Methionine and Guanidinoacetic Acid Metabolism in Yucatan Miniature Piglets

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Abstract

Methionine, an essential amino acid, plays crucial roles beyond its incorporation into proteins. It can be converted into S-adenosylmethionine (SAM), the universal methyl donor involved in over 50 transmethylation reactions. These reactions are essential for creatine and phosphatidylcholine (PC) synthesis and DNA methylation. The first experiment in this thesis investigated the methionine requirement for synthesizing major transmethylated products, whole body proteins and tissue specific proteins. Data from the first experiment demonstrated that DNA methylation is prioritized over hepatic creatine synthesis, while PC synthesis continuously increases with methionine intake. When methionine was limited, liver protein synthesis was prioritized, followed by kidney and muscle protein synthesis. Since different tissues have varying methionine requirements, our data provide insights into why growth is restricted at lower amino acid intakes, to spare limited amino acids for intestinal function and critical metabolic processes in other vital organs.. These findings indicate that using protein synthesis alone to determine whole-body methionine requirements is inadequate, as more methionine is needed to fulfill its non-protein roles. The second major objective of this thesis was to investigate the effect of dietary methionine on supplemental guanidinoacetate (GAA) absorption and creatine synthesis in neonatal piglets. Using a 4-h duodenal infusion with radioisotope tracers and dietary treatments varying in methionine levels, we found that excess dietary methionine increases the portal appearance of GAA and enhances creatine synthesis in piglets. Furthermore, our data revealed that GAA accumulates in the liver when dietary methionine is deficient, although no toxic effect was apparent. Recently, researchers identified that the GAA + creatine mixture enhanced muscle and brain creatine levels in healthy individuals. However, there is little information on how the GAA + creatine mixture affects GAA absorption, transport, and utilization in pigs. Hence, we compared the effectiveness of three supplementation options: GAA alone, GAA + methionine, and GAA +

creatine, in enhancing creatine stores and GAA absorption in neonatal piglets. Moreover, we evaluated the effectiveness of creatine and GAA combinations in enhancing GAA absorption across the gut in neonatal piglets using an ex vivo Ussing chamber model. This study demonstrated that both GAA + methionine and GAA + creatine groups showed increased brain creatine levels, compared to control. Moreover, hepatic creatine concentration was highest in the GAA + creatine group, compared to control and GAA groups, suggesting GAA+ creatine is the best combination to improve hepatic creatine stores. Findings from the Ussing chamber model showed that a higher level of creatine enhanced GAA absorption across the jejunum.

Overall, these studies improve our understanding of methionine metabolism in both protein and non-protein pathways and GAA metabolism and creatine synthesis in neonatal piglets. These findings could significantly impact the animal industry and health and disease management across various populations, including different age groups and animal species.

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I dedicate this thesis to the memory of my loving father, who believed in me more than anyone else.

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List of Abbreviations

5,10-THF - 5,10-methylenetetrahydrofolate

5-methyl-THF - 5-methyltetrahydrofolate

AA - amino acids

AGAT - arginine:glycine amidinotransferase

ANOVA - analysis of variance

Arg - arginine

ATP - adenosine triphosphate

AUC - area under the curve

BHMT - betaine-homocysteine methyltransferase

BP - break point

BW - body weight

CBS - cystathionine β-synthase;

CDP - choline – cytidine diphosphate-choline

CGL - cystathionine γ-lyase

CNS - central nervous system

CpG - Cytosine and Guanine dinucleotide

Cre - creatine

CrT - creatine transporter

d - day

DMG - dimethylglycine

DNA - deoxyribonucleic acid

DNMT - DNA methyltransferase

DPM - disintegrations per minute

EDTA - ethylenediaminetetraacetic acid

F - female

FAA - free amino acids

FCR - feed conversion ratio

FAO - Food and Agriculture Organization

GAA - guanidinoacetate

GABA - γ-aminobutyric acid

GAMT - guanidinoacetate methyltransferase

GC/MS – gas chromatography mass spectrometry

Gly - glycine

GNMT - glycine N-methyltransferase

h - hours

HA - hepatic artery

HPLC - high-performance liquid chromatography

HV - hepatic vein

IAAO - indicator amino acid oxidation

Ks - fractional protein synthesis rates

L - linear effect

M - male

MAT - methionine adenosyltransferase

Met - methionine

MetO - methionine sulfoxide

mRNA - messenger ribonucleic acid

MS - methionine synthase

MTHFR - methylenetetrahydrofolate reductase

NS - not significant

Ox - oxidation

PB - protein breakdown

PC – phosphatidylcholine

PE - phosphatidylethanolamine

PEMT - phosphatidylethanolamine N-methyltransferase

PFBBr - pentafluorobenzyl bromide

PFC - prefrontal cortex

Phe - phenylalanine

PN - parenteral nutrition

PS - protein synthesis

PV - portal vein

q - flux

Q - quadratic effect

SAH - S-adenosylhomocysteine

SAHH - S-adenosylhomocysteine hydrolase

SAM - S-adenosylmethionine

SD - standard deviation

SEM - standard error of the mean

SRA - specific radioactivity

TauT - taurine transporters

TFA - trifluoroacetic acid

THF - tetrahydrofolate.

TLC - thin-layer chromatography

Trt - treatment effect

Tyr – tyrosine

UNU - United Nations University

UPLC - ultra-high performance liquid chromatography

WHO - World Health Organization

Chapter 1: Introduction

This chapter provides a background of the main components of the thesis, along with brief review of the literature that lead into each chapter. The initial sections focus primarily on methionine, its related metabolites, and the significance of methionine-related compounds in breast milk and infant formula. The discussion then highlights the significance of creatine and its precursor, guanidinoacetic acid (GAA). Later sections cover the key roles of GAA in the animal industry and its effects on other precursor amino acids, including methionine and arginine (modified from Asiriwardhana et al., 2022).

1.1 Methionine

Methionine is an indispensable sulfur-containing amino acid required for growth and development (Ball et al., 2006). It is essential for synthesizing protein and several other critical products, including creatine, phosphatidylcholine (PC), and methylated DNA. Methionine is also needed for the biosynthesis of polyamines and carnitine, which is required for fatty acid metabolism (Huang et al., 2012). Methionine serves as the major methyl group donor in vivo.

In growing animals, the metabolism of methionine involves three essential functions, which are represented by three different outlets. The first outlet involves using methionine for protein synthesis, which removes methionine from the methionine cycle. The second outlet involves the use of S-adenosylhomocysteine (SAH) for the formation of polyamines. The third outlet is the irreversible cystathionine synthase reaction, which commits homocysteine to transsulfuration. These three outlets represent the essential functions of methionine in the body (Courtney-Martin et al., 2012). Methionine is also crucial for proper immune system function and antioxidant mechanisms via its metabolites (Sandrielly et al., 2017) from transsulfuration, including cysteine

which is the limiting metabolite for glutathione, a powerful antioxidant, and taurine, which directly impacts immune system function (Blachier et al., 2007).

Methionine undergoes catabolism and recycling through a set of metabolic reactions known as the methionine cycle (Figure 1.1). This cycle is closely connected to the folate cycle, and together they constitute one-carbon metabolism (Aissa et al., 2022). The methionine cycle, which is present in the majority of cells, is comprised of three pathways: transmethylation, transsulfuration, and remethylation. The transmethylation of methionine leads to homocysteine synthesis. Homocysteine can be remethylated to form methionine or catabolized via the transsulfuration pathway to form cysteine (Huang et al., 2012). Those processes are critical during development; thus, it is important to determine how dietary methionine contributes to the non-protein pathways in the neonate.

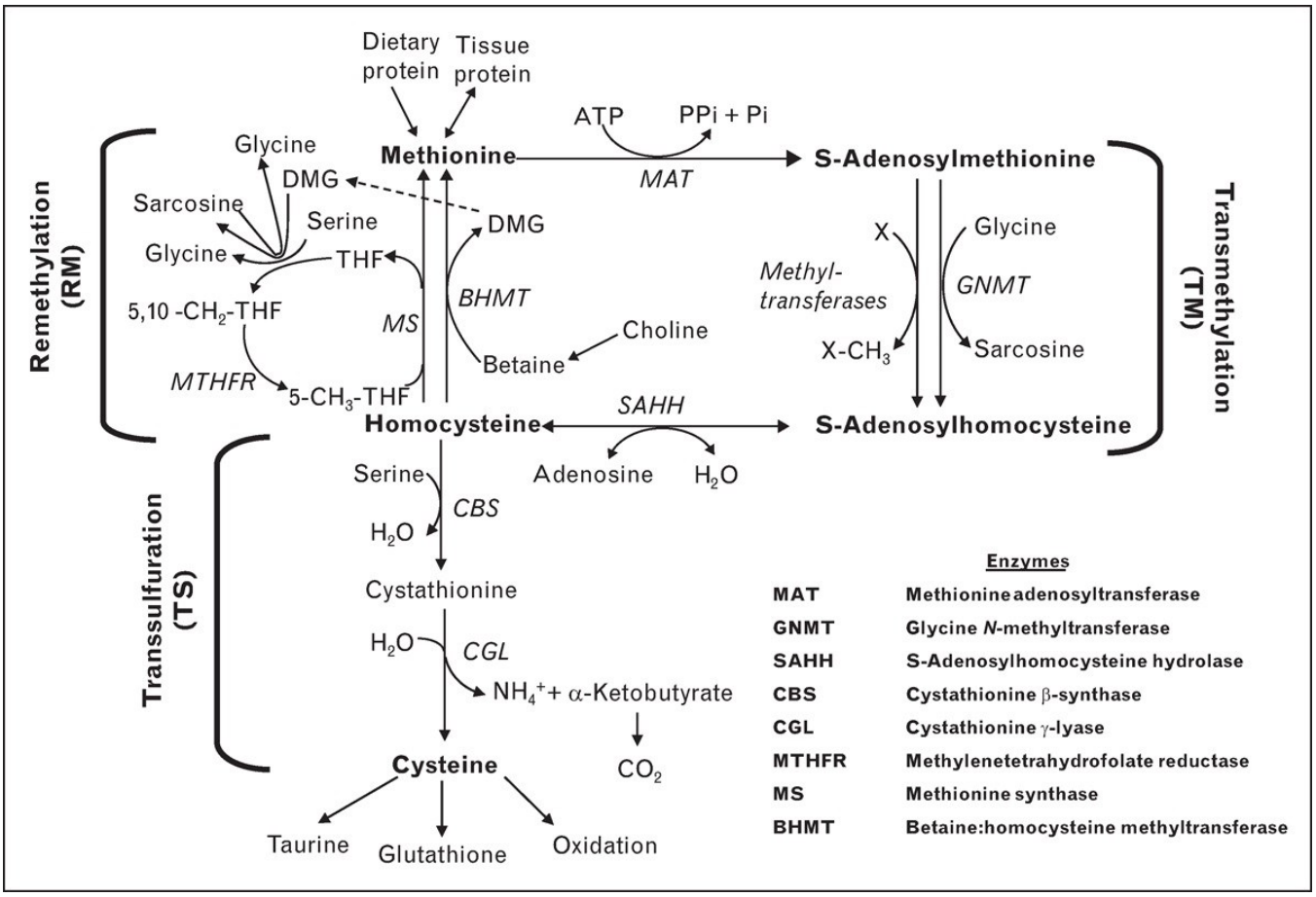

Figure 1.1: Schematic diagram of methionine cycle

BHMT (EC 2.1.1.5), betaine–homocysteine methyltransferase; CBS (EC 4.2.1.22), cystathionine β-synthase; CGL (4.4.1.1), cystathionine γ-lyase; DMG, dimethylglycine; GNMT (EC 2.1.1.20), glycine N-methyltransferase; MAT (2.5.1.6), methionine adenosyltransferase; MS (EC 2.1.1.13), methionine synthase; MTHFR (EC 1.5. 1.20), methylenetetrahydrofolate reductase; SAHH (EC 3.3.1.1), S-adenosylhomocysteine hydrolase; THF, tetrahydrofolate. (Bertolo et al., 2013)

1.1.1 **Transsulfuration and remethylation pathways**

The transsulfuration pathway is only found in certain tissues and is primarily limited to the liver, kidney, intestine, and pancreas (Brosnan et al., 2006). Transsulfuration refers to the process of transferring sulfur from homocysteine to cysteine via cystathionine. It serves as an irreversible pathway for eliminating homocysteine. Methionine is recognized as the metabolic precursor for cysteine. Cysteine is a semi-essential amino acid critical for synthesizing glutathione, as it is the rate-limiting substrate for this process (Lyons et al., 2000). However, cysteine cannot act as a precursor for methionine due to the irreversible nature of the cystathionine β-synthase (CBS) reaction (Ball et al., 2006). Cysteine serves multiple functions in the body, including its involvement in protein synthesis. It also acts as a precursor for the synthesis of several important metabolites, such as taurine, 3'-phosphoadenosine-5'-phosphosulfate, and coenzyme A (Humayun et al., 2006; Martínez et al., 2017).

Indeed, the process of transsulfuration is responsible for the research finding that dietary cysteine can reduce the methionine requirement by approximately 40% in piglets (Shoveller et al., 2003a; 2003b) and 55% in children (Humayun et al., 2006). This suggests that the availability of dietary cysteine can effectively support the remethylation of homocysteine to methionine while reducing its conversion to cysteine through transsulfuration pathway, thereby reducing the overall methionine requirement in these populations. In piglets, the research conducted by Bauchart-Thevret et al. (2009b) demonstrated that transsulfuration was compromised when sulfur amino acids were eliminated from the diet. This suggests that transmethylation and protein synthesis is prioritized over transsulfuration in piglets. Consequently, the partitioning of methyl groups towards transsulfuration is not expected to be influenced by dietary methyl donors until concentrations of S-adenosylmethionine (SAM) accumulate (Finkelstein et al., 1971). Therefore,

dietary methyl donors are anticipated to enhance the diversion of methionine towards transsulfuration only after the demands of transmethylation and protein synthesis have been met.

In the remethylation pathway, homocysteine can act as an acceptor for methyl groups from two sources. Folate (specifically, 5-methyl-THF) is one of these sources, and the conversion process is facilitated by 5-methyltetrahydrofolate homocysteine methyltransferase, which is also referred to as methionine synthase (Figure 1.1). The other methyl source is betaine, which is produced from the oxidation of choline. This reaction is catalyzed by betaine: homocysteine methyltransferase (BHMT), which transfers a methyl group from betaine to homocysteine (Humayun et al., 2006).

In adults under normal dietary conditions, it was estimated that methionine undergoes remethylation approximately 1.5-2.0 times before being oxidized. However, this rate was roughly doubled during methionine-restriction, as reported by Mudd and Poole (1975). Bauchart-Thevret et al. (2009b) found that in neonatal piglets, regardless of the presence or absence of dietary methionine and cysteine, remethylation accounted for approximately 20% of the overall methionine flux in the entire body.

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 (with excess cysteine), and then dietary methionine was reduced to 80% of the requirement in both groups on day six. Transmethylation and remethylation were affected by deficient methionine; however, neither transsulfuration nor protein synthesis was affected. This suggests that 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), remethylation accounts for only 7 - 10% of the whole-body

methionine flux; however, a significant portion of the rate of appearance of methionine was directed towards protein synthesis and transmethylation reactions.

1.1.2 **Transmethylation pathway**

Transmethylation refers to the process of transferring methyl groups from methionine to various methylated products. It can be viewed as the non-protein requirement of methionine (Bertolo et al., 2013).

Methionine is essential for metabolism as it serves as a precursor for transmethylation, producing SAM, a critical component involved in methylation reactions in living organisms (Elango, 2020). Methionine is adenylated by methionine adenosyltransferase (MAT) to form SAM, which serves as the primary biological methyl donor in mammalian cells and a precursor for polyamine synthesis, while also undergoing intracellular transmethylation to produce homocysteine (Bauchart-Thevret et al., 2009a).

