L-arginine:glycine amidinotransferase (AGAT), and the subsequent conversion of GAA to creatine requires methionine and the enzyme guanidinoacetate *N*-methyltransferase (GAMT) (Walker et al, 1979, Wyss et al, 2000). Tissue GAA and creatine concentrations and *in-vitro* activities of AGAT and GAMT have been analyzed to describe the inter-organ system of creatine synthesis (Walker et al, 1979, Wyss et al, 2000, Brosnan et al, 2009, Dinesh et al, 2018). The kidney has been considered the major organ responsible for GAA synthesis, based on arterio-venous balance data determined from rats (Edison et al, 2007). However, in two previous studies in neonatal piglets that assessed creatine status with varying dietary amino acid availability, we demonstrated that pancreatic AGAT specific activity was five-fold higher than kidney AGAT activity when expressed per g of tissue. *In-vitro* data from piglets (Brosnan et al, 2009, Dinesh et al, 2018), as well as data from humans (Wyss et al, 2000), have suggested that the pancreas might be of greater importance than was determined in rodents (da Silva et al, 2014). Although there are *in-vitro* data on creatine synthesis, the quantitative contribution of the pancreas to whole-body GAA and creatine supply is not known. Furthermore, the contributions of the kidney, pancreas and gut likely depends on availability of precursors (i.e., arginine and methionine) or preformed creatine and should be elucidated.

Arginine is the direct precursor for GAA synthesis, but citrulline is converted to arginine in the kidney. Evidence that citrulline may be important for GAA synthesis emerged from a study in piglets that measured citrulline balance across the kidney. Only 74% of citrulline entering the kidney appeared as arginine (Marini et al, 2012). We suggest that some of the citrulline entering the kidney could likely be released as GAA, so this was also investigated.

In this study, the primary objective was to measure the net release or uptake of arginine, citrulline, GAA and creatine across the kidney, pancreas and gut. An in-situ piglet model with multiple catheters and blood flow probes was used to describe the net contributions of each of these organs to GAA and creatine synthesis in sow-reared piglets.

5.3 METHODS

Twenty-five Yucatan miniature piglets (17 – 21 d old, supplied by Animal Care Services, Memorial University of Newfoundland) were transported to the Animal Care Facilities on campus and randomized into one of the four different infusion protocols. Within 2.5 h of removal from the sow, piglets were sedated as described previously (Dinesh et al, 2014). Atropine was also administered prior to endotracheal intubation, and general anesthesia was initiated and maintained with isoflurane (0.8 - 1.5%) delivered with oxygen (1.5 $L.min^{-1}$). Body temperature was regulated with the use of a heating pad. Heart rate and blood

oxygenation were monitored every 15 min throughout the experiment. A jugular vein catheter was advanced to superior vena cava to facilitate infusion of one of four experimental intravenous solutions (described below). The infusions were initiated using a priming bolus followed by a constant infusion that continued for 45 - 60 min. All piglets were fasted for at least 3 h prior to starting the test infusions. Another group of piglets that was not fasted prior to the procedure was designated as sow-fed (SF) (n = 4); these piglets underwent the same experimental protocol but did not receive any intravenous infusions. This group was used as reference data to characterize the normal feeding situation. The entire protocol (described below) was completed within 30 min of the SF piglets' arrival, to minimize effect of fasting. The piglets were weight-matched across the infusion treatments.

Laparotomy and isolation of blood vessels: The carotid artery was isolated by blunt dissection to allow sampling of arterial blood at the termination of the experiment. A laparotomy was then performed to expose the visceral organs and right kidney. To measure renal metabolism, the renal vein was isolated and an ultrasonic perivascular blood flow probe (2 mm) (Transonic Systems Inc., Dutch Mill Road, USA) was secured around the renal vein, or the renal artery if the vein was branched. The splenic vein, which is surrounded by the ring structure of the pancreas, was isolated by blunt dissection, taking care to minimize damage to the pancreatic tissues. (Ferrer et al, 2008). Sixty percent of blood flow from the pancreas drains into the splenic vein, and 40% drains into the superior mesenteric vein (SMV), both of which drain into the portal vein. The stomach and the

spleen also drain into the splenic vein. There are no reports of AGAT activity in the spleen or in the stomach; therefore, we assumed that changes in related metabolites analyzed in the blood sampled from the splenic and portal veins should represent metabolism in pancreas and gut, respectively. A blood flow probe (2 mm) was placed on the splenic vein and a 4 mm flow probe was placed on the portal vein to measure the blood flow in those vessels. The exposed visceral organs were kept moistened with warmed saline and covered with wet gauze and plastic wrap during the entire procedure to prevent dehydration.

Experimental intravenous nutrient solutions: The precursors and products of creatine biosynthesis were measured during acute intravenous infusions of each of the following: 1) arginine and methionine (Arg/Met), 2) creatine added to the same concentration of arginine and methionine (+Cre), 3) citrulline and methionine (Cit/Met), or 4) alanine (Ala). The experimental IV solutions were prepared using sterile saline. The Arg/Met treatment included arginine delivered at a rate of 4.8 µmol (0.83 mg) arginine.kg^{-1}.min^{-1}, which was based on a parenteral arginine intake that was shown to maximize muscle protein synthesis in neonatal piglets (Brunton et al, 2003), and methionine delivered at a rate of 1.4 µmol (0.21 mg).kg^{-1}.min^{-1}, which was chosen to maintain plasma methionine concentrations similar to that of sow-fed piglets (Dinesh et al, 2018). Creatine was infused at a rate of 0.6 µmol (0.08 mg).kg^{-1}.min^{-1} which is the accretion rate of neonatal piglets (Brosnan et al, 2009, Dinesh et al, 2018). The Cit/Met treatment included citrulline at concentrations equimolar to the arginine infusion and methionine at the above infusion rate. Finally, alanine was used as the control condition, and was infused at a concentration that was

equimolar to the arginine plus methionine infusions (6.2 μmol [0.6 mg] alanine.kg^{-1}.min^{-1}). In a rat model, Edison et al (2007) reported that an acute intravenous infusion of alanine resulted in no response in GAA concentration, and was similar to a saline control, demonstrating alanine is an appropriate amino acid control.

All experimental treatments were prepared by dissolving amino acids in sterile saline at 55^{o}C under nitrogen gas. Once dissolved, the solutions were sterile filtered via 0.22 μm filters (PALL Life Science, USA) and stored in a cooler until used. Amino acids were purchased from Ajinomoto Inc., Canada or Sigma-Aldrich, Canada; the creatine monohydrate was purchased from Evonik Industries, Germany.

Experimental protocol: The experimental solutions were infused for 1.5 hours. A bolus priming dose of the experimental treatment was given that was equal to 1.5 x the hourly rate. Following the prime, the experimental IV solutions were infused at a rate of 10 mL.kg^{-1}.h^{-1} via a syringe pump.

Blood flow measurement and blood sampling: Measurement of the blood flow from the renal vein (or renal artery), splenic vein and portal vein was initiated approximately 45 min after the initiation of the IV treatments. The mean flow was recorded during a minimum of two minutes, when the variability in flow measurements was less than 5%. At the end of the infusion, blood was sampled from the carotid artery (representing arterial in-flow to the

kidney, pancreas and gut), and from the renal, splenic and portal veins at the end of the infusion, using a Surflo winged infusion set (23G x 3/4 or 21G x 3/4) (Solmed Pty Limited, Australia) or 24-gauge IV catheter (BD Angiocath, USA). At the end of the experiment, the piglets were killed while under anesthesia by an intra-cardiac injection of sodium pentobarbital (Euthanyl, Biomeda-MTC, Animal Health Ins, ON, Canada).

GAA and creatine analyses: Plasma creatine and GAA concentrations were assayed using an HPLC method modified from Buchberger and Ferdig (2004) using a C18 reverse phase column (Hypersil ODS 5 U 150x4.6 mm column) with ninhydrin derivatization and fluorescence detection (Ex/EM 390/470).

Amino acids: Plasma amino acid concentrations were measured by reverse-phase HPLC (C18 column) following derivatization with phenylisothiocyanate (PITC) (Waters, Woburn, Massachusetts, USA) as per the method of Bidlingmeyer et al (1984).

Arterio-venous balance: Net arterio-venous balances across organs were determined by multiplying the concentration difference between venous and arterial blood by the blood flow and correcting for body weight. The net balances of amino acids, GAA and creatine reported for the kidney have been doubled to represent the contribution of two kidneys. A positive net balance indicates net output while negative net balance indicates net uptake of the nutrient by that organ when mean values were different from zero.

Statistical analysis: Data were analyzed using a two-way ANOVA and treatments within organs and among organs were compared by Bonferroni's multiple comparisons test (GraphPad 7.03, Graph Pad Software Inc. CA, USA) if the interactions were present. The variables analyzed by two-way ANOVA were organs, experimental groups and their interaction. For the data that did not have significant interactions, a one-way ANOVA was performed, and groups were compared by Bonferroni's multiple comparisons test (GraphPad 7.03). All data were expressed as mean ± SD and were considered significantly different if $P < 0.05$. When the group variances were significantly different, ANOVA testing was conducted using log-transformed values, although the values are presented as means ± SD of the non-transformed data. In addition, means were tested to verify whether they were statistically significantly different from zero by analyzing the one-sample t-test (GraphPad 7.03).

5.4 RESULTS

Throughout the in-situ studies under anesthesia, all piglets remained stable, well oxygenated and maintained a body temperature of 37.5 ± 1.5˚C. The body weight on the day of procedure did not differ among treatment groups (**APPENDIX A1**). No significant differences in blood flow were determined among the treatment groups for renal artery/vein, splenic vein or portal vein (**APPENDIX A2**). The blood flow rates measured in SF piglets were also not different than those in the IV treated piglets (**APPENDIX A2**).

GAA balance Independent of the amino acid compositions of the treatments, all piglets had a net release of GAA from the kidneys. Moreover, all treatment groups except Cit/Met

released GAA from the pancreas **(FIGURE 5.1**). The kidneys released three to four times more GAA than the pancreas. GAA balance across the gut was not different than zero in piglets treated with Arg/Met, Cit/Met or Ala; only piglets treated with creatine (+Cre) demonstrated a net release of GAA from the gut. GAA balance across the gut was also not different than zero in SF piglets **(FIGURE 5.1)**.

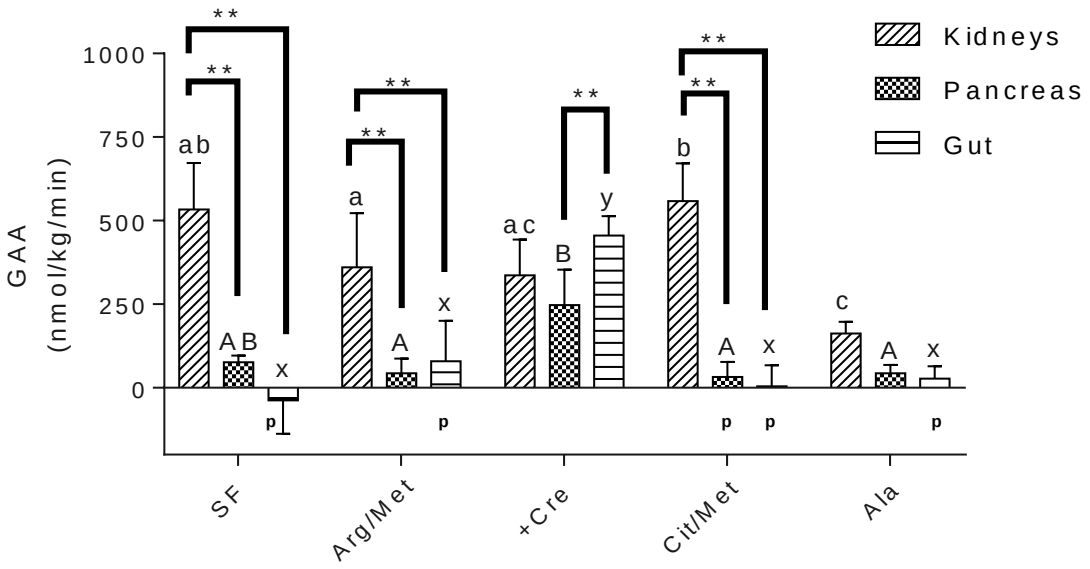

FIGURE 5.1 Net fluxes of GAA across kidneys, pancreatic, and intestinal tissues of sow-fed (SF) non-fasted piglets or fasted piglets that received an infusion of either arginine, citrulline or alanine. Lines represent significant differences between organs within an experimental group (*P < 0.05 or **P < 0.0001). Differing letters represent significant differences within each organ among experimental groups (P < 0.0001). n = 6 per infusion group and n = 4 per SF group. Values are means ± SD of non-transformed data; statistics were determined using log values when variances were different. Arginine and methionine: Arg/Met, creatine in Arg/Met: +Cre, citrulline and methionine: Cit/Met and alanine: Ala. P: Not different from zero.

There were differences among treatments for the GAA released from the kidneys, pancreas and gut, with a significant interaction between organs and treatments. In the kidneys, piglets infused with Cit/Met had the highest net GAA release, while piglets infused with Ala had the lowest; piglets treated with Arg/Met and +Cre were at intermediate levels; the GAA released from the kidneys in SF piglets was as high as the GAA released by the piglets infused with Cit/Met but did not differ from the piglets infused with Arg/Met, +Cre or Cit/Met. **(FIGURE 5.1**). In contrast to the kidneys, the release of GAA from the pancreas and gut demonstrated similar patterns and were only affected by the +Cre treatment, in that +Cre piglets had higher GAA release than Arg/Met, Cit/Met and Ala piglets but was similar to SF **(FIGURE 5.1**).

When comparing the net GAA balances among the three organs, the kidneys released the highest amount of GAA compared to gut or pancreas in the Arg/Met, Cit/Met and SF piglets. Interestingly, with the +Cre treatment, the gut released significantly more GAA than the pancreas with kidneys intermediate. GAA release was not different among the three organs for the Ala treatment **(FIGURE 5.1**).

Creatine balance The creatine balance across the kidney was not different from zero for any of the treatments (Arg/Met, +Cre, Cit/Met and Ala), unlike the SF piglets which demonstrated a significant net release of creatine. Creatine balance across the pancreas was similar to zero for all of the treatments that we investigated; however, the net balance of

creatine across renal and pancreatic tissues was highly variable. For Arg/Met, +Cre and Ala treatments, there was a net creatine release from the gut. For the SF piglets and Cit/Met treated piglets, the net release of creatine from gut was highly variable and resulted in a mean value not different than zero (**FIGURE 5.2**). Notably, when comparing creatine balance among organs, the creatine release was highest across the gut compared to renal and pancreatic tissues with the Arg/Met and +Cre treatments. However, in Cit/Met and Ala piglets, the creatine release from the intestinal, pancreatic and renal tissues did not differ among organs **(FIGURE 5.2**).

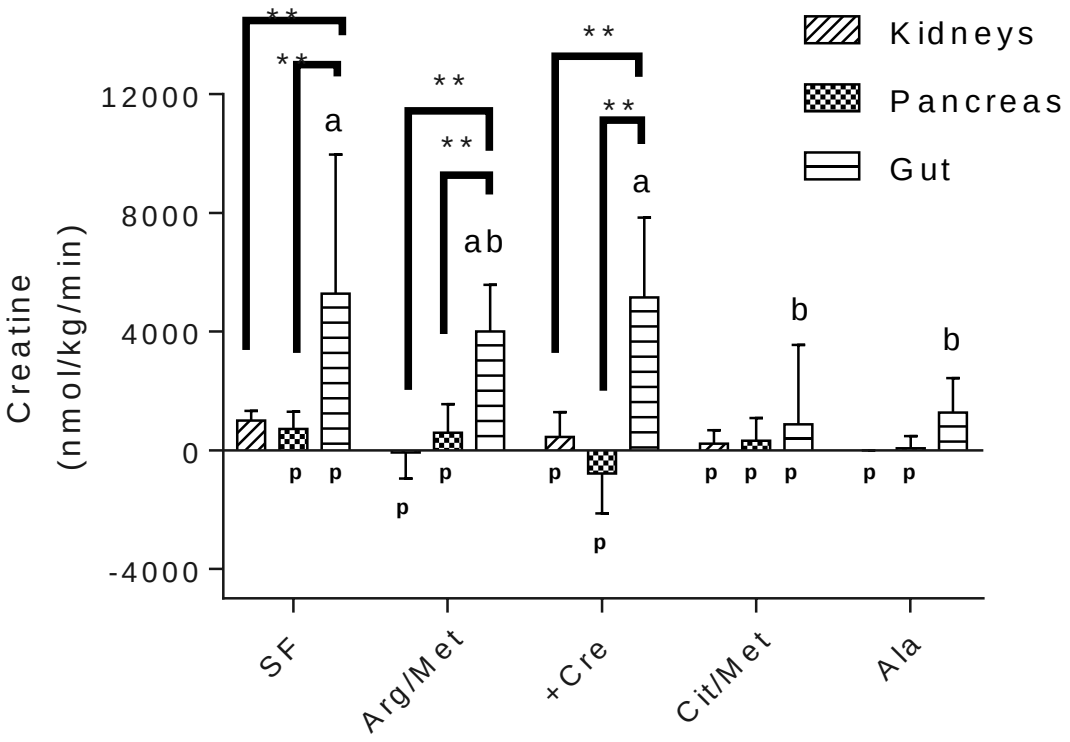

FIGURE 5.2 Net fluxes of creatine across kidneys, pancreatic, and intestinal tissues of sow-fed (SF) non-fasted piglets or fasted piglets that received an infusion of either arginine, citrulline or alanine. Data were analyzed via two-way ANOVA with Bonferroni's multiple comparisons test for post hoc analysis. Lines represent significant differences between organs within an experimental group (**P < 0.0001). Differing letters represent significant differences within each organ among experimental groups ($P < 0.0001$). n = 6 per infusion group and n = 4 per SF group. Values are mean + SD. Values are means ± SD of non-transformed data; statistics were determined using log values when variances were different. Arginine and methionine: Arg/Met, creatine in Arg/Met: +Cre, citrulline and methionine: Cit/Met and alanine: Ala. p: Not different from zero.

Plasma GAA and creatine concentrations There was a significant interaction between treatment and vessel for the plasma GAA data **(TABLE 5.1)**. For arterial GAA concentration, there was no difference among groups. The Cit/Met and SF groups had the highest renal vein GAA concentrations, compared to Ala treatment, with Arg/Met and +Cre intermediate. The creatine treatment (+Cre) resulted in higher GAA concentrations in the splenic and portal veins compared to the Ala treatment, while Arg/Met, Cit/Met or SF piglets were not different from any other treatment group in splenic or portal vein GAA concentrations **(TABLE 5.1).** The renal vein GAA concentration was significantly higher than the GAA concentrations in all other blood vessels for all groups except that treated with alanine; in Ala group, only the carotid artery GAA was lower than the renal vein concentration. In piglets treated with creatine, the lowest GAA concentration was in the plasma sampled from the carotid artery ($P < 0.05$). No other differences were apparent among the vessels.

For the plasma creatine concentrations, no significant interactions occurred between vessel and treatment (**TABLE 5.1)**. In carotid artery and in portal vein, the +Cre treatment resulted in significantly higher creatine concentrations compared to Cit/Met and Ala treatments, with SF and +Arg/Met piglets intermediate. In the renal vein, the +Cre and SF treatments resulted in the highest creatine concentrations that were different from all other treatments, with the Ala demonstrating the lowest creatine concentration (**TABLE 5.1**). No differences in creatine concentrations among groups (including SF) were observed in the plasma sampled from splenic vein.

TABLE 5.1 Plasma GAA and creatine concentrations measured at the termination of the experiments in the carotid artery, renal vein, splenic vein and portal vein of sow-fed (SF) non-fasted piglets or fasted piglets that received an infusion of either arginine, citrulline or alanine.