Transmethylation places a substantial metabolic burden on dietary methionine. Research by McBreairty et al. (2013) indicated that approximately 75% of hepatic methionine is involved in transmethylation. Furthermore, it is estimated that a methionine molecule undergoes transmethylation 2-4 times in adults, depending on the availability of dietary methyl groups, as suggested by Mudd and Poole (1975).

Transmethylation involves a considerable number of reactions, with at least 50 described reactions (Schubert et al., 2003). However, it is assumed that the actual number of methyltransferase enzymes utilizing SAM as a substrate may exceed 300 (Brosnan and Brosnan 2006). Quantifying the minimum proportion of the methionine requirement necessary to support transmethylation in vivo is crucial to determining the overall whole body methionine requirement and how related

nutrients can affect this requirement. Moreover, how transmethylation allocates methyl groups to specific products is unknown. This partitioning of transmethylation reactions may also be adjusted depending on what products and precursors are available. These transmethylation reactions produce various products, including creatine, phosphatidylcholine, DNA, and sarcosine, among others (Stead et al., 2006).

1.1.2.1 Creatine synthesis

Creatine is a naturally occurring nitrogenous compound that is derived from amino acids. It can be found in animal-derived food sources, especially in meat and fish. Endogenous creatine is produced through a two-step enzymatic process (Figure 1.2): firstly, the guanidinoacetate (GAA) is synthesized from arginine and glycine, facilitated by l-arginine: glycine amidinotransferase (AGAT); secondly, GAA is methylated by S-adenosyl-l-methionine: N-guanidinoacetate methyltransferase (GAMT). The methyl group of SAM, originally from methionine, is transferred to GAA to synthesize creatine (Stöckler-Ipsiroglu et al., 2006). The synthesis of creatine is regulated by the activity of the AGAT enzyme (Stöckler-Ipsiroglu et al., 2006; Sipila, 1980; McGuire et al., 1984).

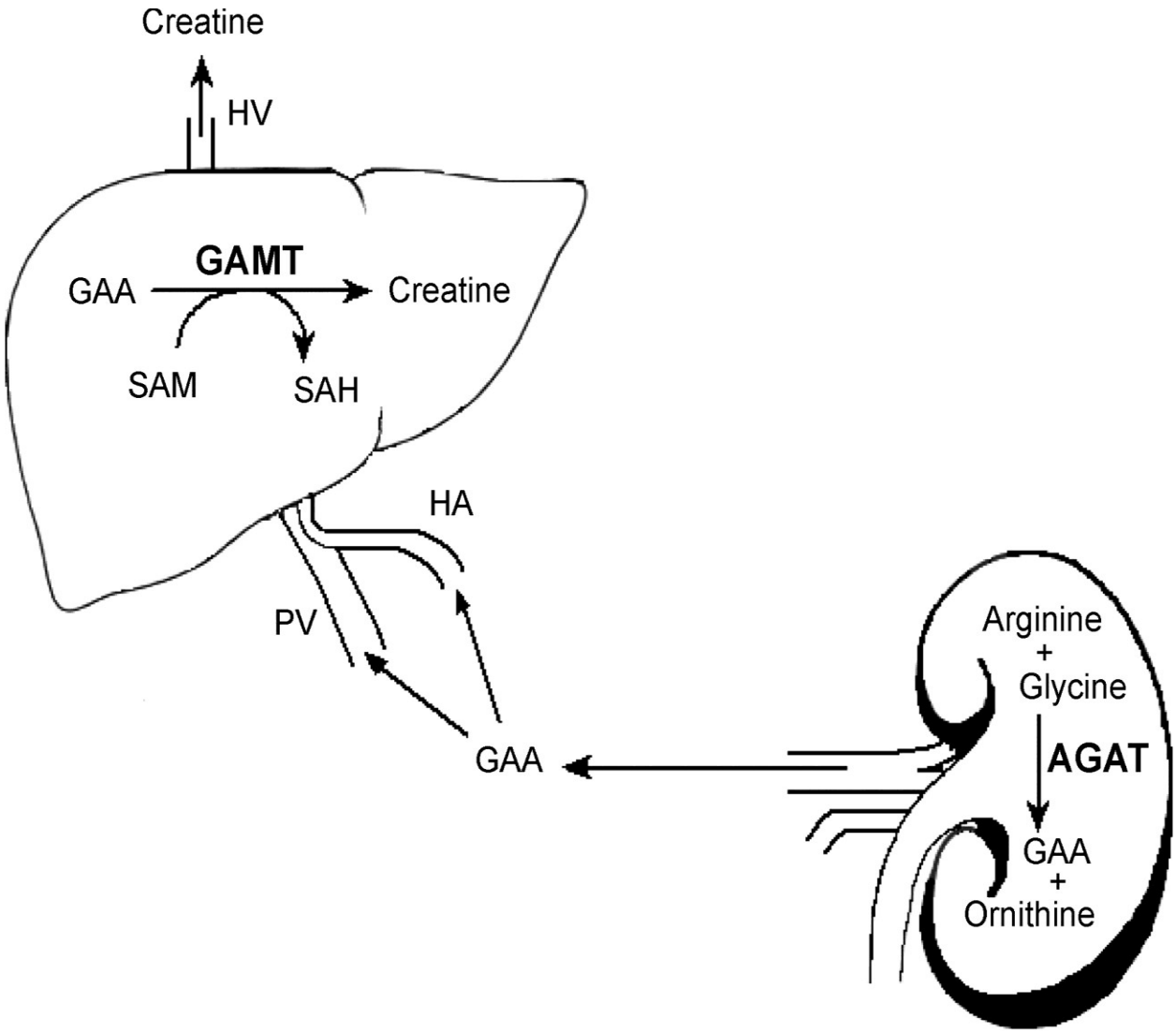

Figure 1.2: Creatine biosynthesis pathway

Abbreviations, AGAT (EC 2.1. 4.1): L-arginine: glycine amidinotransferase, GAA: guanidinoacetic acid, GAMT (EC 2.1. 1.2): 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. (Da Silva et al., 2009

The process of creatine synthesis involves metabolic interactions between different organs (Wyss and Kaddurah-Daouk, 2000). The primary location for GAA production is the kidneys; although AGAT specific activity is also high in pancreas, our data have suggested that the pancreas does not release significant GAA to the circulation (Dinesh et al., 2020). GAA methylation rates vary across different tissues, with the liver exhibiting the highest level of GAA methylation as indicated by the highest specific activity of the enzyme GAMT compared to other tissues in rats (Da Silva et al., 2009), piglets (Brosnan et al., 2009), and other mammalian species (Wyss and Kaddurah-Daouk, 2000). However, lower but still significant levels of GAMT activity have also been reported in the pancreas, kidney, intestine, brain, and muscle in piglets (Brosnan et al., 2009).

In contrast to other organs, the expressions of AGAT, GAMT, and creatine transporters in the central nervous system (CNS) suggest that the CNS can synthesize its own creatine from GAA (Braissant and Henry, 2008, Hanna-El-Daher and Braissant, 2016). However, the expressions of AGAT, GAMT, and creatine transporters vary among different brain regions. For example, AGAT was detected in all major brain cell types, such as neurons, astrocytes, oligodendrocytes, and microcapillary endothelial cells. In contrast, GAMT is expressed by neurons, astrocytes, and oligodendrocytes, with elevated levels observed in both types of glial cells, but it is not found in microcapillary endothelial cells. Most types of brain cells express any of the above enzymes or transporters, while most brain regions do not have AGAT and GAMT together, suggesting the inability of creatine synthesis within the same brain region (Hanna-El-Daher and Braissant, 2016).

Creatine is transported into cells through an X-linked sodium/chloride-dependent creatine transporter known as CrT/SLC6A8. Although the blood-brain barrier contains CrT, enabling the brain to transport creatine from the circulation, this transportation method appears to have limited efficiency (Braissant et al., 2001; Braissant and Henry 2008). Creatine is crucial for storing and

transmitting high-energy phosphates, such as adenosine triphosphate (ATP), through its reversible conversion into phosphocreatine, facilitated by creatine kinases. Creatine may also have a role in neurotransmission. Tissues with substantial energy requirements, such as skeletal muscles, cardiac muscles, and brain tissue, store larger reservoirs of creatine and phosphocreatine (Wyss and Kkaddurah-daouk, 2000). The skeletal muscle contains around 95% of the human body's creatine reserves, and the remaining 5% is distributed primarily in the brain, liver, kidneys, and testes (McCall and Persky, 2007).

Creatine and phosphocreatine within cells are non-enzymatically transformed into creatinine, which is then excreted in the urine. Daily creatinine excretion is directly related to the body's overall creatine content. The average total creatine storage in the adult human body is approximately 120 g, and the daily loss of creatine is estimated to be around 1.7% of the overall body pool (Brosnan et al., 2011). Besides meeting the basic maintenance needs, rapidly developing neonates must also accumulate creatine as their lean tissue stores expand. An evaluation of piglets indicated that the amount of creatine required by growing neonates exceeds what sow milk provides. As a result, suckling piglets depend on endogenous creatine synthesis to fulfill approximately 75% of their daily creatine needs (Brosnan et al., 2009).

An average adult individual requires approximately 2 g of creatine daily to counterbalance the daily creatine loss. Half of this amount (1 g) is synthesized internally, while the remaining half is typically obtained through consumption of a mixed diet (Brosnan and Brosnan, 2007). Recent developments in creatine nutrition and physiology indicate that the amount of creatine naturally produced by the body may not be adequate to fulfill total whole body requirements in humans. Consequently, obtaining sufficient creatine from the diet becomes necessary, making creatine an

essential nutrient under specific circumstances, such as during intense physical training, aging, or certain medical conditions (Ostojic et al., 2022).

The impact of variable creatine supply on in vivo transmethylation partitioning to creatine synthesis is still not fully understood. This regulation of partitioning is especially important because newborns fed creatine-rich cow's milk-based formula only need to synthesize approximately 65% of their creatine requirements, compared to breastfed infants, who are predicted to synthesize about 90% of their creatine requirements (Edison et al., 2013). Based on the available information, it is assumed that creatine synthesis in the body adapts during development, depending on the availability of dietary creatine. Increased creatine demands can be met through dietary methyl donors or by diverting endogenous methyl groups to creatine synthesis, sacrificing other transmethylation reactions. However, whether the body prioritizes creatine synthesis over other transmethylation reactions is currently unknown.

1.1.2.2 Phosphatidylcholine (PC) synthesis

Phosphatidylcholine is another transmethylated product that represents the primary phospholipid constituent in all categories of plasma lipoproteins. It is the sole phospholipid presently recognized as essential for forming and releasing lipoproteins (Cole et al., 2012). The liver serves as a key location for both the production of PC and the creation of plasma lipoproteins (Cole et al., 2012). In mammals, there are two pathways for de novo synthesis of PC. The primary pathway, found in all nucleated cells, is the CDP-choline pathway, also known as the "Kennedy" pathway (Cole et al., 2012). PC can also be produced endogenously through an alternative pathway, which involves three consecutive methylations of phosphatidylethanolamine (PE) by the enzyme phosphatidylethanolamine N-methyltransferase (PEMT). The PEMT pathway is primarily located in the liver, accounting for around 30% of the overall hepatic PC synthesis in human adults (Li

and Vance, 2008). Researchers have proposed that PEMT serves as an evolutionary mechanism to overcome the immediate shortage of choline. In order to produce one mole of PC, PEMT requires three moles of SAM. Consequently, the methyl demand of the PEMT pathway is considered to be quantitatively higher than that of creatine and potentially all other transmethylation reactions, as indicated by studies conducted by McBreairty et al. (2013) in pigs and Stead et al. (2006) in rats.

Under normal conditions, both the PEMT pathway and the Kennedy pathway cooperate to meet the requirements for PC synthesis. They work together to ensure an adequate supply of PC. Walkey et al. (1998) have shown that mice lacking the PEMT gene can still grow at a normal rate when provided with sufficient choline. However, if these mice are fed a choline-deficient diet, it was lethal. Moreover, despite the provision of dietary choline, PEMT knockout mice exhibited hepatosteatosis and lower concentrations of PC in the liver (Zhu et al., 2003). This suggests that the PEMT pathway plays a crucial role in maintaining liver health and adequate PC levels, even when choline is available through the diet. According to Vance et al. (2007), the Kennedy pathway is capable of supplying only 70% of the total PC demands. As a result, the PEMT pathway plays a substantial role in meeting the remaining transmethylation requirement for PC synthesis under all circumstances.

1.1.2.3 DNA methylation

DNA methylation is a process that involves adding a methyl group to cytosine bases at the carbon-5 position in cytosine-guanine (CpG) dinucleotide residues, which modifies DNA and affects gene expression. This addition of methyl groups has been demonstrated to reduce gene expression levels and inhibit protein-coding (Bokor et al., 2022). Methyl group donors in the diet, such as folate, folic acid, betaine, choline, and methionine, contribute to one-carbon metabolism at various points in the methionine cycle, ultimately being transformed into the common methyl group donor SAM.

SAM supplies a methyl group for all DNA methylation processes (Anderson et al., 2012), so can compete with creatine and PC synthesis for limited methyl group supply.

Methyl group contributors play a crucial role in DNA methylation and have been demonstrated to have a significant impact on fetal growth and later life health. The availability of methyl group donors from the maternal source could potentially impact the health of offspring by inducing changes in DNA methylation, serving as a significant connection between early environmental exposures and the risk of developing diseases in the offspring (Bokor et al., 2022). Epigenetic alterations, including DNA methylation, are among the primary mechanisms crucial in health programming (McGee and Bainbridge, 2018). The availability of maternal methyl group donors may be linked to fetal growth and development, as well as various perinatal outcomes like low birth weight, neural tube defects, congenital heart defects, cleft lip and palate deformities, impaired brain development, as well as the development of obesity and related non-communicable diseases later in life (Bokor et al., 2022).

A diet lacking in methyl group donors like methionine, can impact DNA methylation and liver lipid metabolism. Additionally, methionine influences other epigenetic processes, including microRNAs (Aissa et al., 2022). The available evidence from previous studies suggests that reductions in dietary intake of the methyl donors methionine, choline, and folate can lead to a decrease in DNA methylation (Burdge et al., 2009; Cordero et al., 2013; Kotsopoulos et al., 2008; Niculescu et al., 2002). Based on this information, it is plausible to conclude that the availability of dietary methionine and other methyl donors impact how methyl groups are distributed on DNA.

1.1.3 **Methionine metabolism in the gut**

The gastrointestinal tract plays a fundamental role in the metabolism of sulfur-containing amino acids in the body. The small intestine is a metabolically significant site where these amino acids are processed and utilized (Riedijk et al., 2007). Methionine and cysteine are essential for the gut to continue performing all of its essential tasks, such as nutrient digestion, absorption, and metabolism, immunological surveillance of the intestinal epithelial layer, and control of the mucosal response to foreign antigens (Fang et al., 2010). According to research on neonatal pigs, about 20% of the dietary intake of methionine is metabolized in the gastrointestinal tract. This metabolism involves the transmethylation of methionine to homocysteine and the transsulfuration of methionine to cysteine (Riedijk et al., 2007). Additionally, the gastrointestinal tract is responsible for a net release of homocysteine into the bloodstream (Riedijk et al., 2007). In piglets, the net portal balance of methionine accounts for 48% of the intake, suggesting that half of dietary methionine is utilized by the intestine (Stoll et al., 1999).