Metabolite	Blood vessel	SF	Arg/Met	+Cre	Cit/Met	Ala
			($\mu mol.L^{-1}$)			
GAA	Carotid Artery	6 ± 2	8 ± 2	5 ± 1*	10 ± 3	2 ± 0.8]
	Renal Vein	56 ± 13$^{a!}$	36 ± 11$^{b!}$	40 ± 14$^{b!}$	60 ± 13$^{a!}$	16 ± 3^{c}
	Splenic Vein	12 ± 3^{ab}	11 ± 2^{ab}	22 ± 10^{a}	12 ± 4.5^{ab}	6 ± 2^{b}
	Portal Vein	7 ± 0.7^{ab}	10 ± 3^{ab}	20 ± 4^{a}	13 ± 4^{ab}	3 ± 0.7^{b}
Creatine	Carotid Artery	179 ± 101^{ab}	170 ± 70^{ab}	265 ± 34^{a}	125 ± 48^{b}	127 ± 16^{b}
	Renal Vein	270 ± 126^{a}	162 ± 62^{b}	306 ± 81^{a}	146 ± 44^{b}	92 ± 25^{c}
	Splenic Vein	235 ± 97	205 ± 66	212 ± 71	147 ± 59	131 ± 25
	Portal Vein	296 ± 162^{ab}	270 ± 74^{a}	362 ± 65^{a}	149 ± 37^{b}	158 ± 34^{b}

The concentrations of GAA and creatine for the portal vein represent GAA and creatine from the gut and pancreas. Differing superscript letters within a row represent significant differences among groups within each vessel ($P < 0.05$). * or ! indicates significantly different from the rest of the blood vessels while] denotes significant difference between two vessels ($P < 0.0001$). Values are means ± SD of non-transformed data; statistics were determined using log values when variances were different. Sow-fed (SF), arginine and methionine (+Arg/Met), creatine in +Arg/Met infusion (+Cre), citrulline and methionine (+Cit/Met), alanine (Ala). Values are mean ± SD, n = 6 -7 for each bar for infusion piglets, n = 4 for SF.

Amino acid balance A significant interaction between the vessels and treatment was observed only in the citrulline data. Independent of the treatment, all groups had a net uptake of citrulline by the kidneys, and most of the groups demonstrated a net release of citrulline from the gut. One exception was that the net release of citrulline from the gut was highly variable for the Cit/Met piglets (**TABLE 5.2**); as such, the citrulline balance did not differ from zero. Citrulline balance across the pancreas was also not different than zero for most treatments, except for +Cre piglets which demonstrated a net release of citrulline from the pancreas. Across organs**,** a significantly higher net uptake of citrulline by the kidney occurred only in piglets treated with Cit/Met compared to gut and pancreas **(TABLE 5.2**). The Cit/Met infusion resulted in a net release of arginine from the kidneys. Arginine balance across the kidney, pancreas and gut did not differ from zero for any other treatment. The Arg/Met infusion resulted in a net release of methionine from the gut. Methionine balances across organs were highly variable and did not differ from zero for any other infusions. Alanine balances across organs were also highly variable and did not differ from zero in any groups.

TABLE 5.2 Net fluxes of arginine, methionine and citrulline across kidneys, pancreas and gut of sow-fed (SF) non-fasted piglets or fasted piglets that received an infusion of either arginine, citrulline or alanine.

Amino acid	Organ	SF	Arg/Met	+Cre	Cit/Met	Ala
			($nmol.kg^{-1}min^{-1}$)			
Arginine	Kidneys	580 ± 490	-1762 ± 2372	111 ± 211	3184 ± 1608$^{!}$	361 ± 357
	Pancreas	599 ± 1448	-2079 ± 3730	691 ± 1784	849 ± 394	118 ± 443
	Gut	685 ± 1829	-731 ± 4502	-1300 ± 7060	1211 ± 1571	1094 ± 1978
Methionine	Kidneys	48 ± 27	36 ± 65	27 ± 64	11 ± 84	-21 ± 70
	Pancreas	82 ± 122	2 ± 53	41 ± 150	46 ± 120	18 ± 61
	Gut	-11 ± 71	212 ± 224$^{!}$	98 ± 200	-17 ± 129	109 ± 172
Citrulline	Kidneys	-1005 ± 179$^{!}$	-546 ± 287$^{!}$	-583 ± 131$^{!}$	-3711 ± 2611*$^{!}$	-543 ± 202$^{!}$
	Pancreas	227 ± 314	82 ± 265	189 ± 164$^{!}$	1899 ± 2119	-5 ± 184
	Gut	1273 ± 691$^{!}$	817 ± 587$^{!}$	1496 ± 1306$^{!}$	3116 ± 5381	1999 ± 933$^{!}$

Data were analyzed via two-way ANOVA with Bonferroni's multiple comparisons test for post hoc analysis. *significantly different from the citrulline balances of pancreas and gut ($P < 0.01$). n = 6 per infusion group and n = 4 per SF group. Values are means ± SD of non-transformed data; statistics were determined using log values when variances were different. Arginine and methionine: Arg/Met, creatine in Arg/Met: +Cre, citrulline and methionine: Cit/Met and alanine: Ala. "!" Different from zero.

Selected plasma amino acid concentrations As expected, the arginine treatments (as Arg/Met and +Cre) resulted in significantly higher arginine in blood sampled from carotid artery, compared to all other treatments (**TABLE 5.3**). Again, arginine treatments had significantly higher arginine in blood samples from the renal vein, compared to Ala and SF piglets. The Cit/Met piglets appeared to convert citrulline into arginine, as indicated by significantly higher arginine in the renal vein, compared to the Ala and SF piglets. The renal vein arginine in Cit/Met piglets was not different than the Arg/Met and +Cre piglets. Within the Cit/Met treatment group, citrulline was lower in the renal vein compared to all other blood vessels (**TABLE 5.3**).

TABLE 5.3 Selected plasma amino acid concentrations in the carotid artery, renal vein, splenic vein and portal vein of sow-fed (SF) non-fasted piglets or fasted piglets that received an infusion of either arginine, citrulline or alanine.

Amino acid	Vessel	SF	Arg/Met	+Cre	Cit/Met	Ala
			($\mu mol.L^{-1}$)			
Arginine	CA	133 ± 24^{a}	760 ± 239^{b}	652 ± 121^{b}	293 ± 90^{a}	230 ± 180^{a}
	RV	187 ± 37^{a}	620 ± 207^{b}	664 ± 136^{b}	571 ± 154^{b}	242 ± 130^{a}
	SV	178 ± 92^{a}	638 ± 270^{bc}	705 ± 237^{b}	348 ± 50^{ac}	244 ± 199^{a}
	PV	164 ± 44^{a}	715 ± 211^{b}	640 ± 232^{bc}	348 ± 68^{ac}	271 ± 199^{a}
Methionine	CA	25 ± 8	23 ± 6	31 ± 5	24 ± 6	23 ± 5
	RV	30 ± 7^{ab}	26 ± 6^{ab}	34 ± 6^{a}	25 ± 6^{ab}	21 ± 6^{b}
	SV	32 ± 5	24 ± 8	34 ± 15	27 ± 7	24 ± 6
	PV	27 ± 6	30 ±10	34 ± 7	25 ± 6	26 ± 6
Citrulline	CA	142 ± 16^{a}	96 ±18^{a}	100 ± 21^{a}	821 ± 128^{b}	83 ± 20^{a}
	RV	44 ± 8^{a}	53 ± 23^{a}	40 ±18^{a}	557 ± 103^{b*}	36 ± 7^{a}
	SV	160 ± 36^{a}	102 ± 24^{a}	114 ± 29^{a}	949 ± 119^{b}	83 ± 10^{a}
	PV	181 ± 33^{a}	124 ± 31^{a}	135 ± 36^{a}	961 ± 197^{b}	129 ± 29^{a}
Alanine	CA	501 ± 78^{a}	816 ± 233^{a}	859 ± 208^{a}	757 ± 135^{a}	1405 ± 312^{b}
	RV	637 ± 73^{a}	953 ± 288^{ab}	919 ± 222^{a}	669 ± 93^{a}	1358 ± 358^{b}
	SV	672 ± 134^{a}	854 ± 283^{a}	906 ± 247^{a}	801 ± 81^{a}	1424 ± 412^{b}
	PV	648 ± 115^{a}	906 ± 274^{a}	912 ± 231^{a}	842 ± 134^{a}	1456 ± 437^{b}
Ornithine	CA	49 ± 13^{a}	154 ± 43^{b}	164 ± 41^{b}	97 ± 24^{a}	43 ± 9^{a}
	RV	88 ± 21^{a}	181 ± 47^{b}	228 ± 68^{b}	186 ± 28^{b}	52 ± 18^{a}
	SV	48 ± 10^{a}	133 ± 51^{b}	170 ± 61^{b}	105 ± 15^{ab}	40 ± 14^{a}
	PV	51 ± 20^{a}	168 ±51^{b}	164 ± 47^{b}	107 ± 15^{ab}	49 ± 20^{a}
Glycine	CA	1053 ± 292	1015 ± 198	849 ± 97	856 ± 123	893 ± 164
	RV	1221 ± 180	966 ± 203	950 ± 54	960 ± 149	958 ± 227
	SV	1108 ± 188	896 ± 267	880 ± 148	990 ± 127	945 ± 237
	PV	1125 ± 271	975 ± 166	871 ± 146	966 ± 119	981 ± 172
Proline	CA	501 ± 57	407 ± 136	364 ± 91	398 ± 118	358 ± 60
	RV	544 ± 26^{a}	349 ± 106^{b}	370 ± 64^{b}	416 ± 108^{ab}	357 ± 56^{b}
	SV	555 ± 80^{a}	313 ± 96^{b}	367 ± 159^{ab}	442 ± 102^{ab}	370 ± 53^{ab}
	PV	570 ± 43^{a}	375 ± 92^{b}	360 ± 95^{b}	433 ± 119^{ab}	409 ± 50^{ab}

The concentrations of amino acids for the portal vein represents output from the gut and the pancreas. Differing superscript letters within a row represent significant differences

among groups within a vessel ($P < 0.05$). ***** indicates significantly different from the rest of the blood vessels ($P < 0.0001$). Values are means ± SD of non-transformed data; statistics were determined using log values when variances were different. Sow-fed (SF), arginine and methionine (+Arg/Met), creatine in +Arg/Met infusion (+Cre), citrulline and methionine (+Cit/Met), alanine (Ala). Values are mean ± SD, n = 6 - 7 for each bar for infusion piglets, n = 4 for SF. CA; carotid artery, RV; renal vein, SV; splenic vein and PV; portal vein.

5.5 DISCUSSION

Source of GAA for hepatic creatine synthesis One of the outstanding questions from previous studies of GAA and creatine synthesis was whether the high AGAT activity measured *in-vitro* in pancreas tissue translated into a significant source of GAA for hepatic creatine biosynthesis (Dinesh et al, 2018). It is clear from this study, that this is not the case. The GAA released from the renal tissues accounted for the majority (88%) of the total GAA released from the three organs, when measured in suckling piglets. The relative contributions of the kidneys and the pancreas to the GAA supply were also assessed when the precursor availability was manipulated under experimental conditions. The GAA released from the renal tissues accounted for 79 – 94% of the total GAA released from the three organs for most of the experimental conditions. An exception was GAA released by the renal tissues during the infusion of creatine, which was only 32% of the total GAA released from the three organs because other tissues contributed more GAA to the circulation. The kidneys consistently released the most GAA, even in the control (alanine) condition when no exogenous precursor was supplied. The absolute amount of GAA released from the pancreas was more than 75% lower than that from the kidneys but was patterned similarly under most experimental conditions; the one exception was during the infusion of citrulline (Cit/Met), which did not facilitate a net positive GAA balance. The gut also did not demonstrate a net release of GAA under most experimental conditions, including in the sow milk fed state. Thus, it appears that in the neonate, the kidneys are the major source of GAA for hepatic creatine synthesis.

In the Chapter Three (Dinesh et al, 2018), we demonstrated that a dietary supply of creatine led to a reduction in *in-vitro* renal AGAT activity compared to control neonatal piglets. Therefore, we predicted that infusing creatine would result in less GAA released from the kidneys. However, this was not the case. The kidneys released similar amounts of GAA with both the arginine and creatine infusions. The very short timeframe of this study may explain this result, in that it likely requires a longer period of exposure to creatine to lower the enzyme activity.

The kidney, but not the pancreas or gut, has the capacity to convert citrulline into arginine and GAA In a study of arginine metabolism in neonatal piglets, only 74% of citrulline entering the kidney appeared as arginine (Marini et al, 2012). We speculated that some of the citrulline entering the kidney was ultimately released as GAA. In this study, when citrulline was infused, its disappearance across the kidney equaled ~4000 nmol.kg^{-1}.min^{-1} while the arginine appearance was ~3000 nmol.kg^{-1}.min^{-1} and the GAA released was ~500 nmol.kg^{-1}.min^{-1}. Thus, similar to previous tracer studies (Marini et al, 2012), it appears that ~75% of the citrulline entering the kidneys was utilized to supply arginine to the periphery and ~12.5% was converted to arginine and then subsequently GAA; the fate of the remaining citrulline entering the kidneys is uncertain, although some of the arginine synthesized from citrulline in the kidneys may have been utilized for its own protein synthesis. In SF piglets, citrulline disappearance was 1000 nmol.kg^{-1}.min^{-1} while the arginine appearance was 600 nmol.kg^{-1}.min^{-1} and the GAA release was 500 nmol.kg^{-1}.min^{-}

[1]. Therefore, SF piglets appeared to utilize all of the citrulline that disappeared across kidneys to synthesize arginine and GAA. An interesting finding is that the GAA released from the kidney with the citrulline infusion was approximately 1.5-fold greater than the GAA released during an equimolar infusion of arginine. According to recent findings, neonatal piglets express argininosuccinate synthase (ASS) and argininosuccinate lyase (ASL) in the kidneys to convert citrulline into arginine (Marini et al, 2017). We speculate that ASL, ASS and AGAT may be expressed in a manner in the kidney to preferentially channel citrulline to GAA (via arginine).

It is not known whether the pancreas expresses enzymes that convert citrulline into arginine and then GAA, but our results suggest that this does not occur. Piglets infused with citrulline had no net citrulline, arginine or GAA fluxes across the pancreas, suggesting that the pancreas does not have the capacity to use citrulline. Similarly, the gut also does not appear to have the capacity to convert citrulline into GAA. The gut had zero GAA and creatine fluxes when provided with a systemic source of citrulline. Furthermore, the gut had no net release of arginine with the citrulline infusion, suggesting that gut cannot utilize systemically available citrulline to make arginine. Piglets infused with citrulline also had no net release of creatine from pancreatic or gut tissues, which suggests that neither the gut nor the pancreas is capable of utilizing citrulline as a precursor of GAA or creatine.

GAA synthesis in the gut A particularly novel finding of this study was quantifying a net release of GAA from the gut. That the gut has the capacity to synthesize GAA was a surprising outcome, because a previous study of *in-vitro* enzyme activity in small intestinal mucosal tissue from piglets reported that AGAT activity was negligible (Brosnan et al, 2009). Further to this, it was also interesting that a significant release of GAA only occurred when creatine was infused, suggesting that perhaps the gut releases GAA when there is no need to synthesize creatine. That the gut released the GAA when creatine was plentiful suggests that the GAA synthesized by the gut may be utilized for creatine biosynthesis within the gut when creatine is limited in supply.

Creatine synthesis in the gut In sow-reared piglets, small intestinal mucosa has a very low GAMT specific activity, reported as half of that present in the kidneys (Brosnan et al, 2009). Therefore, we expected no net release of creatine across the gut in our piglets. However, piglets infused with arginine had a significant net release of creatine demonstrating the capacity of the gut to synthesize GAA and then creatine, when adequate arginine and methionine are available. It does not appear that the gut could utilize systemically available GAA as the precursor for the creatine that was released, based on a zero net GAA flux across the gut for most of the treatments. The ability of gut to take up creatine from the systemic circulation is not certain. Feeding creatine increased plasma and tissue creatine concentrations in neonatal piglets (Chapter Four), suggesting that small intestine absorbs creatine from the small intestinal lumen and releases that creatine into the portal blood. However, it is not certain whether systemic creatine crosses the basolateral

membrane of the small intestine. The net release of GAA from the gut when creatine was infused suggests that the gut was responding to systemic creatine. However, there was no measurable uptake of creatine by the gut when piglets were infused with creatine; rather there was a net release. Even in the circumstance where the gut was not exposed to GAA precursors, it did not appear to take up creatine from the systemic circulation. Based on the results from this experiment, it is not possible to determine whether the neonatal gut has the ability to take creatine from systemic circulation. Further investigations using isotopic tracers would help to resolve this question.

Pancreatic GAA and creatine synthesis In Chapter Four, we showed that a dietary supply of creatine lowered the *in-vitro* AGAT activity in pancreas. Therefore, a lower GAA release from the pancreas was expected with the creatine infusion. However, that was not the case. A four-fold higher GAA release was induced when exogenous creatine was supplied, suggesting that perhaps the pancreas releases GAA when there is no need to synthesize creatine, similar to the small intestine. The creatine treatment induced a higher net release of GAA from the pancreas than most other treatments, which leads us to speculate that under low creatine conditions, the GAA synthesized in the pancreas may be utilized within the organ to synthesize creatine. Support for this concept is the high GAMT activity in pancreas of piglets, which was reported to be three times higher than in the kidney (Brosnan et al, 2009). Despite this capacity for creatine synthesis, it did not lead to a net release of creatine from the pancreas in any of our treatment groups. A creatine and phosphocreatine shuttle have been reported in the pancreas (Wyss et al, 2000). Therefore, the pancreas may

synthesize and use creatine to support its own secretory functions, ultimately releasing it as creatinine. When comparing the pancreatic capacity to synthesize GAA and creatine, pancreatic GAMT activity per gram of tissue was only 4 percent of the pancreatic AGAT activity in sow reared neonatal piglets (Brosnan et al, 2009). It follows that the net release of creatine must be very low compared to the net release of GAA. Therefore, such low creatine release may not be detectable in this model. Alternatively, the high systemic creatine could have downregulated GAMT in gut and pancreas leading to a higher net release of unconverted GAA from these tissues.

In summary, the results from this study demonstrated that the kidney has the greatest capacity to supply GAA compared to the pancreas and the gut. We also demonstrated that citrulline is a better precursor than arginine for GAA synthesis in the kidneys, but not in the pancreas or gut. We also determined that there is no significant release of GAA or creatine from the pancreas. In conclusion, the neonatal piglet can convert citrulline into arginine and then some of that arginine into GAA in the kidneys. The noteworthy novel finding was that the neonatal gut has the capacity to release GAA and creatine when arginine and methionine are available. Interestingly, an intravenous creatine infusion altered the small intestinal GAA and creatine metabolism in neonatal piglets. Because of the complex regulation of inter-organ synthesis of creatine, the dietary levels of various precursor amino acids need to be considered when designing neonatal diets, especially if organ health is compromised.

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5.7 ACKNOWLEDGEMENTS

J.A.B. and R.F.B. own the research idea. O.C.D. was involved in designing the research; O.C.D., J.A.B. and R.F.B. conducted the research; O.C.D. analyzed the data; O.C.D. drafted the paper. J.A.B. and R.F.B. reviewed the paper. O.C.D, J.A.B. and R.F.B had primary responsibility for final content. All authors read and approved the final manuscript.

CHAPTER SIX

6.0 GENERAL DISCUSSION

The overall goal of my PhD program was to understand and quantify the demand for arginine to support creatine biosynthesis and protein synthesis in neonates. The research presented in this thesis focused on the availability of arginine for creatine and protein synthesis during development under two contrasting conditions: when premade creatine is available and when premade creatine is not available.

The three research experiments in this thesis focused on clinical problems and metabolic questions. The clinical question addressed in the first study was whether PN without creatine affects the plasma and tissue creatine levels of neonates. We approached this question using the neonatal piglet as a relevant translational model. The second and the third studies addressed metabolic questions. The first metabolic question was: how does the availability of arginine impact the whole-body arginine partitioning into GAA/creatine and proteins in piglets fed diets with differing amounts of precursors of creatine? The second metabolic question addressed in my third study was: in a neonatal piglet model, how are different organs involved in GAA/creatine synthesis? Furthermore, the third study was designed to investigate whether these inter-organ pathways were acutely regulated by different precursor amino acids (arginine vs citrulline) or by the presence of creatine.