While methionine breakdown in pig enterocytes is minimal, it undergoes significant catabolism in other cells of the portal-drained viscera and intestinal mucosa (Blachier et al., 2007). Research studies have indicated that the methionine requirement of enterally-fed piglets is ~30% higher than that of parenterally-fed piglets. This finding underscores the significant metabolic demand of the gut, which is atrophied during parenteral feeding, and which metabolizes a substantial portion of dietary methionine through transmethylation and transsulfuration (Shoveller et al., 2003a). Glutathione, homocysteine, and taurine are the main end products of methionine and cysteine metabolism and are crucial for the gut immune response (Wang et al., 2009). Moreover, it is well established that the digestion of proteins and metabolism of amino acids are influenced by the

presence of beneficial microorganisms in the gut (Martínez et al., 2017), which is likely altered during parenteral feeding and other dietary perturbations.

1.1.4 **Methionine deficiency and toxicity**

Insufficient consumption of methionine not only hinders growth, but also affects the sulfur metabolic pathways that are necessary for producing important metabolic intermediates. On the other hand, when provided in excess, methionine is recognized as the most toxic amino acid for animals (Huang et al., 2012; Harper et al., 1970).

Methionine deficiency can have significant consequences on various physiological processes within the body. Insufficient levels of methionine can impair protein synthesis, leading to reduced tissue growth and impaired wound healing. Furthermore, inadequate methionine can lead to oxidative stress and compromised antioxidant defense mechanisms, affecting cellular health (Wu et al., 2004; Elshorbagy et al., 2012).

In newborn animals, a deficiency of methionine inhibits the growth of epithelial cells by reducing intestinal methionine cycle function (Bauchart-Thevret et al., 2009b). The scarcity of sulfur amino acids reduces goblet cells and decreases glutathione content in the small intestine (Bauchart-Thevret et al., 2009b). Maintaining sufficient methionine levels is crucial for proper physiological function and overall health, as a deficiency of methionine leads to a decrease in the proportional weight of lymphoid organs, negatively impacting growth in chickens (Carew et al., 2003).

High methionine may cause cholestatic changes in animal livers similar to those seen in human neonates fed a parenteral nutrition (PN) diet (Moss 1999). Infants who consumed a formula fortified with methionine at a level of 788 $mg \cdot L^{-1}$ or a high-protein formula providing 9 g $protein \cdot kg^{-1} \cdot d^{-1}$ were observed to have elevated levels of methionine and homocysteine (Harper

et al., 1970; Snyderman et al., 1968). Elevated homocysteine levels have been associated with an increased risk of cardiovascular disease and impaired cognitive functions (Selhub, 1999; Boushey et al., 1995). In severe cases, excessive levels of methionine may result in cerebral edema (Harper et al., 1970) and may also negatively affect growth. Elevated levels of methionine and methionine sulfoxide (MetO) in the bloodstream can gradually increase the glomerular filtration rate, which may have detrimental effects on kidney function. These findings allude to the detrimental impacts of hypermethioninemia in hepatic and renal diseases (Sandrielly et al., 2017).

1.1.5 **Homocysteine**

Homocysteine is a sulfur-containing amino acid found in blood and tissues but not utilized in protein synthesis (Bauchart-Thevret et al., 2009a). Increased plasma levels of homocysteine, a crucial by-product of methionine metabolism via the transmethylation route, have been strongly related to cardiovascular disease in adults (Hogeveen et al., 2002; Fang et al., 2010). Plasma homocysteine concentrations are directly affected by dietary methionine deficit or excess (Fang et al., 2010). Furthermore, research has demonstrated a correlation between elevated levels of homocysteine and an increased likelihood of neonatal stroke (Hogeveen et al., 2002). Reducing plasma homocysteine levels by supplementing methyl donors is also a key strategy to reduce the risk for stroke (Spence and Hankey, 2017). Because methionine supplementation increases homocysteine concentrations linearly (Shoveller et al., 2004), supplementation regimes should avoid direct methionine supplementation to avoid increasing homocysteine and risk for cardiovascular events.

1.2 Neonatal methionine requirements

Studies indicate that unlike most amino acids, methionine is predominantly utilized for non-protein pathways rather than for protein synthesis. Therefore, it is crucial to determine the true methionine

requirement for infant nutrition to optimize all metabolic functions including growth, as both inadequate and excessive methionine intake can have harmful impacts.

Consuming cysteine through the diet can fulfill a portion of the requirement for sulfur amino acids, reducing the amount of methionine needed in the diet. This is thought to be due to the suppression of the conversion of methionine to cystathionine by dietary cysteine intake (Finkelstein et al., 1988). When excess dietary cysteine is present, the average methionine requirement for the population of healthy, school-aged children was 5.8 $mg \cdot kg^{-1} \cdot d^{-1}$, and the minimum safe requirement (i.e. upper 95% confidence interval) was 7.3 $mg \cdot kg^{-1} \cdot d^{-1}$ (Humayun et al., 2006). In contrast, when dietary cysteine was absent, the average methionine requirement was 12.9 $mg \cdot kg^{-1} \cdot d^{-1}$, and the safe requirement was 17.2 $mg \cdot kg^{-1} \cdot d^{-1}$. These findings suggest that dietary cysteine spares methionine requirements, with a 55% to 58% reduction for average and safe requirements, respectively (Humayun et al., 2006).

The indicator amino acid oxidation technique has been used to determine the methionine requirement of piglets (Shoveller et al., 2003a). This method quantifies the amount of methionine intake needed to optimize whole body protein synthesis. When methionine intake is deficient, other amino acids (including the indicator amino acid) is oxidized at a high rate. When methionine intake is at requirement, protein synthesis is maximized, and indicator amino acid oxidation is low and constant, as higher intakes of methionine cannot change protein synthesis. In a study using the indicator method, piglets fed parenteral nutrition required less methionine (0.26 $g \cdot kg^{-1} \cdot d^{-1}$) than those fed intragastrically (0.44 $g \cdot kg^{-1} \cdot d^{-1}$) (Shoveller et al., 2003b). This difference indicates that first pass metabolism by the gastrointestinal tracts utilizes around 30% of the dietary methionine in healthy piglets fed enterally. The above methionine requirements for piglets was determined by using a cysteine-free diet. When excess cysteine was added to the diet, the methionine

requirements for both parenteral nutrition (0.18 $g \cdot kg^{-1} \cdot d^{-1}$) and enteral feeding (0.25 $g \cdot kg^{-1} \cdot d^{-1}$) were lower (Shoveller et al., 2003a). These data suggest that an adequate dietary cysteine intake can spare approximately 40% of the methionine requirement in growing piglets (Shoveller et al., 2003a).

Breastfed infants require an estimated intake of ~28 $mg \cdot kg^{-1} \cdot d^{-1}$ of methionine (WHO 2007). As breast milk is deemed the best source of nourishment for infants below the age of six months, the joint expert consultation of World Health Organization/ Food and Agriculture Organization/ United Nations University (WHO/FAO/UNU) recommended a daily methionine intake for infants based on the average methionine intake of breastfed infants (Huang et al., 2012, WHO 2007). However, when determining enteral requirements from infant formula products, the estimated requirement for methionine is much higher, ranging from approximately 38-48 $mg \cdot kg^{-1} \cdot d^{-1}$ (Huang et al., 2012). The difference in the estimated methionine requirement for breastfed infants versus those fed with formula may be attributed to the unique composition of human milk, which contains a different combination of methyl donors/acceptors, such as choline, betaine, creatine, folate, and cysteine. These components play crucial roles in methionine metabolism and are present in different concentrations in human milk compared to formula products, which may account for the variation in estimated methionine requirements (Elango, 2020). Just as cysteine in the diet can reduce the requirement for methionine, several important methyl nutrients and 1-carbon metabolites have been proposed to similarly affect the availability of methionine (Robinson et al., 2016). During periods of active growth, the demands for transmethylation products (e.g. creatine, PC) and for protein synthesis are high, requiring significant amounts of methionine. However, a portion of this demand can be met through remethylation, where the body recycles methionine from homocysteine to produce additional transmethylation products and protein (Elango, 2020).

Therefore, when determining the methionine requirement, it is essential to consider that methionine metabolism serves protein synthesis, transmethylation, and transsulfuration processes. Furthermore, the availability of precursors or products involved in transmethylation and transsulfuration pathways can affect their overall functioning. Thus, the requirement for methionine must consider the interplay between these different metabolic pathways and the availability of their respective precursors or products (Bertolo et al., 2013).

1.3 Breast milk amino acid (AA) composition

During the initial months of an infant's life, rapid growth and significant physiological development take place. As a result, it is crucial to ensure an adequate supply of energy and essential nutrients, including amino acids and methyl donors/acceptors. Sufficient quantities and quality of these nutrients are necessary for tissue synthesis and to support the necessary morphological and physiological changes during this critical period of infancy (Bosch et al., 2006). Although breast milk is the ideal nutrition during this stage of development, even breast milk composition is variable and influenced by various factors, such as the stage of lactation, the genetic background and dietary habits of the mothers, the gestational age at delivery, and the geographical location (Stam et al., 2013). For example, maternal choline status can influence the choline levels in breast milk, subsequently impacting infants' choline status. Additionally, diets deficient in choline can affect the activity of PEMT and thereby methionine cycle metabolism (Caudill et al., 2010; Bertolo et al., 2013).

As an infant grows, their body weight, body composition, growth rate, and volume of milk intake tend to change. Consequently, the nutrient requirements of infants, including the protein and individual amino acid composition, also vary across different stages of lactation. The majority of amino acids present in breast milk are incorporated into milk proteins. However, there is also a

small proportion of free amino acids (FAA), typically making up less than 10% of the total amino acid levels (Garcia-Rodenas et al., 2016). The primary differences between human and bovine milk appear to be higher levels of cysteine and tryptophan, and lower methionine concentrations in human milk (Davis et al., 1994).

Most neonates meet their amino acid requirements through proteins obtained from the breast milk of nursing mothers or infant formulas (Viadel et al., 2000). According to the WHO, it is advised that infants should be given only breast milk for the initial six months after birth (Martin et al., 2016). In certain circumstances, breastfeeding may not be feasible, appropriate, or sufficient, requiring a pause or discontinuation of breastfeeding. Factors such as maternal health problems, inadequate milk supply, and specific medications can contribute to this decision.

1.4 Formula AA composition

The composition of human milk serves as the benchmark for creating infant formulas in the manufacturing process. While it is not possible to create a replica of breast milk, significant efforts have been made to replicate the nutritional composition of human breast milk to support healthy growth and development in infants ((Martin et al., 2016). Unfortunately, there are instances where infant formulas become necessary to provide the essential nutrients needed by infants when human milk is not available or is insufficient (Bosch et al., 2006). Commercial infant formulas are typically formulated to have comparable levels of major and minor nutrients. The protein sources commonly used in infant formulas include modified cow's milk, casein or whey hydrolysates, or soy protein.

Among milk-based formulas, there are two subtypes distinguished by the main protein fraction, casein and whey proteins. The protein content is carefully adjusted to approximate human milk's

essential amino acid composition regardless of the formula type. This ensures that the formulas provide a similar protein profile to human milk. Cow's milk has a casein-to-whey protein ratio of 82:18, whereas human milk has a ratio of 20:80 (Liao et al., 2017). Variances in the protein composition can lead to slight differences in the amino acid profiles between infant formulas and human milk.

Additionally, a casein-based formula contains a significantly higher amount of methionine and lower cysteine levels than a whey-based formula or breast milk (Bertolo et al., 2013). Soy-based formulas address the lower levels of specific amino acids (methionine/cysteine, tryptophan, threonine, and lysine) with higher protein content, typically ranging from 1.9 to 2.2 g of protein·100 mL^{-1}. In contrast, cow milk-based infant formulas generally contain 1.2 to 1.9 g of protein·100 mL^{-1}. This compensatory adjustment in protein content helps ensure an adequate supply of essential amino acids in soy-based formulas (Bosch et al., 2006). In the early stages of life, the composition of the infant diet will also impact the specific needs of methionine cycle pathways. For example, a diet devoid of creatine, such as a soy-based formula or a vegan diet, necessitates synthesizing the complete amount of creatine needed for maintaining and promoting growth from dietary amino acids, potentially diverting methionine from transmethylation pathways. Ultimately, the functioning of these pathways relies on the availability of methyl donors (methionine, betaine, choline, folate), which are obtained from both dietary sources and de novo synthesis (Bertolo et al., 2013). Moreover, bovine milk-based and soy-based formulas contain lower levels of choline than breast milk (Holmes-McNary et al., 1996; Zeisel et al., 1986). If PC synthesis from dietary choline is limited, then more methyl groups must be used to synthesize PC via the PEMT pathway. When one or more of these methyl nutrients are deficient, the remaining nutrients must compensate to ensure an adequate supply. Moreover, it is crucial to consider the

activity of these pathways when determining the requirement for methionine, which contributes to protein synthesis and cysteine production as well as transmethylation pathways. This consideration should also include the abundance of precursors necessary for remethylation back to methionine (Bertolo et al., 2013).

1.5 Creatine supplementation

Creatine is a popular supplement among athletes and in the commercial animal industry. The use of creatine as a performance-enhancing supplement has been extensively studied, and results consistently demonstrate the positive effects of creatine supplementation on energy metabolism, body composition, muscle strength, and exercise capacity (Wyss and Kaddurah-Daouk, 2000; Buford et al., 2007; Rae et al., 2003; Volek et al., 1997). Moreover, creatine supplementation has been shown to promote muscle hypertrophy and increase lean body mass (Kreider et al., 2003). These positive effects can be attributed to creatine's role in ATP regeneration, the primary energy source for muscle contractions. By increasing muscle creatine stores, supplementation allows for greater ATP availability during intense exercise, leading to improved performance and enhanced muscular adaptations (Branch, 2003; Casey et al., 1996). However, because creatine is relatively unstable in food sources (Baker, 2009), researchers have investigated guanidinoacetic acid (GAA) as a potential alternative to creatine supplements.

1.6 Guanidinoacetic acid (GAA)

1.6.1 GAA biosynthesis and excretion

GAA, also called glycocyamine or guanidinoacetate, is a substance found naturally in the tissues of the body (Mori et al., 1996). The endogenous synthesis of GAA involves two amino acids, arginine, and glycine. Through the action of AGAT, the amidino group of arginine is transferred

to glycine, producing ornithine and GAA. Following this, GAA undergoes methylation by incorporating a methyl group from SAM, which comes from methionine (Wyss and Kaddurah-Daouk, 2000). GAA synthesis predominantly occurs in the kidney and GAA methylation primarily occurs in the liver (Wyss and Kaddurah-Daouk, 2000).

The production of GAA appears varies among different species. For instance, while GAA synthesis takes place in the kidneys of rats, pigs and humans, the capacity for GAA production differs among them (Edison et al., 2007). In rats and piglets, the kidney is considered the primary site for GAA production, whereas, in humans, the kidney contributes only 20% of GAA production (Edison et al., 2007). Regarding enzyme distribution in tissues, rats show the highest AGAT activity in their kidneys (Da Silva et al., 2009), while piglets exhibit considerable AGAT activity in the kidney and the pancreas (Brosnan et al., 2009). However, recent research conducted by our laboratory indicates that although piglets have higher pancreatic AGAT specific activity, pancreatic GAA does not significantly contribute to GAA production for the rest of the body (Dinesh et al., 2020). The study revealed that more than 80% of the total GAA released in the entire body originates from the kidney, suggesting that the kidney is the most important quantitative source of GAA for creatine synthesis in piglets (Dinesh et al., 2020). Additionally, Dinesh et al. (2020) discovered a net release of GAA from the piglet gut, indicating that intestinal cells also possess a significant capacity for GAA synthesis, despite having a modest specific activity of AGAT. However, there is a lack of comparable data on other species regarding these findings.