6.1 Creatine bio-synthesis in PN fed neonates

Suckling neonatal piglets get only 23% of their creatine requirement from milk, but they can endogenously synthesize the remainder of their creatine requirement. Following preterm birth, PN is often required as a means of nutritional support for infants with gastrointestinal disorders or prolonged intolerance of enteral feeding. However, creatine is not a component of pediatric PN products. Therefore, the entire creatine requirement needs to be synthesized endogenously. Since arginine is one of the precursor amino acids required for creatine synthesis, synthesis of arginine and then creatine may be compromised during PN feeding. Whether this affects the optimal creatine accretion in neonates was unknown. Chapter Three of my thesis was designed to address whether creatine biosynthesis is affected by the reduced de novo arginine synthesis as a consequence of gut atrophy during PN feeding in neonates. The data from this study determined that the addition of creatine into PN increased the creatine concentrations in the plasma and most of the tissue analyzed. The enzyme activities were lower in piglets fed creatine compared with piglets fed no creatine in PN. That led us to ask: is creatine synthesis limited by substrate availability or alternatively by the maximal capacity of the enzymes involve in creatine biosynthesis? *In-vitro* enzyme activities and tissue GAA and creatine concentrations may not necessarily mimic the in-vivo regulation of creatine biosynthesis. In Chapter Four, my second study, this question was addressed by measuring the whole-body precursor to product conversion rates via isotope kinetics.

In Chapter Three of this thesis, creatine supplementation increased the hepatic and kidney fractional protein synthesis rate compared to that in piglets fed a creatine-free diet. The amino acid concentrations in the plasma, liver and kidney did not differ between piglets fed creatine and control diets. However, the lack of differences in arginine concentrations in the plasma and tissues may be explained by the diversion of spared arginine towards protein synthesis. Despite no differences in the concentrations of the plasma amino acids, the greater tissue protein synthesis suggests that creatine supplementation spared arginine for protein synthesis. The changes in the concentrations of the plasma and tissue amino acids would not be detectable if the differences in the concentrations are small and amino acid fluxes are high. When the fluxes are high, amino acids enter and exit the body pool rapidly and a slight change in concentrations of free amino acids would not be detectable without isotope tracers.

According to the literature (Wilkinson et al, 2004, Beaumier, 1998), arginine turnover in neonates is quite high, such that plasma and tissue free amino acid concentrations may not show minor changes during a short study period. Therefore, the measurement of free amino acid concentrations in tissues and/or plasma cannot be sensitive enough to demonstrate changes in fluxes through the metabolic pathways. Consequently, an isotope kinetics investigation was warranted to quantify such changes *in-vivo*.

6.2 Arginine partitioning into GAA/creatine and whole-body proteins

The research presented in Chapter Four was designed to address some of the uncertainties arising from the work presented in Chapter Three. Specifically, we wanted to determine whether the availability of arginine, GAA, GAA with excess methionine, or creatine affects creatine and/or protein synthesis in neonates. We also wanted to determine whether it is creatine bio-synthesis or protein synthesis that is prioritized under changing conditions of arginine availability in neonatal piglets. We further wanted to quantitatively determine *in-vivo*, how much arginine was partitioned into GAA/creatine versus proteins when differing amounts of precursors of creatine were available.

Dietary creatine has been shown to repress endogenous GAA/creatine biosynthesis in rodent models and in humans (McGuire et al, 1984; Derave et al, 2004; da Silva et al, 2009; 2014, Deminice et al, 2011). *In-vitro* enzyme activity (AGAT) involved in creatine bio-synthesis has been shown to be down regulated with dietary creatine provision. Similar to those data, we observed the reduction in *in-vitro* AGAT enzyme activity in our piglets. However, our *in-vivo* data did not reflect *in-vitro* enzyme activities. Therefore, this raised the question of whether the amount of dietary GAA/creatine that we provided maximized the creatine requirement of our piglets. Furthermore, is there a level of creatine that could minimize the conversion of arginine into GAA/creatine in neonatal piglets? Therefore, future studies should focus on determining what amount of creatine could spare arginine from creatine synthesis, such that arginine can be partitioned into other arginine-requiring metabolic pathways. The arginine that was directed towards creatine was

high when large amounts of arginine and methionine were provided. However, what has not been determined so far is the amount of arginine that would maximize the creatine synthesis. Does this level fall somewhere between 0.6 $g.kg^{-1}.d^{-1}$ to 1.8 $g.kg^{-1}.d^{-1}$? To address this question, future work should focus on whether the amount of arginine that is partitioned towards creatine synthesis peaks at a specific level of dietary arginine and then plateaus, despite increasing the amounts of arginine in the diet.

The arginine requirement for whole-body protein synthesis has been investigated via the phenylalanine oxidation technique (Brunton et al, unpublished data). The data from that study could not be utilized to estimate a break point from dietary arginine intakes that could minimize phenylalanine oxidation which, consequently, would have shown the level of arginine that could maximize whole-body protein synthesis. We used our *in-vivo* data (Chapter Four, phenylalanine and arginine tracer data) to estimate the rate of arginine partitioning into proteins. The rates did not differ between low arginine and high arginine. The minimum amount that could achieve this level of whole-body protein synthesis requires further investigation. Furthermore, we do not know whether this is the maximum level of whole-body protein synthesis in neonatal piglets or if the whole-body protein synthesis could further increase with very high arginine delivered in the diet.

Phenylalanine and tyrosine kinetics allow us to determine whole-body protein synthesis. However, whole-body protein synthesis is not indicative of the changes in the synthesis of individual proteins or in specific tissues. For example, piglets fed low arginine may express more AGAT protein than piglets fed high arginine if the piglets are attempting

to maximize GAA and creatine synthesis. This situation would lead to an increase in kidney AGAT protein synthesis. However, this specific increase in AGAT synthesis would not necessarily correlate with an increase in whole-body protein synthesis. This issue might be able to be resolved if we measured the labelled phenylalanine incorporated into specific proteins.

In our study, for 4 days, we fed diets with different levels of arginine and methionine. We determined on the fourth day that under the condition of low arginine, there was no change in whole-body protein synthesis, however, there was lower creatine biosynthesis. What we could not determine from this study was whether the whole-body protein synthesis was low prior to the fourth day of feeding. Changes in protein synthesis in response to the route of feeding (whether intravenous or intragastric) appeared by 24 hours of the initiation of feeding (Niinikoski et al, 2004). However, there have been no studies monitoring the changes in protein synthesis over a period of time. In terms of metabolism, three days of life in piglets represents approximately one month for human infants. A month is a significant duration for neonates as they are in peak growth phase. Low protein synthesis for a one-month period could cause significant impact on growth and development of neonates. Therefore, it is important to address whether the protein synthesis is not low at any particular time during feeding low concentrations of arginine.

Plasma glutamine concentrations were higher in piglets fed low versus high arginine. This is quite normal in an animal fed a diet with a low concentration of an essential amino acid. In the condition when one essential amino acid is inadequate, the whole-body

protein synthesis will be low. Now, with low whole-body protein synthesis, the other essential amino acids will be in excess. These amino acids, to some extent, may be utilized by other metabolic pathways in the body. Any excess amounts of essential amino acids need to be disposed of via the urea cycle. Arginine is a critical intermediate in the urea cycle. The experimental diet is low in arginine; therefore, impaired urea cycle function might impede excess nitrogen disposal by that route, and lead to the formation of glutamine as a secondary method of regulating ammonia accumulation. Therefore, I expect that the plasma glutamine level would go down with the increasing levels of arginine. I speculate that the glutamine level in the diet will plateau at the point when arginine in the diet maximizes the whole-body protein synthesis. Further investigation will reveal whether there is such a relationship between dietary arginine and plasma glutamine.

We determined that dietary GAA can be utilized to meet the creatine needs in neonatal piglets when arginine is limited in the diet, but only when GAA is delivered with excess dietary methionine. Interestingly, we did not see any increase in plasma or tissue GAA concentrations in piglets fed GAA alone. This raised the question of whether GAA uptake by the gut was affected by the amount of methionine in the diet. There is no information available on the regulation of GAA uptake by gastrointestinal tissues or whether it involves a transporter in neonatal piglet. This needs to be addressed in future studies. An ex-vivo experiment using an Ussing chamber apparatus would facilitate an exploration into the mechanisms of GAA uptake by the gut.

6.3 Inter-organ pathways of GAA and creatine bio-synthesis in neonatal piglets

The experiment presented in Chapter Four investigated the effect of arginine availability on whole-body GAA and creatine bio-synthesis. GAA/creatine synthesis is an inter-organ pathway in adults; therefore, one of my thesis objectives was to investigate whether GAA/creatine synthesis occurs as a similar process in neonates. This line of inquiry was also driven by the differences in rate of enzyme activities measured in two different tissues; although the kidney was identified as the most important organ for GAA synthesis in rodents, we measured about double the AGAT activity in the pancreas tissue of our piglet. To further investigate this outcome, in Chapter Five we focused on the inter-organ pathways of GAA and creatine synthesis in neonatal piglets to elucidate the contribution of the pancreas to whole-body GAA/creatine supply. We measured the net flux of creatine and its precursors to describe *in-vivo* the inter-organ synthetic pathways of creatine in sow-reared piglets. The novel aspect of this study was investigating the role of citrulline as a precursor for GAA and creatine. Citrulline can be converted into arginine in the kidney. Therefore, we hypothesized that some of the citrulline utilized by the kidney can be converted to GAA. We concluded that citrulline is a more effective precursor in the kidney for GAA release than arginine from our third study described in Chapter Five of this thesis. However, we did not know whether citrulline can be utilized to release GAA/creatine in the pancreas or gut. It is an interesting and novel finding that the pancreas and gut cannot use citrulline to release GAA and creatine. It is also interesting and novel that the gut has the greatest release of creatine among the tissues that we studied.