The synthesis of GAA is controlled by a negative feedback mechanism that involves the regulation of AGAT by concentrations of creatine and ornithine (Wyss and Kaddurah-Daouk, 2000). For example, the administration of creatine supplements can inhibit AGAT activity and mRNA

expression in rat kidneys, resulting in the downregulation of GAA synthesis (Edison et al., 2007). In piglets, creatine supplementation also leads to reduced AGAT activity in both the kidneys and pancreas, while GAA supplements do not have the same effect (Dinesh et al., 2021). On the other hand, there is no evidence of similar feedback regulation on the activity of GAMT. However, GAMT activity appears to be induced when the precursors GAA and methionine are provided in the diet (Dinesh et al., 2021). Moreover, the availability of precursors for these enzymes may also influence the flux through them, as demonstrated by the regulation of a key creatine transporter (SLC6A8) by extracellular creatine concentration (Loike et al., 1988; Wyss and Kaddurah-Daouk, 2000).

The primary route of GAA disposal involves its conversion into creatine. However, GAA can also be directly excreted by the kidneys, alongside the major losses through creatine and creatinine synthesis. Tossenberger et al. (2016) demonstrated that broiler chickens possess an effective mechanism to compensate for excessive GAA intake; this mechanism involves increasing the conversion of GAA into creatine and creatinine, which are then excreted, along with any excess GAA, through urine. These findings indicate that the excretion of GAA can rapidly increase in response to higher dietary GAA levels. Indeed, the literature currently provides limited information regarding the specific pathways and mechanisms of GAA excretion. Further studies are necessary to investigate the effects of GAA supplementation on GAA excretion pathways, elucidate the factors influencing GAA excretion rates, and provide a more detailed understanding of the dynamics of GAA elimination from the body.

1.6.2 **GAA transportation**

After GAA is synthesized, it must be transported to its target cells for further metabolism. GAA transport relies on various types of transporters, and their distribution throughout the body varies.

Although a complete description of GAA transport mechanisms is lacking, some potential transporter candidates have been proposed. These include creatine transporters (CRT/Solute carrier SLC6A8), taurine transporters (TauT/SLC6A6), γ-aminobutyric acid (GABA) transporter (SLC6 A13), and passive diffusion through the cell membrane (Tachikawa et al., 2009). For example, in rat livers, hepatic uptake of GAA appears to predominantly occur through the GABA transporter (Tachikawa et al., 2012). While GAA can be transported via creatine transporters, it seems to have a lower affinity than creatine (Tachikawa et al., 2009). Consequently, supplementing GAA along with creatine may saturate the creatine transporters, leading to increased GAA transport through other transporters, thereby synergistically enhancing cellular concentrations of both GAA and creatine (Ostojic, 2017). However, further research is required to examine the interactions between GAA and creatine during transport in various tissues and verify the transporters involved.

1.6.3 **GAA supplementation in animal industry**

The potential therapeutic applications of GAA have been under investigation since the early 1950s (Borsook and Borsook, 1951). However, the interest in GAA supplementation increased significantly in the poultry industry following Europe's ban in 2001on meat and bone meal, the main source of creatine prior to the ban. The prohibition of animal by-products, which served as a source of creatine, resulted in reduced performance in poultry (Córdova-Noboa et al., 2018a). Furthermore, most plant-based growing-finishing pig diets in Europe lack sufficient levels of creatine (He et al., 2018). Consequently, GAA supplementation has been explored as an alternative approach to enhance growth performance in the poultry and swine industry, addressing the absence of dietary creatine. In livestock, creatine supplements have been utilized to enhance growth performance, primarily due to their involvement in muscle metabolism. However, the high cost of

supplemental creatine has prompted the testing of GAA in animal diets, particularly during the later stages of growth when feed consumption is highest (He et al., 2018). In addition to cost considerations, creatine supplements have other drawbacks, such as instability during manufacturing (Baker, 2009) and relatively low bioavailability (Alraddadi et al., 2018). On the other hand, GAA supplementation has been recognized as a stable feed additive for animals by the European Food Safety Authority (2009; 2022). Furthermore, a recent study investigating the stability of GAA in canine food during manufacturing and storage (van der Poel et al., 2019) found that granulated and crystallized GAA exhibited high stability compared to added creatine. GAA supplements have demonstrated twice the solubility of creatine supplements and are 40% less costly (Ostojic and Vojvodic-Ostojic, 2015). Therefore, as the only natural precursor for creatine, GAA represents a potentially safe and beneficial alternative to creatine supplementation.

GAA has been investigated as a potential supplement in animals to improve various aspects of growth performance, including feed conversion ratio (FCR; used in animal research to assess the efficiency with which animals convert feed into body weight), meat yield and quality, and reproductive performance. While GAA is primarily known as a precursor for creatine, its supplementation can also impact other metabolic pathways. For example, GAA can directly influence endocrine functions, neuromodulation, and oxidant-antioxidant processes. However, the main mechanism of action for GAA is its conversion into creatine, which effectively increases creatine stores in the body. Numerous studies have demonstrated the effectiveness of GAA supplementation in enhancing creatine concentrations in muscles (Liu et al., 2011), liver, kidney, and plasma (McBreairty et al., 2013; Tossenberger et al., 2016), resulting in improved growth and performance outcomes.

1.6.3.1 Effects of GAA supplementation on growth performance

The primary goal of the commercial animal sector is to optimize growth performance while keeping costs low and minimizing environmental impact. Consequently, there is a strong emphasis on maximizing feed efficiency to prevent unnecessary expenses and minimize environmental pollution from overfeeding nutrients.

Supplementing GAA in broiler diets has shown promise in improving both growth performance and gastrointestinal health (Ahmadipour et al., 2018). Recent studies have highlighted the positive effects of GAA supplementation on broiler growth performance (Zhao et al., 2021). With the adoption of pure plant-based diets in broilers, a decline in performance has been observed. To address this issue, GAA has been proposed as a potential supplement to enhance broiler performance (Ringel et al., 2007). The supplementation of GAA in all-plant diets during the finishing period has been shown to improve feed-to-gain ratios and overall performance in broilers (Michiels et al., 2012; Córdova-Noboa et al., 2018b). Furthermore, growing-finishing pigs supplemented with GAA improved average daily gain and feed-to-gain ratio as the dietary GAA dosage increased. Among these studies, a GAA dosage of 0.3 $g \cdot kg^{-1}$ was identified as the optimal concentration for maximizing growth performance by enhancing muscle creatine and ATP concentrations in pigs (He et al., 2018).

1.6.3.2 Effects of GAA supplementation on meat quality

GAA supplements have drawn attention as potential feed additives for improving meat quality in the animal industry due to their involvement in muscle metabolism. Several studies have investigated the effects of GAA supplementation on various meat-related parameters, such as pH, meat yield, and carcass traits in poultry and swine. For example, in broiler chickens, dietary supplementation of GAA at a concentration of 1.8 $g \cdot kg^{-1}$ of diet increased meat pH, contributing

to improved meat quality (Mohebbifar et al., 2019). Additionally, GAA supplementation in the diet of broilers resulted in increased creatine concentrations in breast meat, leading to enhanced energy provision for muscle performance and growth (Michiels et al., 2012). These findings suggest that GAA supplementation has the potential to positively influence meat quality by affecting meat pH and increasing creatine levels in animal muscle. Limited research has been conducted on the effects of dietary GAA on carcass traits in swine. However, the available studies indicate that incorporating GAA into the diet improves hot carcass weight, carcass length, and lean percentage in pigs. These findings suggest that GAA could be a promising feed additive for enhancing carcass quality in swine (He et al., 2018).

1.6.3.3 GAA supplementation and reproduction

As a precursor for creatine, GAA has shown the potential to improve energy metabolism in the reproductive system. Research indicates that dietary supplementation of GAA can positively affect reproductive performance in poultry and swine. In broiler breeder hens, adding GAA to the diet has improved fertility rates and sperm penetration, particularly in aged hens (Sharideh et al., 2016). This improvement in reproductive performance may be attributed to the increased availability of ATP in sperm mitochondria, leading to enhanced sperm motility and ultimately increasing fertility rates. These findings suggest that GAA supplementation has the potential to positively impact reproductive outcomes in poultry and swine by enhancing energy metabolism in the reproductive system. Moreover, feeding GAA to swine during gestation and lactation has been found to enhance the performance of multiparous sows and gilts, as well as improve production, and the growth performance of litters (Panisson et al., 2019). On the other hand, Mendonca et al. (2019) reported that supplementing sows and their offspring with 1 g GAA per kg of feed did not have any notable

effects on growth performance during the nursery phase. However, further studies are required to confirm the efficacy of GAA as a feed additive for improving reproductive parameters in swine.

1.6.3.4 GAA supplementation and brain functions

The role of GAA in brain metabolism has attracted attention due to the well-established role of creatine in brain function. Including GAA in the diet can significantly affect the concentrations of creatine and GAA in brain tissue. The supplementation of GAA may increase brain creatine concentration, potentially enhancing energy metabolism in the brain. Studies have investigated whether GAA supplementation impacts cognitive function, particularly in swine. For example, in Yucatan miniature pigs, daily supplementation of GAA at a dose of 157 $mg \cdot kg^{-1}$ body weight for two weeks did not improve memory performance. No relationship was found between GAA and spatial memory in these pigs (Robinson et al., 2020). However, GAA supplementation did increase the concentration of GAA in the cerebellum while simultaneously increasing creatine concentration and decreasing GAA concentration in the prefrontal cortex (PFC) of these pigs. These findings suggest that while GAA supplementation may alter GAA and creatine concentrations in specific brain regions, its direct impact on cognitive function may be limited, at least based on the study conducted in Yucatan miniature pigs.

1.6.4 **GAA and arginine metabolism**

The effects of dietary GAA supplementation may be achieved by preserving arginine for important health and growth-related functions, regardless of its involvement in creatine synthesis. Arginine is crucial for poultry and essential for neonatal pigs under certain conditions. Numerous studies have indicated that arginine enhances functional health and promotes growth in various animal species (Wu et al., 2009; Wu et al., 2016; Yu et al., 2018). Additionally, a significant amount of arginine is utilized in the synthesis of creatine, with approximately 20% of dietary arginine intake

being allocated for GAA synthesis in suckling piglets, despite the presence of considerable amounts of creatine in milk (Brosnan et al., 2009).

In the poultry industry, arginine has been used as a supplement to improve overall performance (Yu et al., 2018). Several studies have demonstrated that GAA can serve as an arginine-saving agent in animals. For example, broiler chickens fed an arginine-deficient diet along with supplemental GAA (1.2 $g \cdot kg^{-1}$) exhibited enhanced growth, feed efficiency, and overall performance, indicating that GAA supplementation could potentially replace arginine in the diet of young chicks (Dilger et al., 2013).

However, in the case of piglets, it was found that GAA supplementation did not conserve arginine for whole-body protein synthesis, even though the diets provided insufficient arginine (Dinesh et al., 2021). Currently, there is a lack of studies conducted on pigs regarding the sparing effect of GAA on arginine, although the concept seems plausible considering arginine's beneficial impact on protein synthesis, nitric oxide production, and the release of growth hormone (Ostojic, 2015). Future research should focus on investigating changes in the activity of the AGAT enzyme in a dose-dependent manner to understand better the arginine-sparing effects of GAA (DeGroot et al., 2019).

1.6.5 **GAA and methionine metabolism**

The process of converting GAA to creatine in adult humans has been estimated to require approximately 70% of labile methyl groups, making it the transmethylation reaction with the highest demand (Stead et al., 2006). Moreover, due to the lack of feedback regulation in the enzyme GAMT, supplemental GAA leads to a proportional increase in the utilization of methyl groups from SAM to produce more creatine (Da Silva et al., 2009; McBreairty et al., 2013). As a

result, when GAA is supplemented, the increased consumption of methyl groups to synthesize creatine may lead to limited availability of methyl groups, which may negatively impact other crucial methylation reactions that compete for SAM, such as DNA methylation and the conversion of PE to PC. Additionally, the increased usage of methyl groups may reduce the amount of available methionine for protein synthesis. For example, studies conducted on both suckling and weaned Yucatan miniature pigs demonstrated that intraportal infusion (McBreairty et al., 2013) or daily feeding (McBreairty et al., 2015) of GAA significantly enhanced creatine synthesis, while concurrently reducing methionine incorporation into hepatic protein and methyl incorporation into PC.

Methylated DNA is also a product of transmethylation that, although quantitatively minor, plays a critical role in the epigenetic regulation of genes (Randunu and Bertolo, 2020). However, there is limited research investigating the relationship between GAA supplementation and DNA methylation. A study conducted by McBreairty et al. (2015) in young pigs demonstrated that supplementation of GAA for 18-19 days did not result in any changes in the rate of methyl incorporation into DNA, despite a four-fold increase in the utilization of methyl groups for the conversion of supplemented GAA to creatine. In contrast, the diversion of methyl groups to form creatine led to 80% less methyl incorporation to synthesize PC (McBreairty et al., 2015).

Supplemental GAA also notably affects other metabolites and enzymes within the methionine cycle. For example, because GAA is rapidly converted to creatine through transmethylation, its supplementation in rats (at doses of 5 and 10 $g \cdot kg^{-1}$) leads to a significant reduction in hepatic SAM concentration and an increase in SAH and homocysteine concentration, which enhances the activity of CBS, an enzyme responsible for removing homocysteine (Fukada et al., 2006). CBS is

likely induced to cope with the higher concentrations of homocysteine resulting from GAA supplementation.

Interestingly, while GAA supplementation increases the activity of methionine synthase in rats (Stead et al., 2001), the remethylation process through the alternate pathway involving hepatic BHMT is likely reduced due to decreased enzyme activity (Liu et al., 2011). According to Liu et al. (2011), dietary supplementation of GAA in rats decreases hepatic betaine levels due to increased betaine consumption caused by the accelerated methionine cycle. Furthermore, a low concentration of SAM may impede the synthesis of PC through the PEMT pathway, while a higher concentration of SAH may inhibit transmethylation reactions. These effects are likely due to the altered methionine cycle resulting from enhanced GAA methylation to creatine, which generates excess homocysteine (Liu et al., 2011).

Furthermore, insulin may play a role in mediating the changes observed in the methionine cycle due to GAA supplementation. For example, GAA supplementation has been found to increase insulin concentration while decreasing homocysteine concentration (Ostojic, 2014). It is possible that GAA directly stimulates insulin secretion, which in turn may positively impact the activity of glycine N-methyltransferase (GNMT), a key regulatory enzyme in methyl group metabolism (Ostojic, 2014). However, further studies are needed to fully understand the precise mechanisms through which GAA affects the flux of the methionine cycle, subsequently influencing the concentrations of various metabolites.

Despite the close relationship between methionine and GAA metabolism, only a limited number of studies have investigated the effects of dietary methionine and GAA supplementation in poultry and swine. For example, in finishing broilers, the impact of dietary GAA supplementation was found to be influenced by the concentration of dietary methionine, highlighting the importance of

maintaining adequate sulfur amino acid composition in broiler diets when incorporating GAA supplementation (Majdeddin et al., 2019). Additionally, the effects of dietary GAA supplementation in growing broilers were found to be influenced by the overall concentration of sulfur amino acids in the diet, indicating that the composition of sulfur amino acids plays a role in determining the effectiveness of GAA supplementation (Zarghi et al., 2020). However, further research is needed to determine the specific amount of methionine required to enhance the effectiveness of GAA supplementation.

1.7 References

Ahmadipour B, Khajali F, Sharifi MR. Effect of guanidinoacetic acid supplementation on growth performance and gut morphology in broiler chickens. Poult. Sci. J. 2018;6 (1): 19–24.

Aissa AF, Tryndyak VP, de Conti A, Machado AR, Tuttis K, da Silva Machado C, Hernandes LC, da Silva Santos PW, Serpeloni JM, Pogribny IP, Antunes LM. Epigenetic changes induced in mice liver by methionine-supplemented and methionine-deficient diets. Food Chem Toxicol. 2022;163:112938.