The net release of creatine from the gut indicates that either 1) release is higher than uptake or 2) there is no uptake and the release is solely due to endogenous synthesis in gut tissues from arginine or from GAA. Unfortunately, we could not determine which is most likely from our net balance study. Therefore, we cannot say whether the gut has the capacity to take up GAA or creatine from the arterial circulation. A better approach would be to use isotope-labelled precursors of GAA and creatine. Isotopic kinetics would allow us to measure the disappearance of a precursor isotope (e.g., intravenous arginine tracer) across the gut which would be an indicator of arginine uptake from arterial blood. Isotopic kinetics then would allow us to determine whether the gut has the capacity to take up, synthesize and release GAA and creatine from the systemic circulation.

What could not be compared was the proportion of creatine released via the gut versus liver. The liver is considered to be the major organ synthesizing creatine. This comparison would help to determine the significance of the gut in creatine synthesis. We did not collect the hepatic venous blood to measure the creatine flux across the liver. Future studies should focus on determining the quantitative role of the gut for creatine synthesis.

We used an intravenous route for the infusion of the experimental solutions, so the first pass intestinal metabolism of dietary derived arginine and GAA was bypassed. The next level of study should incorporate this gut metabolism. That would allow us to determine how dietary precursors would respond to fluxes of the precursor amino acids, GAA and creatine across gastrointestinal tissues.

Dietary arginine increases ornithine concentrations in mucosal tissues suggesting that either arginase or AGAT activities are significantly higher in the small intestinal mucosa (Tennakoon et al, 2013). However, we do not know the contribution of either arginases and AGAT for the synthesis of ornithine in the gut. Furthermore, what we do not know is whether there is a significant amount of AGAT activity in the neonatal gut. According to *in-vitro* enzyme activity, gut mucosa AGAT activity is insignificant (Brosnan et al, 2009). However, *in-vitro* enzyme activities represent enzyme capacities and do not represent *in-vivo* fluxes. Therefore, future studies need to focus on the *in-vivo* flux through AGAT in the gut, possibly via isotope kinetics.

Dietary citrulline bypasses the splanchnic citrulline conversion into arginine and a portion of whole-body endogenous arginine synthesis occurs in the renal tissues from citrulline (Marini et al, 2012). Therefore, arginine synthesized via the kidney from dietary citrulline bypasses the first-pass splanchnic arginine catabolism (possibly arginase activity, NOS and/or AGAT). Therefore, intragastrically fed citrulline is largely available to systemic metabolism. For this reason, feeding citrulline may be more beneficial than feeding arginine to neonatal piglets. We also determined that intravenous citrulline is more beneficial than arginine as citrulline is a better precursor for GAA/creatine synthesis in the kidney than arginine. However, feeding citrulline also bypasses other fates of arginine during splanchnic metabolism, such as arginine incorporation into splanchnic proteins, nitric oxide synthesis and polyamines synthesis. Therefore, citrulline feeding may be detrimental to gut metabolism in neonates (given that the gut is one of the most metabolically active organs). We know that the gut also takes up amino acids from the

arterial circulation. However, it has been shown that bypassing the first pass metabolism by the gut during intravenous feeding causes significant gut atrophy and also that intravenous delivery of arginine in neonates does not ameliorate gut atrophy. Therefore, supplementation of citrulline enterally needs further investigation to clarify whether a consequent lack of arginine in the diet would affect protein synthesis, polyamine synthesis and/or nitric oxide synthesis in splanchnic organs.

6.4 Brain creatine synthesis

We analyzed the brain GAA and creatine concentrations in both our IV and enteral (Chapter Three and Four) studies. In both these studies, we found no differences in brain GAA or creatine concentrations with or without supplementation of creatine or GAA in the diets. However, both of our studies were short term. The brain may be taking priority of available creatine over other organs to meet its creatine requirement. Therefore, short term changes of arginine, methionine, GAA or creatine may not affect brain creatine levels. Long term studies are needed in order to determine whether creatine supplementation is effective at increasing brain creatine levels. Studies conducted with humans who were suffering from creatine deficiency syndrome suggest that the brain takes up arterial creatine, but only very slowly. Therefore, changes in brain creatine and GAA concentrations may be undetectable (very low) during a short term feeding trials. Under this circumstance, even though the changes are very small, such changes are still very important for neonates as brain development during the neonatal period is at peak phase. Such short term changes would perhaps be visible with more sophisticated analytical methods such as isotopic kinetics.

Here, we are limited by the impossibility of measuring precursors and products of GAA and creatine across the brain. Isotopically labelled precursors and products of GAA and creatine can be measured in cerebrospinal fluid and in brain tissues. Therefore, the relationship between brain creatine concentration and dietary and endogenous creatine supply via isotopic kinetics are possible and warrant more investigation.

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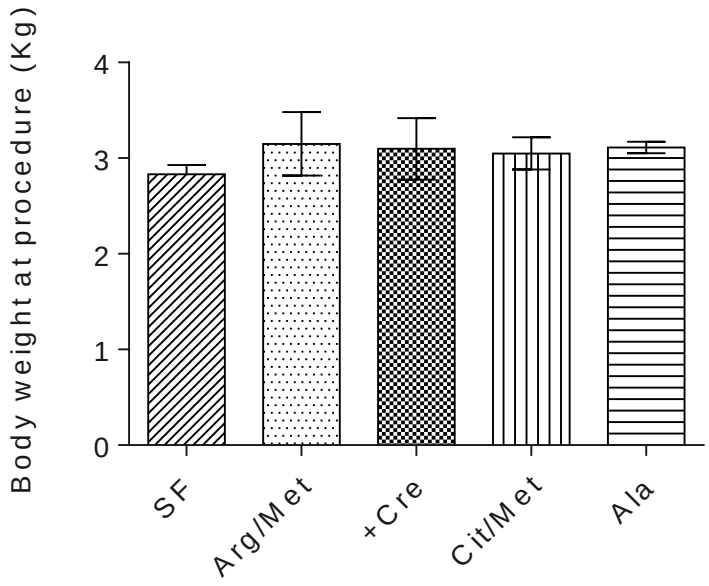
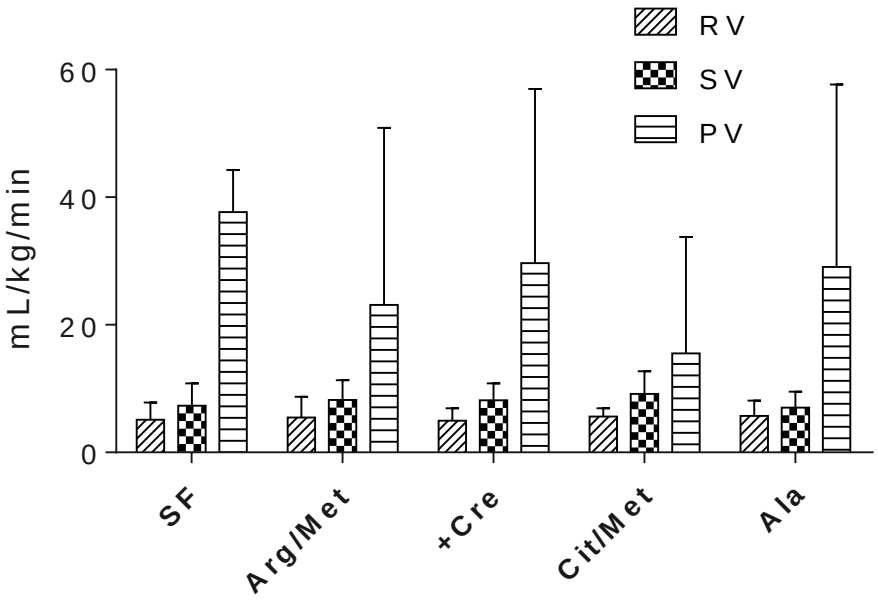
APPENDIX A: Body weight and blood flow for the study described in Chapter Five



APPENDIX A1 Body weight of sow-fed (SF) non-fasted piglets or fasted piglets that received an infusion of either arginine, citrulline or alanine. Data were analyzed one-way ANOVA with Bonferroni's multiple comparisons test for post hoc analysis. n = 6 per infusion groups and n = 4 per SF group. Values are means ± SD of non-transformed data. Arginine and methionine: Arg/Met, creatine in Arg/Met: +Cre, citrulline and methionine: Cit/Met, alanine: Ala



APPENDIX A2 Renal, Splenic and portal blood flow in sow-fed (SF) non-fasted piglets or fasted piglets that received an infusion of either arginine, citrulline or alanine. Data were analyzed one-way ANOVA with Bonferroni's multiple comparisons test for post hoc analysis. n = 6 per infusion groups and n = 4 per SF group. Values are means ± SD of non-transformed data. Arginine and methionine: Arg/Met, creatine in Arg/Met: +Cre, citrulline and methionine: Cit/Met, alanine: Ala

Appendix B: Methods

B.1 Determination of tissue and plasma amino acid concentrations

Amino acids concentrations were determined according to the method of Bidlingmeyer *et al.*, 1984.

B.1.1 Tissue preparation

Liver tissues and previously pulverized kidney tissue were homogenized with 2% cold perchloric acid (1:3 w/v) at 50% speed using a mechanical homogenizer for ~45 s and centrifuged at 3000 x g for 15 min to separate tissue-free amino acids from tissue-bound amino acids. The supernatant was collected into a scintillation vial. This step was repeated 3 times to total 1:9 wt./vol. This step facilitated complete separation of tissue free amino acids. Norleucine (25 µmol/mL) was added to the scintillation vial as an internal standard (1:0.1 w/v) and stored at -80^{o}C until needed.

B.1.2 Tissue free amino acid preparation

The supernatant was neutralized with 2.0 M K_2CO_3 (12.5% v/v) in a plastic tube and centrifuged at 5000 x g for 3 min to separate the supernatant from the precipitate. One mL of supernatant was added to another plastic tube which was flash frozen in liquid nitrogen and placed on a freeze dryer overnight. The dried samples were stored in -20^{o}C freezer until the derivatization was performed. Then samples were derivatized as described in **B.1.4.** when ready to run in the HPLC.

B.1.3 Plasma preparation

A 100 µL of aliquot plasma was mixed with 20 µL of 2.5 µmol/mL norleucine internal standard (Sigma Aldrich, Oakville, Canada) and 1 mL of 0.5% trifluoroacetic acid (TFA) (Sigma Aldrich, Oakville, Canada) in methanol (MeOH) (Fisher Scientific, Whitby, Canada) in a micro centrifuge tube, to precipitate proteins. The samples were vortexed and centrifuged at 2655 x g for 5 min. Then the supernatant was poured into a plastic tube and flash frozen in liquid nitrogen to dry down overnight in a freeze dryer (Thermo Savant, Canada).