Alraddadi EA, Lillico R, Vennerstrom JL, Lakowski TM, Miller DW. Absolute oral bioavailability of creatine monohydrate in rats: debunking a myth. Pharmaceutics. 2018;10(1):31.

Anderson OS, Sant KE, Dolinoy DC. Nutrition and epigenetics: an interplay of dietary methyl donors, one-carbon metabolism and DNA methylation. J Nutr Biochem. 2012;23(8):853–859.

Baker DH. Advances in protein-amino acid nutrition of poultry. Amino Acids 2009; 37:29–41.

Ball RO, Courtney-Martin G, Pencharz PB. The in vivo sparing of methionine by cysteine in sulfur amino acid requirements in animal models and adult humans. J Nutr. 2006;136(6):1682S-1693S.

Bauchart-Thevret C, Stoll B, Burrin DG. Intestinal metabolism of sulfur amino acids. Nutr Res Rev. 2009a;22(2): 175-187.

Bauchart-Thevret C, Stoll B, Chacko S, Burrin DG. Sulfur amino acid deficiency upregulates intestinal methionine cycle activity and suppresses epithelial growth in neonatal pigs. Am J Physiol Endocrinol Metab. 2009b;296(6):E1239-E1250.

Bertolo RF, McBreairty LE. The nutritional burden of methylation reactions. Curr Opin Clin Nutr Metab Care. 2013;16(1):102-108.

Blachier F, Mariotti F, Huneau JF, Tomé D. Effects of amino acid-derived luminal metabolites on the colonic epithelium and physiopathological consequences. Amino acids. 2007;33:547-562.

Bokor S, Vass RA, Funke S, Ertl T, Molnár D. Epigenetic effect of maternal methyl group donor intake on offspring' s health and disease. Life. 2022:1–13.

Borsook ME, Borsook H. Treatment of cardiac decompensation with betaine and glycocyamine. Ann. West. Med. Surg. 1951;5(10):830–855

Bosch L, Alegría A, Farré R. Amino acid contents of infant foods. Int J Food Sci Nutr. 2006;57(3–4): 212–218.

Boushey CJ. A quantitative assessment of plasma homocysteine as a risk factor for vascular disease: probable benefits of increasing folic acid intakes. Jama. 1995;274(13):1049-1057.

Braissant O. and Henry H. AGAT, GAMT and SLC6A8 distribution in the central nervous system, in relation to creatine deficiency syndromes: a review. J. Inherit. Metab. Dis. 2008;31: 230–239

Braissant O, Henry H, Loup M, Eilers B, Bachmann C. Endogenous synthesis and transport of creatine in the rat brain: an in-situ hybridization study. Mol. Brain Res. 2001;86(1-2):193–201

Branch JD. Effect of creatine supplementation on body composition and performance: a meta-analysis. Int J Sport Nutr Exerc Metab. 2003;13(2):198-226.

Brosnan JT, Da Silva RP, Brosnan ME. The metabolic burden of creatine synthesis. Amino acids. 2011;40:1325–1331.

Brosnan JT, Wijekoon EP, Warford-Woolgar L, Trottier NL, Brosnan ME, Brunton JA, Bertolo RF. Creatine synthesis is a major metabolic process in neonatal piglets and has important implications for amino acids metabolism and methyl balance. J Nutr. 2009;139(7): 1292-7.

Brosnan JT, Brosnan ME. The sulfur-containing amino acids: an overview. J Nutr. 2006;136: 1636S–1640S.

Buford TW, Kreider RB, Stout JR, Greenwood M, Campbell B, Spano M, Ziegenfuss T, Lopez H, Landis J, Antonio J. International Society of Sports Nutrition position stand: creatine supplementation and exercise. J Int Soc Sports Nutr. 2007; 4(1):6.

Burdge GC, Lillycrop KA, Phillips ES, Slater-Jefferies JL, Jackson AA, Hanson MA. Folic acid supplementation during the juvenile-pubertal period in rats modifies the phenotype and epigenotype induced by prenatal nutrition. J Nutr. 2009;139:1054–1060.

Carew LB, McMurtry JP, Alster FA. Effects of methionine deficiencies on plasma levels of thyroid hormones, insulin-like growth factors-I and-II, liver and body weights, and feed intake in growing chickens. Poult Sci. 2003;82(12):1932-1938.

Casey A, Constantin-Teodosiu D, Howell S, Hultman EG, Greenhaff PL.Creatine ingestion favorably affects performance and muscle metabolism during maximal exercise in humans. Am J Physiol Endocrinol Metab. 1996;271:E31–E37

Caudill MA. Pre and postnatal health: evidence of increased choline needs. J Am Diet Assoc 2010;110:1198–1206.

Cole LK, Vance JE, Vance DE. Phosphatidylcholine biosynthesis and lipoprotein metabolism. Biochim Biophys Acta Mol Cell Biol Lipids. 2012;1821(5):754-61.

Cordero P, Gomez-Uriz AM, Campion J, Milagro FI, Martinez JA. Dietary supplementation with methyl donors reduces fatty liver and modifies the fatty acid synthase DNA methylation profile in rats fed an obesogenic diet. Genes Nutr. 2013;8:105–113.

Córdova-Noboa HA, Oviedo-Rondón EO, Sarsour AH, Barnes J, Sapcota D, López D, Gross L, Rademacher-Heilshorn M, Braun U. Effect of guanidinoacetic acid supplementation on live performance, meat quality, pectoral myopathies and blood parameters of male broilers fed corn-based diets with or without poultry by-products. Poult Sci 2018a;97(7):2494–2505.

Córdova-Noboa HA, Oviedo-Rondón EO, Sarsour AH, Barnes J, Ferzola P, Rademacher-Heilshorn M, Braun U. Performance, meat quality, and pectoral myopathies of broilers fed either corn or sorghum-based diets supplemented with guanidinoacetic acid. Poult Sci. 2018b; 97(7): 2479–2493.

Courtney-Martin G, Ball RO, Pencharz PB. Sulfur amino acid metabolism and requirements. Nutr Rev. 2012;70(3):170-175.

Da Silva RP, Nissim I, Brosnan ME, Brosnan JT. Creatine synthesis: hepatic metabolism

of guanidinoacetate and creatine in the rat in-vitro and in-vivo. Am J Physiol Endocrinol Metab. 2009;296:E256–61.

Davis TA, Nguyen HV, Garcia-Bravo R, Fiorotto ML, Jackson EM, Lewis DS, Lee DR, Reeds PJ. Amino acid composition of human milk is not unique. J Nutr. 1994;124(7):1126-32.

DeGroot AA, Braun U, Dilger RN. Guanidinoacetic acid is efficacious in improving growth performance and muscle energy homeostasis in broiler chicks fed arginine-deficient or arginine-adequate diets. Poult Sci. 2019 ;98 (7) :2896–2905.

Dilger RN, Bryant-Angeloni K, Payne RL, Lemme A, Parsons CM. Dietary guanidino acetic acid is an efficacious replacement for arginine for young chicks. Poult Sci 2013 ;92 (1):171–177.

Dinesh OC, Brunton JA, Bertolo RF. The kidneys are quantitatively more important than pancreas and gut as a source of guanidinoacetic acid for hepatic creatine synthesis in sow-reared Yucatan miniature piglets. J Nutr 2020;150: 443-9.

Dinesh OC, Kankayaliyan T, Rademacher M, Tomlinson C, Bertolo RF, Brunton JA. Neonatal piglets can synthesize adequate creatine, but only with sufficient dietary arginine and methionine, or with guanidinoacetate and excess methionine. J Nutr 2021;151(3):531-9.

Edison EE, Brosnan ME, Meyer C and Brosnan JT. Creatine synthesis: production of guanidinoacetate by the rat and human kidney in-vivo. Am J Physiol Renal Physiol.2007; 293: F1799-F1804

Edison EE, Brosnan ME, Aziz K, Brosnan JT. Creatine and guanidinoacetate content of human milk and infant formulas: implications for creatine deficiency syndromes and amino acid metabolism. Br J Nutr. 2013;110:1075– 1078.

Elango R. Methionine nutrition and metabolism: insights from animal studies to inform human nutrition. J Nutr. 2020;150(1):2518S-2523S.

Elshorbagy AK, Kozich V, Smith AD, Refsum H. Cysteine and obesity: consistency of the evidence across epidemiologic, animal and cellular studies. Curr Opin Clin Nutr Metab Care. 2012;15(1):49-57.

European Food Safety Authority (EFSA). Safety and efficacy of guanidinoacetic acid as feed additive for chickens for fattening. EFSA J. 2009;7 (3): 988.

Fang Z, Yao K, Zhang X, Zhao S, Sun Z, Tian G, Yu B, Lin Y, Zhu B, Jia G, Zhang K. Nutrition and health relevant regulation of intestinal sulfur amino acid metabolism. Amino acids. 2010;39: 633-640.

Finkelstein JD, Martin JJ, Harris BJ. Methionine metabolism in mammals. The methionine-sparing effect of cystine. J Biol Chem. 1988;263(24):11750-4.

Finkelstein JD, Kyle WE, Harris BJ. Methionine metabolism in mammals. Regulation of homocysteine methyltransferases in rat tissue. Arch Biochem Biophys. 1971;146(1):84–92

Fukada SI, Setoue M, Morita T, Sugiyama K. Dietary eritadenine suppresses guanidinoacetic acid-induced hyperhomocysteinemia in rats. J Nutr. 2006;136 (11):2797–2802.

Garcia-Rodenas CL, Affolter M, Vinyes-Pares G, De Castro CA, Karagounis LG, Zhang Y, Wang P, Thakkar SK. Amino acid composition of breast milk from urban Chinese mothers. Nutrients. 2016;8(10):606.

Hanna-El-Daher L, Braissant O. Creatine synthesis and exchanges between brain cells: what can be learned from human creatine deficiencies and various experimental models?. Amino Acids. 2016;48:1877-1895.

Harper AE, Benevenga NJ, Wohlhueter RM. Effects of ingestion of disproportionate amounts of amino acids. Physiol Rev. 1970;50(3):428-558.

He DT, Gai XR, Yang LB, Li JT, Lai WQ, Sun XL, Zhang LY. Effects of guanidinoacetic acid on growth performance, creatine and energy metabolism, and carcass characteristics in growing-finishing pigs. J Anim Sci. 2018;96(8):3264–3273.

Hogeveen M, Blom HJ, Van Amerongen M, Boogmans B, Van Beynum IM, Van De Bor M. Hyperhomocysteinemia as risk factor for ischemic and hemorrhagic stroke in newborn infants. J Pediatr. 2002;141(3):429–431.

Holmes-McNary MQ, Cheng WL, Mar MH, Fussell S, Zeisel SH. Choline and choline esters in human and rat milk and in infant formulas. Am J Clin Nutr. 1996;64:572–576

Huang LiSha HL, Hogewind-Schoonenboom JE, Dongen MV, Groof FD, Voortman GJ, Schierbeek H, Twisk JW, Vermes A, Chen Chao CC, Huang Ying HY, Goudoever JV. Methionine requirement of the enterally fed term infant in the first month of life in the presence of cysteine. Am J Clin Nutr. 2012;95(5):1048–1054.

Humayun MA, Turner JM, Elango R, Rafii M, Langos V, Ball RO, Pencharz PB. Minimum methionine requirement and cysteine sparing of methionine in healthy school-age children. Am J Clin Nutr. 2006;84(5):1080-1085.

Kotsopoulos J, Sohn KJ, Kim YI. Postweaning dietary folate deficiency provided through childhood to puberty permanently increases genomic DNA methylation in adult rat liver. J Nutr 2008;138:703–9.

Kreider RB. Effects of creatine supplementation on performance and training adaptations. Mol Cell Biochem. 2003;244:89-94.

Liao Y, Weber D, Xu W, Durbin-Johnson BP, Phinney BS, Lönnerdal B. Absolute quantification of human milk caseins and the whey/casein ratio during the first year of lactation. J Proteome Res. 2017;16(11):4113-4121.

Loike JD, Zalutsky DL, Kaback E, Miranda AF, Silverstein SC. Extracellular creatine regulates creatine transport in rat and human muscle cells. Proc Natl Acad Sci. 1988;85(3):807–811.

Lyons J, Rauh-Pfeiffer A, Yu YM, Lu XM, Zurakowski D, Tompkins RG, Ajami AM, Young VR, Castillo L. Blood glutathione synthesis rates in healthy adults receiving a sulfur amino acid-free diet. Proc Natl Acad Sci. 2000;97(10):5071-6.

Majdeddin M, Golian A, Kermanshahi H, Michiels J, De Smet S. Effects of methionine and guanidinoacetic acid supplementation on performance and energy metabolites in breast muscle of male broiler chickens fed corn-soybean diets. Br Poult Sci. 2019;60(5):554–563.

Martin CR, Ling PR, Blackburn GL. Review of infant feeding: key features of breast milk and infant formula. Nutrients, 2016;8(5):279.

Martínez Y, Li X, Liu G, Bin P, Yan W, Más D, Valdivié M, Hu CA, Ren W, Yin Y. The role of methionine on metabolism, oxidative stress, and diseases. Amino Acids. 2017;49:2091-2098.

McBreairty LE, McGowan RA, Brunton JA, Bertolo RF. Partitioning of [methyl-3H] methionine to methylated products and protein is altered during high methyl demand conditions in young Yucatan miniature pigs. J Nutr. 2013;143(6):804-809.

McBreairty LE, Robinson JL, Furlong KR, Brunton JA, Bertolo RF. Guanidinoacetate is more effective than creatine at enhancing tissue creatine stores while consequently limiting methionine availability in Yucatan miniature pigs. PLoS One. 2015;10(6): e0131563.

Mccall W, Persky AM. Pharmacokinetics of creatine. Creatine and creatine kinase in health and disease. 2007. In: Salomons, G.S., Wyss, M. (eds) Creatine and Creatine Kinase in Health and Disease. Subcellular Biochemistry, vol 46. Springer, Dordrecht.

McGee M, Bainbridge S, Fontaine-Bisson B. A crucial role for maternal dietary methyl donor intake in epigenetic programming and fetal growth outcomes. Nutr Rev. 2018;76(6):469-78.

McGuire DM, Gross MD, Van Pilsum JF, Towle HC. Repression of rat kidney L-arginine: glycine amidinotransferase synthesis by creatine at a pretranslational level. J Biol Chem. 1984;259(19):12034-12038.

Mendonça IB, Watanabe PH, Silva BA, Boiago MM, Panisson JC, Andrade TS, Campos AC, Mello MA. Dietary supplementation of guanidinoacetic acid for sows and their progenies: performance, blood parameters and economic viability at nursery phase. Livest Sci. 2019;227: 105–110.

Michiels J, Maertens L, Buyse J, Lemme A, Rademacher M, Dierick NA, De Smet S. Supplementation of guanidinoacetic acid to broiler diets: effects on performance, carcass characteristics, meat quality, and energy metabolism. Poult Sci. 2012;91(2):402–412.

Mohebbifar A, Torki M, Abdolmohammadi A. Effects of dietary guanidinoacetic acid supplementation on performance, blood parameters and meat quality of male broilers with cold-induced ascites. Iran J Appl Anim Sci. 2009;9 (1):125–133.

Mori A, Kohno M, Masumizu T, Noda Y, Packer L. Guanidino compounds generate reactive oxygen species. IUBMB Life. 1996;40(1):135–143.

Moss RL, Haynes AL, Pastuszyn A, Glew RH. Methionine infusion reproduces liver injury of parenteral nutrition cholestasis. Pediatr Res. 1999;45(5):664-668.