Plasma and tissue-free amino acid concentrations were measured by reverse-phase HPLC (C18 column) following derivatization with phenylisothiocyanate (PITC) (Waters, Woburn, MA) as per the method of Bidlingmeyer et al (1984).

B.1.4 Derivatization of plasma and tissue free supernatants

100 µL of 2:2:6 TEA (Sigma Aldrich, Oakville, Canada): MeOH: water was added to each dried plasma or tissue-free sample followed by freeze drying for ~1 h. Plasma samples were then incubated for 35 min at room temperature with 50 µl of 1:1:7:1 solution of water: TEA: MeOH: PITC. The labelling with PITC was stopped by flash freezing with liquid nitrogen and then placed on a freeze dryer overnight. Plasma samples were re-suspended in 200 µL of sample diluent (710 mg of Na_2HPO_4 (Sigma Aldrich, Oakville, Canada) in 1 liter of water, pH 7.4 with 10% H_3PO_4 acid; 5% of the volume was replaced with acetonitrile (Fisher Scientific, Whitby, Canada)) while tissue-free samples were resuspended in 300 µl of the same sample diluent.

B.1.5 Amino acids by HPLC

Plasma and tissue free amino acid concentrations were measured by reverse-phase HPLC following derivatization with phenylisothiocyanate (PITC) (Thermo Scientific, USA) (Bidlingmeyer *et al.*, 1984).

A 40 µL of aliquot each plasma or tissue free sample was injected into a reverse-phase C18 Pico-Tag column (Waters, 60Å, 4 µm 3.9 X 300 mm) connected to a HPLC system. The HPLC system consisted of a Waters 1525 Binary HPLC pump, Waters 2487 Dual λ absorbance detector and Waters 717 plus Auto sampler (Waters Corporation, Milford, MA, USA). The column temperature was maintained at 46 ^{o}C to facilitate the separation of amino acids. Buffer A consisted of 70 mM sodium acetate (Fisher Scientific, Whitby, Canada) and 2.5% acetonitrile (Fisher Scientific, Whitby, Canada) at pH 6.55 and buffer B consisted of 45% acetonitrile and 15% methanol. Buffer was filtered through a 0.45 µm MAGNA nylon filter (Canadian Life Science, Peterborough, ON, Canada) and degassed using a Waters In Line-Degasser AF. The column was allowed to equilibrate with buffer A at a rate of 1 mL/min. The phenylthiocarbamyl amino acids were detected at 254 nm. Peaks were integrated using Breeze Software (Waters, version 3.3, 2002, Waters Corporation, Woburn MA, USA) and the amino acid concentrations were determined by comparing to the area produced by the internal standard.

B.2 Tissue and plasma guanidinoacetate (GAA) and creatine

B.2.1 Tissue preparation for GAA and creatine

Tissue (kidney, pancreas, brain, and liver) GAA and creatine concentrations were assayed using an HPLC method according to Buchberger and Ferdig (2004) using a C18 reverse

phase column (Hypersil ODS 5 U 150x4.6 mm column) with ninhydrin (Sigma Aldrich) derivatization and fluorescence detection (Ex/EM 390/470).

Tissue was homogenized with cold 1 M perchloric acid (1:4 w/v) followed by centrifugation at 4ºC for 20 min at 15,250 x g. Supernatant was transferred to a tube quantitatively followed by addition of 50% K_2CO_3 and 20% KOH to neutralize (pH 6.5 - 7) the samples. Samples were centrifuged again for 10 min at 10,600 x g at 4ºC. Two-hundred microliters of supernatant was used for derivatization with ninhydrin (see section **B.2.3**).

B.2.2 Plasma preparation for GAA and creatine

Two-hundred microliters of plasma was mixed with 14 µL of 30% perchloric acid and placed on ice for 15 min followed by centrifugation at 15,000 x g for 8 min at 4ºC. One-hundred and fifty microliters of supernatant was transferred to a micro centrifuge tube and 22.4 µL of 20% KOH was added. Then samples were placed on ice for 15 min followed by centrifugation at 10,000 x g for 5 min. After centrifugation, 150 µl of supernatant was used for derivatization with ninhydrin (see section **B.2.3**).

B.2.3 Derivatization with ninhydrin

To both plasma and tissue samples, 1.3 M KOH and 0.9% ninhydrin (4:3:1.5) were added and mixed together and left at room temperature for 15 min. Five percent ascorbic acid and

5 M phosphoric acid (1:1) were added to samples and mixed followed by incubation in a 90^{O}C water bath for 30 min. After derivatization, samples were cooled and filtered using a 0.45 µm PTFE syringe filter (Canadian Life Science, Peterborough, Canada).

B.2.4 Ninhydrin HPLC separation of GAA and creatine

Ninhydrin derivatised plasma and tissue (kidney, pancreas, brain, and liver) samples were run in the HPLC using a C18 reverse phase column (Hypersil ODS 5 U 150x4.6 mm column) with fluorescence detection (Ex/EM 390/470) to analyze the GAA and creatine concentrations. Detailed method was as follows.

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) and derivatized GAA and creatine was separated via reverse-phase HPLC using a dual buffer system (Mobile phase A: 50 mM formic acid and mobile phase B: 100% methanol). Samples were detected by Waters 474 scanning fluorescence detector at 470 nm emission and 390 nm excitation. The run time for each sample was 32 min with a flow rate of 1 mL/min. A standard curve was used for quantification and peaks were determined using Breeze Software (Waters, version 3.3, 2002, Waters Corporation, Woburn, MA, USA).

B.3 Determination of total creatine in gastrocnemius muscle

B.3.1 Tissue preparation

Concentration of gastrocnemius muscle was determined using the simplified method of Lamarre et al (2010).

Tissues were homogenized in a 50 mmol.L^{-1} Tris buffer (pH 7.4) and kept for 30 min at room temperature to convert phosphocreatine into free creatine. The homogenates were then deproteinized with trifluoroacetic acid (TFA) in methanol and micro-centrifuged at 3825 x g at 4^{o}C for 10 min. Samples were then filtered using a Bond-Elut C18 solid phase extraction cartridge.

B.3.2 HPLC separation of total creatine

The total creatine was separated via HPLC using porous graphitized carbon column (Hypercarb, 7 µm, 100 X 4.6 mm, Thermo Scientific, Canada). The peak was detected using a Waters 2487 Dual λ absorbance detector at an absorbance of 210 nm. An isocratic mobile phase of 0.1% TFA and 3% methanol was used with a flow rate of 1 mL/min and run time of 20 min. A standard curve was used for quantification and peaks were determined using Breeze Software (Waters, version 3.3, 2002, Waters Corporation, Woburn, MA, USA).

B.4 *In-vitro* enzyme activities

B.4.1 AGAT activity

AGAT activity was assayed using a modified method of Van et al (1970) using frozen tissues. The assay measured the amount of ornithine converted from arginine due to AGAT activity (transamidinase). Detailed methods were as follows.

Whole kidney and pancreas tissues were pulverized, and a representative sample was used to measure transamidinase activity. Pulverized frozen kidney tissues were placed in 50 mM Potassium Phosphate buffer solution (1:5 w/v) consisting of 0.2 M potassium phosphate monobasic and 0.2 M dipotassium phosphate (pH 7.4). Samples were homogenized with polytron for 25 seconds. The resulted sample preparation was dilute 1/20 with phosphate buffer to make 1% homogenate. The tissue homogenate prepared from homogenized frozen kidney or pancreas samples were incubated at 37^{o}C in a shaking water bath with different substrate buffers containing either arginine (15 mM arginine, 17 mM Sodium Fluoride in 50mM Potassium Phosphate) or glycine (23 mM glycine, 17 mM Sodium Fluoride in 50mM Potassium Phosphate) or arginine and glycine 15 mM arginine, 23 mM glycine and 17 mM Sodium Fluoride in 50mM Potassium Phosphate) or phosphate buffer with no substrates (17 mM Sodium Fluoride in 50mM Potassium Phosphate). Each preparation was duplicated for the reactions and blank tubes. One of the blank reactions was stopped at the beginning of the incubation by adding 1 M PCA (stopped reaction). The

remaining blank and the reactions were stopped at the end of 60 minutes of incubation. All the tubes were covered with marbles during incubation at 37^{o}C to minimise the evaporation. The resulting reactions and the blank and stop reaction were swirled to mix once stopped. The content was transferred to a new eppendorf and cooled on ice followed by centrifugation at 10,000 rpm for 5 minutes at 4^{o}C. A 0.5 mL aliquot resulting supernatants were incubated in a 92^{o}C water bath with ninhydrin colour reagent (6 g) dissolved in 1-propanol anhydrous (100 mL) (Sigma Aldrich) in order to perform the ornithine assay for 25 minutes. The preparation of ornithine assay was as follows;

Blank contains 1 M PCA, 100 mM Potassium phosphate, Sodium fluoride, and ninhydrin solution while standard contains all the solutions in the blank with ornithine standard. The reactions and stopped reactions contain 0.5 mL of the supernatant and the ninhydrin solution. The absorbance of the final incubations was read at 505 nm in a spectrophotometer.

The remaining tissue homogenates were stored at -70^{o}C for total protein assay. The protein content of homogenates was assayed using the Pierce^{TM} BCA protein assay kit (Thermo Fisher Scientific, Mississauga, Canada). The ornithine produced by AGAT was presented as nmol of ornithine per mg of protein per min.