Mudd SH, Poole JR. Labile methyl balances for normal humans on various dietary regimens. Metab. 1975;24(6):721-735.

Niculescu MD, Zeisel SH. Diet, methyl donors and DNA methylation: interactions between dietary folate, methionine and choline. J Nutr. 2002;132:2333S– 2335S

Ostojic SM. An alternative mechanism for guanidinoacetic acid to affect methylation cycle. Med Hypotheses. 2014;83(6):847–848.

Ostojic SM. Advanced physiological roles of guanidinoacetic acid. Eur J Nutr. 2015;54 (8): 1211–1215.

Ostojic SM. Co-Administration of creatine and guanidinoacetic acid for augmented tissue bioenergetics: A novel approach? Biomed Pharmacother. 2017;91: 238–240.

Ostojic SM. and Forbes SC. Perspective: Creatine, a conditionally essential nutrient: building the case. Adv Nutr. 2022;13(1):34–37.

Ostojic SM and Vojvodic-Ostojic A. Single-dose oral guanidinoacetic acid exhibits dose-dependent pharmacokinetics in healthy volunteers. Nutr Res. 2015 ;35 (3) :198–205.

Panisson JC, Silva BA, Oliveira SG, Maiorka A, Azevedo AM, Mendonça IB, Silva KF, Rebordões FI, Luspa MA, Rademacher M. Guanidinoacetic acid: how can sows benefit from this novel feed additive. EAAP Sci Ser. 2019:8–22.

Rae C, Digney AL, McEwan SR, Bates TC. Oral creatine monohydrate supplementation improves brain performance: a double–blind, placebo–controlled, cross–over trial. B Biol Sci. 2003;270(1529):2147-2150.

Randunu RS and Bertolo RF. The effects of maternal and postnatal dietary methyl nutrients on epigenetic changes that lead to non-communicable diseases in adulthood. Int J Mol Sci. 2000;21(9):3290.

Riedijk MA, Stoll B, Chacko S, Schierbeek H, Sunehag AL, van Goudoever JB, Burrin DG. Methionine transmethylation and transsulfuration in the piglet gastrointestinal tract. Proc Natl Acad Sci. 2007;104(9):3408-3413.

Ringel J, Lemme A, Knox A, McNab J, Redshaw MS. Effects of graded levels of creatine and guanidino acetic acid in vegetable-based diets on performance and biochemical parameters in muscle tissue. Proceedings of the 16th European symposium on poultry nutrition. 2007:387–390.

Robinson JL, Bertolo RF. The pediatric methionine requirement should incorporate remethylation potential and transmethylation demands. Adv Nutr. 2016;7(3):523-534.

Robinson,JL, Bartlett RK, Harding SV, Randell EW, Brunton JA, Bertolo RF. Dietary methyl donors affect in vivo methionine partitioning between transmethylation and protein synthesis in the neonatal piglet. Amino Acids. (2016)b;48(12):2821-2830.

Robinson JL, McBreairty LE, Ryan RA, Randunu R, Walsh CJ, Martin GM, Brunton JA, Bertolo RF. Effects of supplemental creatine and guanidinoacetic acid on spatial memory and the brain of weaned Yucatan miniature pigs. PLoS One. 2020;15(1):e0226806.

Soares MS, Oliveira PS, Debom GN, da Silveira Mattos B, Polachini CR, Baldissarelli J, Morsch VM, Schetinger MR, Tavares RG, Stefanello FM, Spanevello RM. Chronic administration of methionine and/or methionine sulfoxide alters oxidative stress parameters and ALA-D activity in liver and kidney of young rats. Amino Acids. 2017;49(1):129-38.

Schubert HL, Blumenthal RM, Cheng X. Many paths to methyltransfer: a chronicle of convergence. Trends Biochem Sci. 2003;28:329–335.

Selhub, J. Homocysteine metabolism. Annu Rev Nutr. 1999;22(1):217-246.

Sharideh H, Esmaeile Neia L, Zaghari M, Zhandi M, Akhlaghi A, Lotfi L. Effect of feeding guanidinoacetic acid and l-arginine on the fertility rate and sperm penetration in the perivitelline layer of aged broiler breeder hens. J Anim Physiol Anim. Nutr. (Berl) 2016;100(2):316–322.

Shoveller AK, Brunton JA, Pencharz PB, Ball RO. The methionine requirement is lower in neonatal piglets fed parenterally than in those fed enterally. J Nutr. 2003;133(5):1390-7.

Shoveller AK, Brunton JA, House JD, Pencharz PB, Ball RO. Dietary cysteine reduces the methionine requirement by an equal proportion in both parenterally and enterally fed piglets. J Nutr 2003b;133(12):4215-4224.

Shoveller AK, House JD, Brunton JA, Pencharz PB, Ball RO. The balance of dietary sulfur amino acids and the route of feeding affect plasma homocysteine concentrations in neonatal piglets. J Nutr 2004; 134:609 –12

Sipilä I. Inhibition of arginine-glycine amidinotransferase by ornithine. A possible mechanism for the muscular and chorioretinal atrophies in gyrate atrophy of the choroid and retina with hyperornithinemia. Biochim Biophys Acta Enzymol. 1980;613(1): 79-84.

Snyderman SE, Holt LE, Norton PM, Roitman E, Phansalkar SV. The plasma aminogram. I. Influence of the level of protein intake and a comparison of whole protein and amino acid diets. Pediatr Res. 1968;2(2):131-44.

Spence JD, Yi Q, Hankey GJ. B vitamins in stroke prevention: time to reconsider. Lancet Neurol. 2017;16(9):750-60.

Stam J, Sauer PJ, Boehm G. Can we define an infant's need from the composition of human milk?. Am J Clin Nutr. 2013;98(2):521S-8S.

Stead LM, Au KP, Jacobs RL, Brosnan ME, Brosnan JT. Methylation demand and homocysteine metabolism: effects of dietary provision of creatine and guanidinoacetate. Am J Physiol Endocrinol Metab. 2001;281(5):E1095-100.

Stead LM, Brosnan JT, Brosnan ME, Vance DE, Jacobs RL. Is it time to reevaluate methyl balance in humans?. Am J Clin Nutr. 2006;83(1):5-10.

Stöckler-Ipsiroglu S, Stromberger C, Item CB, Mühl A. Disorders of creatine metabolism. In Physician's Guide to the Laboratory Diagnosis of Metabolic Diseases. 2003:467-480.

Stoll B, Burrin DG, Henry J, Yu H, Jahoor F, Reeds PJ. Substrate oxidation by the portal drained viscera of fed piglets. Am J Physiol Endocrinol Metab. 1999;277(1):E168-75.

Tachikawa M, Ikeda S, Fujinawa J, Hirose S, Akanuma SI, Hosoya KI. γ-Aminobutyric acid transporter 2 mediates the hepatic uptake of guanidinoacetate, the creatine biosynthetic precursor, in rats. PloS one. 2012;7(2):e32557.

Tachikawa M, Kasai Y, Yokoyama R, Fujinawa J, Ganapathy V, Terasaki T, Hosoya KI. The blood–brain barrier transport and cerebral distribution of guanidinoacetate in rats: involvement of creatine and taurine transporters. J Neurochem. 2009;111(2):499-509.

Tossenberger J, Rademacher M, Németh K, Halas V, Lemme AJ. Digestibility and metabolism of dietary guanidino acetic acid fed to broilers. Poult Sci. 2016;95(9):2058–2067.

Van der Poel AF, Braun U, Hendriks WH, Bosch G. Stability of creatine monohydrate and guanidinoacetic acid during manufacture (retorting and extrusion) and storage of dog foods. J Anim Physiol Anim Nutr. 2009;103(4),1242– 1250.

Vance DE, Li Z, Jacobs RL. Hepatic phosphatidylethanolamine N methyltransferase, unexpected roles in animal biochemistry and physiology. J Biol Chem. 2007; 282:33237–33241.

Viadel A. Alegriá R. Farré P. Abellán F. Romero B. Amino acid profile of milk-based infant formulas. Int J Food Sci Nutr. 2000;51(5):367-72.

Volek JS, Kraemer WJ, Bush JA, Boetes M, Incledon T, Clark KL, Lynch JM. Creatine supplementation enhances muscular performance during high-intensity resistance exercise. J Am Diet Assoc. 1997;97(7):765-770.

Walkey CJ, Yu L, Agellon LB, Vance DE. Biochemical and evolutionary significance of phospholipid methylation. J Biol Chem. 1998;273:27043–27046.

Wang WW, Qiao SY, Li DF. Amino acids and gut function. Amino acids. 2009;37:105-10.

World Health Organization. Protein and amino acid requirements in human nutrition. World Health Organization technical report series. 2007(935):1.

Wu G, Bazer FW, Davis TA, Kim SW, Li P, Marc Rhoads J, Carey Satterfield M, Smith SB, Spencer TE, Yin Y. Arginine metabolism and nutrition in growth, health and disease. Amino acids. 2009;37:153-68.

Wu G, Lupton JR, Turner ND, Fang YZ, Yang S. Glutathione metabolism and its implications for health. J Nutr. 2004;134(3):489-92.

Wu Z, Hou Y, Hu S, Bazer FW, Meininger CJ, McNeal CJ, Wu G. Catabolism and safety of supplemental L-arginine in animals. Amino acids. 2016;48:1541-52.

Wyss M, Kaddurah-Daouk R. Creatine and creatinine metabolism. Physiol Rev. 2000;80(3):1107-213.

Yi-qun LI, Ying LI, Tatsuya MO. Methionine and serine synergistically suppress hyperhomocysteinemia induced by choline deficiency, but not by guanidinoacetic acid, in rats fed a low casein diet. Biosci Biotechnol Biochem. 2011;75(12):2333–2339.

Yu J, Yang H, Wang Z, Dai H, Xu L, Ling C. Effects of arginine on the growth performance, hormones, digestive organ development and intestinal morphology in the early growth stage of layer chickens. Ital J Anim Sci. 2018;17(4),1077–1082.

Zarghi H, Golian A, Tabatabaei Yazdi F. Effect of dietary sulphur amino acid levels and guanidinoacetic acid supplementation on performance, carcase yield and energetic molecular metabolites in broiler chickens fed wheat-soy diets. Ital J Anim Sci. 2020;19(1):951–959.

Zeisel SH, Char D, Sheard NF. Choline, phosphatidylcholine and sphingomyelin in human and bovine milk and infant formulas. J Nutr. 1986;116:50–58

Zhao W, Li J, Xing T, Zhang L, Gao F. Effects of guanidinoacetic acid and complex antioxidant supplementation on growth performance, meat quality, and antioxidant function of broiler chickens. J Sci Food Agric. 2021;101(9):3961–3968.

Zhu X, Song J, Mar MH, Edwards LJ, Zeisel SH. Phosphatidylethanolamine N-methyltransferase (PEMT) knockout mice have hepatic steatosis and abnormal hepatic choline metabolite concentrations despite ingesting a recommended dietary intake of choline. Biochem J. 2003;370(3):987– 993.

Chapter 2: Thesis rationale, objectives and hypotheses

2.1 Thesis rationale

The purpose of this chapter is to describe the thesis rationale, objectives, hypotheses, and experimental approaches. The global objective of this dissertation was to elucidate the role of precursors in the synthesis of creatine in neonates. More specifically, the objectives were to determine the effects of dietary methionine on the synthesis of major methylated products, the fate of GAA supplementation, and endogenous creatine synthesis in neonatal piglets.

Methionine is essential in various biological processes, serving as a critical component for protein synthesis and serving as a precursor for several vital nutrients. Additionally, it acts as a precursor for over 50 transmethylation reactions, producing important metabolites such as creatine, PC, and methylated DNA (Bertolo et al., 2013). Hence, the primary purpose of the initial section of the thesis was to investigate the utilization of dietary methionine in these metabolic pathways, as it plays a crucial role in determining the specific nutritional needs of neonates. Although the methionine requirement has been established in the human infant and piglet based on whole-body protein synthesis, methionine requirements may vary depending on the requirements for other non-protein pathways. Therefore, this research aimed to re-evaluate the methionine recommendations for infants and adjust the methionine requirement estimate to accommodate both protein and non-protein demands for methionine.

The second experiment of the thesis aimed to provide clarity on the influence of dietary methionine on GAA absorption in neonatal pigs. Studies have shown that GAA readily converts to creatine, but only when sufficient methionine is available to transmethylate GAA to creatine in the liver (McBreairty et al., 2013). Furthermore, supplementing pigs with GAA and excess methionine

enabled creatine synthesis without feedback regulation; however, if dietary methionine is limited, creatine synthesis is limited, yet GAA did not accumulate in plasma, liver or other tissues (Kankayaliyan 2014). One possibility is that adequate dietary methionine is necessary for the absorption of supplemental GAA in the gut; however, the impact of dietary methionine on GAA absorption in the gut remains unknown. Therefore, exploring the effects and distribution of supplemented GAA in conjunction with varying dietary methionine levels would be beneficial.

Moreover, creatine and GAA supplements are popular in increasing muscle mass and physical performance. Recent studies have shown that co-administration of GAA and creatine enhances body creatine stores in healthy individuals and is superior to creatine alone in enhancing exercise performance (Semeredi et al., 2019). Furthermore, administering GAA with creatine may reduce side effects such as hyperhomocysteinemia. However, there are no reported findings available regarding the impact of the GAA and creatine mixture on GAA absorption, transportation, and utilization in pigs. Additionally, the mechanism behind the synergistic effect of creatine and GAA on creatine stores has not been investigated. It is possible that GAA enhances creatine transport, or that creatine improves GAA absorption and its conversion to creatine. Therefore, it is crucial to investigate and compare the effects of different combinations of GAA supplements to improve body creatine stores in neonatal piglets. Additionally, understanding the absorption kinetics of the GAA and creatine mixture at the gut level would help identify the most effective nutritional supplement for enhancing creatine synthesis, muscle energy capacity, and performance in piglets.

Objectives

The goals and objectives of the entire thesis are divided into separate chapters. For the first experimental chapter (**Chapter Three**, "The amount of dietary methionine required to maximize synthesis of transmethylated products is higher than that needed for protein synthesis in neonatal piglets"), the objectives were to:

a) determine how much dietary methionine is required to maximize the synthesis of tissue-specific protein, whole-body protein, as well as key transmethylated products, including creatine, PC, and DNA;

b) determine which tissues are prioritized for protein synthesis when dietary methionine supply is limited.

The second study (**Chapter Four**, "Dietary methionine enhances portal appearance of guanidinoacetate and synthesis of creatine in Yucatan miniature piglets") aimed to achieve the following objective:

a) to determine the effects of dietary methionine on supplemental GAA absorption and creatine synthesis in neonatal piglets.

The objectives of the third study (**Chapter Five**, "Guanidinoacetic acid transport across the small intestine is enhanced by dietary creatine in neonatal piglets") were:

a) to compare the effectiveness of three supplementation options: GAA alone, GAA + methionine, and GAA + creatine, in enhancing creatine stores and GAA absorption in neonatal piglets;

b) to determine the effectiveness of creatine and GAA combinations in enhancing GAA absorption in neonatal piglets.

2.2 Hypotheses

The hypotheses for the entire thesis are organized and presented separately in each chapter.

For the first experimental chapter (**Chapter Three**, "The amount of dietary methionine required to maximize synthesis of trans methylated products is higher than that needed for protein synthesis in neonatal piglets"), the hypotheses were:

a) protein synthesis will be maximized at the lowest intakes of dietary methionine, while creatine synthesis will be maximized at a higher intake;

b) PC synthesis will be maximized only at the highest level of methionine intake.

The hypothesis for the second study (**Chapter Four**, "Dietary methionine enhances portal appearance of guanidinoacetate and synthesis of creatine in Yucatan miniature piglets") was:

a) dietary GAA appearance in the portal vein will require a sufficient amount of dietary methionine.

The hypotheses for the third study (**Chapter Five**, "Guanidinoacetic acid transport across the small intestine is enhanced by dietary creatine in neonatal piglets") were:

a) GAA absorption will be more effective when combined with either creatine or methionine compared to GAA alone, and the GAA + creatine combination will result in the largest creatine stores;

b) GAA absorption across the gut will be highest in a 1: 3 GAA to creatine ratio.

2.3 The piglet model

The neonatal piglet is widely regarded as the closest non-primate model to the human infant and is an ideal animal model for studying nutritional metabolism in human neonates because it shares similarities in terms of gastrointestinal tract morphology, physiology, and metabolic changes during development (Miller et al., 1987, Pond et al., 1978). When compared to rodent models, piglets offer several notable advantages. One advantage is their larger size, which allows easier handling and surgical procedures and offers favorable conditions for studying metabolic kinetics that involve the collection of multiple blood samples. Piglets also demonstrate tolerance to multiple catheterizations, enabling rapid blood sampling and the administration of experimental diets. This versatility and adaptability make piglets a valuable model for studying various aspects of infant physiology and nutrition (Bertolo et al., 1999).

The utilization of neonatal miniature piglets in studies of nutrient metabolism has been limited compared to their domestic counterparts. Yucatan miniature piglets fed by sows experience a slower average growth rate during the first month of life than domestic piglets. According to Myrie et al. (2012), the growth rate of miniature piglets is approximately 45 $g \cdot kg^{-1} \cdot d^{-1}$, whereas Wykes et al. (1993) reported a growth rate of 79 $g \cdot kg^{-1} \cdot d^{-1}$ for domestic piglets. However, the miniature pig model may be more appropriate when investigating infant nutrient metabolism. This is because miniature piglets grow slower, which is closer to that of human infants, at approximately half the growth rate of domestic strains of piglets.

The piglet has been extensively employed in studies examining the requirements of amino acids, and their metabolism has been shown to respond to rapid dietary modifications, including changes in the composition of amino acids in their diet (Brunton et al., 2007). Several studies on piglets

have determined specific amino acid requirements for enteral and parenteral nutrition (House et al., 1997a; 1997b). Subsequent studies in human infants have validated and confirmed the findings from the piglet studies, further supporting the suitability of the piglet model for studying amino acid metabolism and requirements in human infants (Roberts et al., 2001). The absolute values obtained from piglet data were divided by five to estimate the amino acid requirements for human infants, considering the differing growth rates between the two species. This correction factor has allowed for comparing the predicted requirements based on piglet data and the measured requirements in humans. Studies on 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) have shown a close similarity between the predicted requirements from piglet data and the measured requirements in humans. Whether this conversion factor applies to the slower growing Yucatan miniature pig strain is unclear. Regardless, these findings have established that amino acid requirement estimates derived from piglet data can be applied to human infants (Chapman et al., 2009).

2.4 References

Bertolo RF, Chen CZ, Pencharz PB, Ball RO. Intestinal atrophy has a greater impact on nitrogen metabolism than liver by-pass in piglets fed identical diets via gastric, central venous or portal venous routes. J Nutr. 1999;129(5):1045-52.

Bertolo RF, Chen CZ, Law G, Pencharz PB, Ball RO. Threonine requirement of neonatal piglets receiving total parenteral nutrition is considerably lower than that of piglets receiving an identical diet intragastrically. J Nutr. 1998;128(10):1752-9.

Bertolo RF, McBreairty LE. The nutritional burden of methylation reactions. Curr Opin Clin Nutr Metab Care. 2013;16(1):102-108.

Brunton JA, Shoveller AK, Pencharz PB, Ball RO. The indicator amino acid oxidation method identified limiting amino acids in two parenteral nutrition solutions in neonatal piglets. J Nutr 2007;137:1253–1259.

Chapman KP, Courtney-Martin G, Moore AM, Ball RO, Pencharz PB. Threonine requirement of parenterally fed postsurgical human neonates. Am J Clin Nutr. 2009;89(1):134-41.

Courtney-Martin G, Chapman KP, Moore AM, Kim JH, Ball RO, Pencharz PB. Total sulfur amino acid requirement and metabolism in parenterally fed postsurgical human neonates. Am J Clin Nutr. 2008 Jul;88(1):115-24.

House JD, Pencharz PB, Ball RO. Phenylalanine requirements determined by using L-[1-14C] phenylalanine in neonatal piglets receiving total parenteral nutrition supplemented with tyrosine. Am J Clin Nutr. 1997a;65(4):984-993.

House JD, Pencharz PB, Ball RO. Tyrosine kinetics and requirements during total parenteral nutrition in the neonatal piglet: the effect of glycyl-L-tyrosine supplementation. Pediatr Res. 1997b;41(4):575-583.

Kankayaliyan T, Dinesh OC, Rademacher M, Brunton JA, Bertolo RF. Creatine de novo synthesis and methionine metabolism in neonatal piglets. 2014: Appl Physiol Nutr Metab, Vol. 39.

McBreairty LE, McGowan RA, Brunton JA, Bertolo RF. Partitioning of [methyl-3H] methionine to methylated products and protein is altered during high methyl demand conditions in young Yucatan miniature pigs. J Nutr 2013;143(6):804-9.

Miller ER, Ullrey DE. The pig as a model for human nutrition. Annu Rev Nutr. 1987 ;7 :361–382.

Myrie SB, MacKay DS, Van Vliet BN, Bertolo RF. Early programming of adult blood pressure in the low birth weight Yucatan miniature pig is exacerbated by a post-weaning high-salt-fat-sugar diet. Br J Nutr. 2012;108(7):1218-25.

Pond WG, Houpt KA. The Biology of the Pig. Ithaca, NY: Comstock. 1978; 371.

Roberts SA, Ball RO, Moore AM, Filler RM, Pencharz PB. The effect of graded intake of glycyl-L-tyrosine on phenylalanine and tyrosine metabolism in parenterally fed neonates with an estimate of tyrosine requirement. Pediatr. Res. 2001;49(1):111-9.

Semeredi S, Stajer V, Ostojic J, Vranes M, Ostojic SM. Guanidinoacetic acid with creatine compared with creatine alone for tissue creatine content, hyperhomocysteinemia, and

exercise performance: A randomized, double-blind superiority trial. Nutrition. 2019;57:162-6.

Shoveller AK, Brunton JA, Pencharz PB, Ball RO. The methionine requirement is lower in neonatal piglets fed parenterally than in those fed enterally. J Nutr. 2003;133(5):1390-7.

Shoveller AK, Brunton JA, House JD, Pencharz PB, Ball RO. Dietary cysteine reduces the methionine requirement by an equal proportion in both parenterally and enterally fed piglets. J Nutr 2003b;133(12):4215-4224.

Wykes LJ, Ball RO, Pencharz PB. Development and validation of a total parenteral nutrition model in the neonatal piglet. J Nutr. 1993;123(7):1248-59.

Chapter 3: The amount of dietary methionine required to maximize synthesis of transmethylated products is higher than that needed for protein synthesis in neonatal piglets.

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Chapter 3 was primarily designed by R. Bertolo. M. Asiriwardhana conducted the research, analyzed the data and prepared the manuscript with guidance from R. Bertolo. The final manuscript was read and approved by all authors.

3.1 Abstract

Methionine is required for synthesizing protein and other critical metabolites, acting as a precursor for >50 transmethylation reactions to produce metabolites such as creatine, phosphatidylcholine (PC), and methylated DNA. In neonatal piglets, because a lower fraction of dietary methionine is incorporated into protein (~1/3) compared to methylated products (~2/3), both protein and non protein demands need to be considered when determining the methionine requirement. Our major objective was to quantify how much dietary methionine is required to maximize protein synthesis and key transmethylated products (creatine, PC, methylated DNA). Twenty surgically altered Yucatan miniature piglets (16 M, 4 F; mean ± SD age, 8 ± 2 d; mean ± SD weight, 1.61 ± 0.14 kg) were fed complete diets for 5 d and then randomized to 20 test diets with methionine intakes ranging from 20%-220% of requirement (0.32 – 3.55 g methionine /100 g total AAs). Stable isotopes of phenylalanine and tyrosine was administered to determine whole-body protein synthesis. [^{3}H-methyl]-methionine was infused to measure methyl incorporation into transmethylated products, and ^{3}H-phenylalanine flooding dose was given to measure tissue-specific protein synthesis. Break-point analysis indicated a whole-body requirement of 1.73 ± 0.27 g methionine /100 g total AAs. [^{3}H-methyl] incorporation to PC increased linearly with increasing dietary methionine, while hepatic creatine synthesis maximized at 1.84 ± 0.22 g methionine /100 g total AAs. DNA methylation rate was maximized at 1.52 ± 0.38 g methionine /100 g total AAs. Plasma concentrations of homocysteine were positively correlated with dietary methionine levels. These data suggest that DNA methylation is prioritized over hepatic creatine synthesis, while PC synthesis continuously increases with methionine intake with no discernible maximum rate. When methionine intake was limited, liver protein synthesis was prioritized, followed by kidney and muscle protein synthesis. Since not all tissues have the same methionine requirement, our data

shed light on why growth is constrained before intestinal function reaches full capacity. These data also suggest that protein synthesis should not be used to determine whole-body requirements since more methionine is required to meet non-protein demands for methionine.

3.2 Introduction

Methionine is an indispensable sulfur-containing amino acid required not only for protein synthesis but also as a methyl donor for over 50 transmethylation reactions, generating critical metabolites (Bertolo et al., 2013). The requirements for these pathways are particularly profound in neonates undergoing rapid growth. Methionine is generally partitioned between protein synthesis and the methionine cycle, which can be summarized by three pathways: transmethylation, transsulfuration, and remethylation. The transmethylation pathway involves the conversion of methionine to homocysteine. In the transsulfuration pathway, homocysteine irreversibly converts into cysteine, also known as the methionine disposal pathway. Alternatively, homocysteine can be remethylated back to methionine through the remethylation pathway (Bertolo et al., 2013).

Transmethylation plays a significant role in synthesizing specific products, including creatine, phosphatidylcholine (PC), and methylated DNA. These pathways consume a significant amount of the methionine pool, emphasizing the importance of considering non-protein roles when establishing the methionine requirement. Among the products of transmethylation, creatine is particularly notable. Creatine can be synthesized endogenously from guanidinoacetate (synthesized from arginine) or acquired through dietary intake. However, even in suckling piglets, only 25% of creatine comes from sow milk, and 75% must be synthesized endogenously, using the equivalent of 35% of dietary methionine (Brosnan et al., 2009). This obligatory synthesis represents a considerable metabolic burden on the precursor amino acids arginine and methionine.

Another crucial metabolite, PC, also requires a significant amount of methyl groups from methionine for its synthesis. The conversion of phosphatidylethanolamine (PE) to PC via the PEMT pathway requires three methyl groups from S-adenosylmethionine (SAM), which results in a higher demand for methyl groups compared to the synthesis of creatine (Stead et al., 2001). PC

can also be synthesized from choline via the CDP-choline pathway, so if choline intake is low, the demand for methyl groups rises dramatically.

Furthermore, another vital transmethylation process, DNA methylation, entails the transfer of a methyl group from SAM to cytosine residues within CpG dinucleotide sequences by DNA methyltransferase (DNMT). These methylated sequences, particularly in a promoter region, can govern gene expression (Bird et al., 2002), emphasizing the importance of providing the necessary amount of methyl groups to ensure appropriate gene regulation. Indeed, reduced intake of dietary methionine and methyl donors has been shown to lead to a decrease in DNA methylation (Niculescu 2002, Kotsopoulos 2008, McMillen 2005, Cordero 2013). Therefore, the dietary methionine requirement must also ensure sufficient methyl groups for DNA methylation.

However, the complexity of these pathways and their metabolic demands highlights the complexity and critical nature of methionine's requirement beyond mere protein synthesis. Therefore, methionine requirements should be established by carefully considering its non-protein metabolic pathways as well (Bertolo et al., 2013). Studies have shown that in the liver of neonatal piglets, a lower fraction of dietary methionine is incorporated into protein (~1/3), while methylation reactions demand the majority of dietary methionine (~2/3) (McBreairty et al., 2013). However, most dietary methionine requirement studies use protein synthesis as the sole outcome when determining the whole-body methionine requirement. For example, Shoveller et al., 2003a described dietary methionine requirements based on indicator amino acid oxidation rate, which is dictated by whole-body protein synthesis. However, transmethylation reactions seem to consume a greater amount of dietary methionine than protein synthesis, so this requirement is likely underestimated. Few studies have described how transmethylated reactions compete for methionine and methyl groups during various dietary situations. According to Robinson et al.,

2016b, transmethylation is sacrificed to maintain protein synthesis when methionine and methyl donors are limited in neonatal pigs.

Moreover, PC and creatine syntheses are sensitive to methionine availability. However, there is no evidence of the actual amount of dietary methionine that is required to maximize the synthesis of each methylated product. Although transmethylation and protein synthesis are fundamental components of the methionine requirement (reviewed by Bertolo et al., 2013), the specific individual demands of these processes for methionine have not been quantified. The main objective of this study was to determine how much dietary methionine is required to maximize the synthesis of tissue-specific protein, whole-body protein, as well as key transmethylated products including creatine, PC, and DNA. We tried to determine which tissues are prioritized for protein synthesis when dietary methionine supply is limited. We hypothesized that protein synthesis will be maximized at the lowest intakes of dietary methionine while creatine synthesis will be maximized at a higher intake. PC synthesis will be maximized only at the highest level of methionine intake. By comparing these tissue-specific and transmethylated product breakpoint "requirements" in young pigs, we can determine which tissues or methylated products are prioritized or spared when dietary methionine is limiting.

3.3 Methods

3.3.1 Reagents.

[^{3}H-Methyl]-methionine and L-[4-^{3}H]-phenylalanine were obtained from American Radiochemicals, Inc. and Moravek Biochemicals and stable isotopes were obtained from

Cambridge Isotope Laboratories. All other chemicals were of analytical grade and were from Fisher Scientific or Sigma.

3.3.2 **Animals and surgical procedure**

All procedures conducted on piglets received approval from the Animal Care Committee of the Memorial University of Newfoundland, which adhered to the guidelines established by the Canadian Council on Animal Care. Yucatan miniature piglets (n = 20; 16 M, 4 F; age, 8 ± 2 d) were removed from the sows at the Memorial University of Newfoundland breeding colony and immediately underwent surgical procedures. These procedures involved the implantation of a jugular vein catheter (advanced to the superior vena cava) and the femoral vein catheter (advanced to the inferior vena cava), following a previously described method (Dodge et al., 2012). Additionally, a gastric feeding catheter was inserted using a Stamm gastrostomy technique (Bertolo et al., 1999).

3.3.3 **Piglet housing and diets**

The piglets were individually housed in circular metabolic cages equipped with a swivel-and-tether system (Lomir Biomedical). This setup allowed them to move freely within the cage while enabling a continuous infusion of their diet. The animal housing room light cycled from 0800 to 2000 and was kept at 28°C. To provide additional warmth, heat lamps were used. The piglets received a continuous gastric infusion of a sterile adaptation diet designed to provide sufficient amounts of all essential nutrients. This diet consisted of 1.1 MJ of metabolizable energy· kg body weight $(BW)^{-1}\cdot d^{-1}$ with glucose contributing 24.5 g ·kg $BW^{-1}\cdot d^{-1}$ and lipid (20% Intralipid; Pharmacia), with both glucose and lipid supplying 50% of the non-protein energy. The diet also included a total of 15.5 g of free L-amino acids (L-AAs) ·kg $BW^{-1}\cdot d^{-1}$. The amino acid profile of the adaptation diet was based on human milk protein and was formulated to meet 120% of the

amino acid requirements for neonatal piglets (NRC 2012); for methionine, the requirement for gastrically fed piglets determined by Shoveller et al. (2003a) was used (i.e., 100% = 0.25 g methionine· kg BW^{-1} ·d^{-1} = 1.62 g methionine /100 g total AAs in the diet).