Calculation

- ΔOD due to AGAT = ΔOD (+arg/+gly) - [{ΔOD (-arg/+gly) + ΔOD (+arg/-gly)} - ΔOD (-arg/-gly)]
- (ΔOD AGAT) x nmol ornithine std x 5/ ΔOD x 60 minutes x 5 mg of sample = nmol ornithine/min/mg kidney

B.4.2 Hepatic GAMT activity

GAMT activity was assayed using fresh liver samples as described previously by da Silva et al (2009) which was modified from Ogawa et al (Ogawa *et al.*, 1983). The reactions stop reactions and blanks were prepared from fresh liver samples immediately following necropsy and frozen at -80ºC for the later analysis of resulting creatine via the ninhydrin HPLC method. Liver tissue was homogenized with a buffer solution (1:5 w/v) consisting of 0.25 M sucrose, 1 mM EDTA and 10 mM HEPES (pH 7.2). Homogenates were centrifuged at 100,000 x g at 4 ºC for 1 h and the supernatant was used to prepare the reactions and stop reactions.

The reactions stop reactions and blank preparation was as follows. Briefly, 10 µl supernatant was added to 350µl of tris-β-mercaptoethanol solution (100 mM Tris buffer (pH 7.4), 20 mM 2-mercaptoethanol) and 50 µL S-adenosylmethionine solution (8.8 mg SAM in 1 mL of water and 1 mL of tris-β-mercaptoethanol buffer). The assay was

started by addition of 10 µl of GAA (4.68 mg of GAA in 1 mL of water and 1 mL of tris-β-mercaptoethanol buffer) followed by incubation at 37^{O}C for 20 min. Blanks for the assay did not contain supernatant. At the end of 20 min of incubation, the assay was stopped by addition of 750 µL of 15% (w/v) trichloroacetic acid and samples were kept on ice for 10 min. Then 720 µL of 1 M Tris (pH 7.4) was added to neutralize the solutions. The resulting solutions were centrifuge at 10,000 x g for 5 min. Creatine synthesized by the enzyme GAMT was measured via HPLC and ninhydrin derivatization using 400 µL of supernatant as described above **(B.2.3 & B.2.4)** and activity was expressed per mg of protein per min. The protein content in each sample was determined via the biuret reaction.

B.5 Measurement of tissue-specific fractional protein synthesis by gas chromatography mass spectrometry (GC-MS)

B.5.1 Tissue preparation

Liver, small intestinal mucosa and kidney samples were prepared for the extraction of cold and deuterated phenylalanine as per the method of Lamarre et al (2015).

A 100 mg sample of frozen tissue was homogenized in 2 mL of cold dH_2O at 50% speed using mechanical homogenizer for ~45 seconds. The resulting homogenate was vortexed with 500 µL of cold 2 M perchloric acid (PCA) thoroughly. The homogenates

were centrifuged at 3000 rpm for 20 minutes at 4^{o}C to separate acid soluble, tissue-free amino acids from acid-insoluble, protein-bound amino acids.

B.5.2 Tissue free amino acid preparation

The supernatant (tissue-free) fraction was applied to a pre-conditioned hydrophobic solid phase extraction cartridge (Bond Elute C18, 100 mg 1 mL; Agilent Technologies, Santa Clara, CA) to extract phenylalanine. The procedure is as follows. The Bond Elute C18 was conditioned with 1 mL of 100% Methanol. One mL of 0.2 M PCA was applied to pre-conditioned Bond Elute C18. A 0.5 mL sample was applied to the Bond Elute C18. The Bond Elute was acid washed with 1 mL of 0.2M PCA. Tissue-free phenylalanine was eluted with 1 mL of 100% methanol into a glass tube. This was freeze dried overnight. The dried bond eluted tissue-free samples were capped with nitrogen gas and stored in -20^{o}C until ready for PFBBR derivatization.

B.5.3 Tissue protein-bound amino acid preparation

The pellet was washed three times as follows to remove all possible tissue-free amino acids from the pellet. The washing procedure was as follows. The pellet was homogenized in 5 mL of 0.2 M PCA and the resulting solution was centrifuged at 3000 rpm for 20 min at 4^{o}C. This step was repeat for three times and the supernatant was discarded each time. The washed pallet was re-suspended in 4 mL of 6N HCL and a glass rod was used to disrupt the pellet, followed by transfer of all contents to a Pyrex tube. Then the solution was placed

in a 110°C oven for 18 h to hydrolyze the protein. The resulting hydrolysate solution was left to cool under the fume-hood. Hydrolysates were transferred into a new screw-capped plastic tube using a 0.45 µm PTFE syringe filter (Canadian Life Sciences, Canada).

The hydrolysed (tissue-bound) fraction was applied to a pre-conditioned hydrophobic solid phase extraction cartridge (Bond Elute C18, 100 mg 1 mL; Agilent Technologies, Santa Clara, CA) to extract bound phenylalanine. The procedure was as follows. The Bond Elute C18 was conditioned with 1 mL of 100% Methanol. One mL of 0.2 M PCA was applied to pre-conditioned Bond Elute C18. One mL tissue-bound sample was applied to the Bond Elute C18. The Bond Elute was acid washed with 1 mL of 0.2 M PCA. Tissue-free phenylalanine was eluted with 1 mL of 100% methanol into a glass tube. The last step was repeated twice. Therefore, the eluted sample was 2 mL in volume. The glass tubes were placed in a vacuum oven overnight to dry. The dried bond eluted tissue-bound samples were capped with nitrogen gas and stored at -20^{o}C until ready for PFBBr derivatization.

B.5.4 PFBBr derivatization

The dried tissue-free and tissue-bound eluents were re-suspended in 500 µL of HPLC water for the derivatization. The derivatization procedure was as follows.

50 µL of resuspended tissue-free or tissue-bound samples were transferred into a glass vial containing 20 µL of 0.5 M phosphate buffer (pH 8.00 and 130 µL of 100 mM

pentafluorobenzyl bromide (PFBBr) derivative (Sigma Aldrich) solution in acetone. The vial was tightly capped, thoroughly mixed for 1 min and incubated at 60^{o}C for an hour. After cooling at room temperature for 5 min, 330 µL of hexane was added and the vial was immediately re-capped. The hexane water sample was mixed for 1 min and the organic phase was removed and placed into a low volume insert fitted with a GC-MS glass vial for mass spectrometry analysis.

B.5.5 GC-MS analysis

The isotopic enrichment of L-[ring-$^{2}H_5$] phenylalanine in tissue-free and protein-bound fractions was determined by GC-MS with a model 6890 GC linked to a 5976N quadrupole MS (Agilent Technologies) operating in the electron ionization mode (Lamarre et al, 2015). A mixed sample of L-[ring-$^{2}H_5$] phenylalanine and unlabeled phenylalanine was run in scan mode, in which 91 and 96 ions or 300 and 305 were identified as potential ions. A standard curve was run before analyzing samples to identify the linear ranges. Ions with mass-to-charge ratio of 91 and 96 ions were monitored via selected ion monitoring for liver, small intestinal mucosa and muscle tissues; for kidney and pancreas samples, 300 and 305 ions were monitored. Percent molar enrichment (mol%) was determined, and fractional synthesis rate (FSR, %/d) of protein was calculated as follows:

$$FSR = IE_{bound} / IE_{free} \cdot 1440/t \cdot 100$$

Where IE_{bound} and IE_{free} are the isotopic enrichments (mol%) of L-[ring-$^{2}H_{5}$] phenylalanine of the PCA-insoluble (protein bound) and PCA-soluble (tissue free) phenylalanine pool; t is time of labeling in min and 1440 is the number of minutes per day.

B.6 Cholesterol and triglyceride analysis

B.6.1 Tissue cholesterol and triglyceride analysis

Total lipids were extracted from the liver tissues using the method of Folch et al (1957). Extracted lipids were used to analyze hepatic cholesterol and triglyceride (TG) concentrations via spectrophotometer assays (505 nm and 520 nm respectively) using commercially available kits (Genzyme DC-CAL, Triglyceride-SL and Cholesterol-SL) from Sekisui Diagnostic (Charlottetown, Canada).

B.6.2 Plasma triglyceride and cholesterol analysis

Plasma samples were also analyzed via spectrophotometer assays (505 nm and 520 nm respectively) using a commercially available glycerol standard (Sigma Aldrich) and cholesterol standard (Pointe Scientific, Canton, MI), and the same commercial kits as used for liver samples (Triglyceride-SL and Cholesterol-SL, Sekisui Diagnostic, Charlottetown, Canada). Cholesterol

B.7 Plasma homocysteine and cysteine

Plasma homocysteine was determined using a modified method by Vester and Rasmussen (1991). A 150 μL aliquot of plasma was combined with 20 μL of tris-(2-carboxyethyl) phosphine (100mg/mL) which was vortexed and left at room temperature for 30 minutes. 50 μL of 0.2 mM 8-amino-naphthalene-1,3,6-trisulfonic acid disodium salt (ANTS) (dissolved in 0.1 mM borate buffer with 2mM EDTA at pH = 9.5) and 125 μL of 0.6 M perchloric acid were added to samples which were left at room temperature for 10 minutes followed by centrifugation at 4°C at 6000 rpm for 5 minutes. 100 μL of supernatant was then transferred to dark Eppendorf tubes followed by addition of 200 μL of borate buffer (2 M at pH = 10.5) and 100 μL of derivatizing agent 7-flurobenzo-2,1,3-oxadiazole-sulfonic acid ammonium salt (SBD-F) solution (1 mg/mL SBDF in 0.1 M borate buffer with 2 mM EDTA at pH = 9.5). Samples were covered in aluminum foil and vortexed followed by incubation at 60°C in a water bath for 60 minutes. Samples were then removed and placed on ice for 5 minutes followed by centrifugation at 7000 rpm for 5 minutes. Samples were injected into a Waters YMC column (Milford, MA) and homocysteine and cysteine were separated via reverse phase HPLC using a dual buffer system (Buffer A: and Buffer B:) with a run time of 20 minutes at 1mL/minute. Internal standard ANTS was used for quantification and Empower 2 software (Waters, Milford, MA) was used to determine area under the curve.

Solution preparation:

Solution 1: Dissolve 6.8 g of sodium acetate trihydrate in 500 mL of HPLC water

Solution 2: Add 11.5 mL glacial acetic acid (99.9%, 17.4 M) to 2 L of HPLC water

Solution 3: Add Solution 1 for Solution 2 until pH = 4.0 (approximately 400 mL)

HPLC Buffer A: Combine 980 mL of Solution 3 with 20 mL of methanol

HPLC Buffer B: Combine 800 mL of Solution 3 with 200 mL of methanol

B.8 REFERENCES

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