The AA composition was as follows (expressed as g·kg BW^{-1}·d^{-1}): alanine, 1.66; arginine, 0.94; aspartate, 0.94; cysteine, 0.22; glutamate, 1.63; glycine, 0.36; histidine, 0.48; isoleucine, 0.72; leucine, 1.62; lysine hydrochloride, 1.61; methionine, 0.30; phenylalanine, 0.61; proline, 1.29; serine, 0.87; taurine, 0.08; threonine, 0.50; tryptophan, 0.33; tyrosine, 0.12; and valine, 0.82 (mass represents free L-AAs); glycyl-tyrosine (as dihydrate), 0.44. Immediately prior to feeding, vitamins (Multi-12K1 Pediatric; Sabex), trace minerals, lipids, and iron dextran (3.0 mg· kg BW^{-1}·d^{-1}; Vetoquinol Canada, Inc.) were added (Supplemental Table 1). The diets were administered through continuous infusion using pressure-sensitive peristaltic pumps. Immediately after the surgical procedure (day 0), piglets received parenteral feeding through the jugular catheter at 50% of their nutritional requirement. On the morning of day 1, the rate of diet delivery was raised to 75% of the required amount. By the evening of day 1, diet delivery was changed to enteral feeding through the IG catheter at 100% of nutrition requirement rate. Piglets were weighed each morning, and the rate of diet infusion was adjusted accordingly. Piglets were administered the complete diet for four days intragastrically. On the evening of day 4, each piglet randomly received one of 20 test diets for 48 h, which is sufficient to observe metabolic changes, each containing a different methionine concentration ranging from 0.32 – 3.55 g methionine /100 g total AAs, or 20 – 220% of the requirement: 100% = 0.25 g methionine· kg BW^{-1} ·d^{-1} (Shoveller et al., 2003a). All diets were adjusted to have the same nitrogen content by altering alanine concentration. The composition of all other amino acids and nutrients remained consistent with the adaptation diet.

3.3.4 **Tracer infusion protocols to measure whole-body and tissue-specific protein synthesis and transmethylated product synthesis**

Whole-body protein synthesis was determined using stable isotopes of phenylalanine and tyrosine, following the methods described earlier (Dinesh et al., 2021). On day 5, a primed, constant infusion of L-[D_5]-phenylalanine (prime 6.39 μmol·kg^{-1}; constant 20 μmol·kg^{-1} ·h^{-1}), L-[3,5-D_2]- tyrosine (prime 2.74 μmol·kg^{-1}; constant 9 μmol·kg^{-1} ·h^{-1}) and L-[D_4]-tyrosine (prime 2.74 μmol·kg^{-1}) (Cambridge Isotope Laboratories, Inc., USA) were given via gastric catheter. (Thivierge et al., 2005; Dinesh et al., 2021). In the 6 h experiment, a constant supply of isotopes was administered through repeated half-hourly doses. Blood samples were collected at 30 min intervals to assess the whole-body protein dynamics.

On day 6, piglets received a primed constant infusion of 20 μCi·kg^{-1} L- [^{3}H-methyl]-methionine via the gastric catheter for 6 h to measure ^{3}H methyl incorporation to transmethylated products. Every 30 min, blood samples were collected from the femoral catheter. After the constant infusion, a flooding dose of labeled L- [4-^{3}H] phenylalanine (0.87 mCi·kg^{-1}) and 1.5 mmol·kg^{-1} unlabelled phenylalanine was infused intravenously over 5 min to measure the tissue-specific protein synthesis (Garlick 1980). The piglets were anesthetized immediately following the last blood sample, and tissue samples (liver, kidney, biceps femoris muscle, mid jejunum mucosa) were harvested. The intestinal segments were washed with sterile 0.9% saline at a cold temperature, and the mucosa was gently scraped onto a chilled glass plate. All samples were freeze-clamped and stored at -80°C until further analysis. After the removal of tissues, piglets were euthanized by exsanguination (Supplemental Figure 1).

3.3.5 **Whole-body Protein synthesis determination**

Plasma samples were prepared for GC/MS (5976N quadrupole MS, Agilent Technologies) analysis following a procedure previously described (Lamarre et al., 2014), with slight adjustments. In summary, 50 µL of plasma was transferred to 2 mL GC vials with screw caps (lined with silicon/PTFE). Then, 20 µL of 0.5 mol·L^{-1} phosphate buffer (pH 8.0), along with 130 µL of a 100 mmol·L^{-1} solution of pentafluorobenzyl bromide (PFBBr; Sigma Aldrich, Canada) in acetone (Sigma Aldrich, Canada), was introduced. The vial was sealed tightly and vigorously mixed for a duration of 1 minute. Subsequently, the vial was incubated at 60°C for 1 hour using a block heater (Fisher Scientific, USA), facilitating the alkalization process via PFBBr. The samples were permitted to cool down at room temperature for 5 minutes. Then, 335 µL of n-hexane was added and mixed thoroughly for 1 minute to stop the reaction. The organic phase at the upper layer was carefully transferred into a vial equipped with an insert designed for mass spectrometry analysis. The oven of the GC (6890N Network GC System) was initially brought to a temperature of 50°C. Subsequently, 2 µL of the sample was injected into a DB-5MS column (0.25 mm x 30 m x 0.22 µm) from Agilent Technologies: Mississauga, ON. Helium was employed as the carrier gas, maintaining a consistent flow rate of 1.1 mL·min^{-1}. The GC parameters were set as follows: The initial oven temperature was sustained at 50°C for a duration of 3 minutes. Afterward, the temperature was accelerated to 280°C at a speed of 30°C·min^{-1} and then maintained at 280°C for 4 minutes. The total runtime for the program was 14.67 minutes. The samples were ionized using electron impact collision energy of 70eV upon entering a quadrupole mass spectrometer. The mass selective detector was configured to operate in selected ion monitoring (SIM) mode, targeting mass-to-charge ratios (m/z) of 300 and 305 for Phe and D_5-Phe, and m/z 316, 318, and 320 for Tyr,

D_2-Tyr, and D_4-Tyr. Isotope enrichment was determined by subtracting the background measurement at baseline from the enrichment observed at steady state.

The whole-body flux of each isotope was calculated using the equation (Tomlinson 2011):

$q=i[(Ei/Ep)-1]$

In this equation, q represents the flux of the amino acid in the pool, *i* stands for the rate of tracer infusion (μmol·kg^{-1}·h^{-1}), E*i* is the tracer's enrichment in the infusion, and E*p* signifies the tracer's enrichment in the plasma under steady-state conditions.

The whole-body protein synthesis was calculated using the following equation (de Betue 2017).

$q_{Phe} = PS + Ox = I + PB$

In this equation, PS represents the rate of protein synthesis, Ox represents Phe oxidation, as estimated by the rate of phenylalanine conversion to tyrosine, PB is the rate of phenylalanine release from protein breakdown, and I is the rate of phenylalanine intake from the diet.

The conversion of phenylalanine to tyrosine was determined using the following formula.,

$Ox = q_{Tyr(M+2)} [E_{Tyr(M+4)} / E_{Phe(M+5)}]$

In this equation,$q_{Tyr(M+2)}$ represents the tyrosine flux in the plasma, while $E_{Tyr(M+4)}$ and $E_{Phe(M+5)}$ denote the enrichments of the product D_4-tyrosine and D_5-phenylalanine, respectively. Protein synthesis (μmol·kg^{-1}·h^{-1}) was determined by subtracting the phenylalanine oxidation rate (Ox) from the phenylalanine flux (q_{Phe}) [$PS = q_{Phe} – Ox$]. This value was then converted to g of protein·kg^{-1}·BW^{-1}·d^{-1}, following the previously established method (Thompson et al., 1989)

3.3.6 **Plasma and tissue AA analyses.**

The specific radioactivity (SRA) of the free and protein-bound phenylalanine in tissues was assessed using established methodologies (Nichols 2008). Methionine concentrations in tissues and plasma samples were assessed through phenylisothiocyanate (PITC) derivatization followed by separation using high-performance liquid chromatography (HPLC) (Bidlingmeyer et al., 1984; Robinson et al., 2016b). Norleucine was used as the internal standard. The fractions corresponding to phenylalanine were collected, and the level of radioactivity was quantified using liquid scintillation counting. SAM and SAH concentrations were also assessed using HPLC according to the method outlined by Ratnam et al., 2006.

3.3.7 **Homocysteine, cysteine and glutathione analyses**

Plasma homocysteine, cysteine and glutathione were measured using an adapted procedure based on the methods introduced by Vester and Rasmussen 1991 (Vester and Rasmussen, 1991; Pfeiffer et al., 1999). Homocysteine, cysteine and glutathione were analyzed via ultra-high performance liquid chromatography (UPLC) following a reducing step with tris (2-carboxyethyl) phosphine (TCEP), deproteinization with perchloric acid and derivatization with a 7-flurobenzo-2,1,3-oxadiazole-sulfonic acid ammonium salt.

3.3.8 **Transmethylated product analyses**

Hepatic creatine concentration was determined using a modified method by Lamarre et al., 2010; by HPLC. Briefly, the tissue was homogenized using a 50 $mmol \cdot L^{-1}$ Tris buffer (pH 7.4), and the resulting homogenates were deproteinized using trifluoroacetic acid (TFA). Creatine was separated using an isocratic mobile phase comprising 0.1% TFA and 3% methanol. The SRA of creatine was

determined by fraction collecting the respective peak, and DPM was determined by scintillation counting (Perkin Elmer Canada).

For PC analyses, lipids were extracted from the liver by using the Folch method (Folch et al., 1957) and separated via thin-layer chromatography (TLC). Lipids were isolated using a silica G-60 TLC plate and a solvent system consisting of mixture of chloroform:methanol: acetic acid: water (25:15:4:2). Subsequently, visualization was achieved through iodine staining. The modified Bartlett method (Bartlett, 1959) was used to quantify PC concentration by measuring total phosphate. Each sample was treated with perchloric acid and subsequently heated at 180°C for a duration of 2 hours. Following this, a solution of 5% ammonium molybdate and 1-amino-2-naphthol-4-sulfonic acid combined with sodium metabisulfite, and sodium sulfite was added to each tube. These tubes were then subjected to a boiling water bath for a period of 12 minutes. To quantify phosphorus, absorbance was measured at 815 nm. A portion of the sample was allocated for scintillation counting and the radioactivity associated with PC was presented in terms of SRA ($DPM.mmol^{-1}$).

DNA extraction was conducted utilizing the phenol extraction technique. In summary, tissues were homogenized in a solution containing 50 $mmol \cdot L^{-1}$ Tris (pH 8), 1% SDS, 100 $mmol \cdot L^{-1}$ EDTA, and 100 $mmol \cdot L^{-1}$ NaCl. Proteinase K was introduced, reaching a final concentration of 0.8 $g \cdot L^{-1}$, and the samples were left to incubate overnight at 56°C. After this incubation, phenol extraction was carried out. The precipitated DNA was obtained by using isopropanol and sodium acetate. The DNA was then reconstituted in a 10 $mmol \cdot L^{-1}$ Tris buffer (pH 8) containing 0.1 $mmol \cdot L^{-1}$ EDTA. The DNA concentration was assessed using a NanoDrop spectrophotometer, and DPM was measured with a liquid scintillation counter to calculate SRA ($DPM \cdot mg^{-1}$).

3.3.9 **Calculations**

Fractional protein synthesis rates (Ks) were calculated in accordance with prior methods (Nicholas 2008) using the following equation:

$Ks = (SRA_b/SRA_f)*100/t$

In this equation, Ks represents the fractional rate of protein synthesis, represented as a percentage of the respective protein pool synthesized per day. SRA_b signifies the specific radioactivity of protein-bound phenylalanine, SRA_f stands for the specific radioactivity of phenylalanine in the precursor pool (free amino acids), and t represents time (in days) for the incorporation of radioactive phenylalanine into protein.

^{3}H-methyl incorporation into methylated products was calculated as follows:

Rate of ^{3}H-methyl $incorporation_{product} = (SRA_{product} /SRA_{precursor})/time*100$

Fraction of ^{3}H-methyl $incorporation_{creatine} = (SRA_{creatine} /SRA_{precursor}) *100$

In this equation, hepatic free methionine was the precursor used for calculating the rate or fraction of ^{3}H-methyl incorporation into methylated products (such as PC, DNA and creatine).

3.4 Statistical analyses.

All data are presented as mean ± SD, except breakpoint analyses, which are presented as breakpoint ± SE. The methionine requirement was assessed by employing one-slope, dual linear regression, using SAS 9.4 (SAS Institute Inc), following methods previously described (Shoveller et al., 2003a; Munasinghe et al., 2017). This model identifies the optimal division of data points between two regression lines by progressively minimizing the SE in a stepwise manner. Subsequently, methionine requirement or the breakpoint was confirmed by using the highest values

of r^2 and adjusted r^2. If no breakpoint was detected and the best fit was with single linear regression, then the data were presented with a single linear regression. Regression analysis variables were dietary concentration of methionine as the independent variable and methionine incorporation rate into tissue-specific protein or whole-body protein synthesis or transmethylated products as the dependent variable. Our strategy of employing 20 distinct diets to characterize the metabolic reaction to varying dietary methionine concentrations is highly effective in determining the breakpoint or describing the response curve (Courtney-Martin et al., 2008; Elango et al., 2012; Munasinghe et al., 2017). However, this approach has its limitations in that we are unable to quantify a response at a specific dietary level. With each treatment group containing only a single animal, it is not possible to statistically compare one methionine level to another; however, the response over methionine intakes is quantifiable.

Comparative statistical results for dual linear models, in contrast to simple linear models, are provided in Supplemental Table 2. Methionine breakpoints for specific tissues were indicated using the mean ± SE. Once the maximum rate of protein synthesis was achieved at the breakpoint, the fractional protein synthesis rate (Ks) typically entered a plateau phase. In cases where the data exhibited a significant breakpoint ($P < 0.05$), the plateau was modeled with a forced slope of zero. Conversely, data without a breakpoint were subjected to linear regression analysis. A significance level of 0.05 was used to assess the statistical significance of all conducted tests.

3.5 Results

3.5.1 Health and performance of the piglets

The piglets were in good health and were lively starting from the day of the surgery. Their initial weight was 1.61 ± 0.15 kg (mean ± SD), and by the time the experimental diet was introduced on day 4, their weight had increased to 2.35 ± 0.29 kg. On average, they were gaining weight at a rate

of 0.15 ± 0.03 $kg \cdot d^{-1}$. Throughout the 48 hours of test diet feeding, none of the dietary methionine concentrations led to any indications of distress.

3.5.2 **Plasma methionine responses to dietary methionine**

Plasma methionine concentrations were low and constant from 0.32 to 1.42 g methionine /100 g total AAs intake and gradually increased above 1.42 ± 0.24 g methionine /100 g total AA, which fit a dual linear regression model ($r^2 = 0.80$; $P < 0.001$) (Figure 3.1). According to these breakpoint graphs, methionine concentrations plateaued from 1.94 to 2.75 g methionine /100 g total AAs and increased linearly with dietary methionine concentrations >2.75 g/100 g AAs (Figure 3.1).

3.5.3 **Plasma homocysteine, cysteine, and glutathione responses to dietary methionine**

Plasma concentrations of homocysteine ($r^2 = 0.87$; $P < 0.0001$) were positively correlated with dietary methionine levels (Figure 3.2A). Plasma concentrations of cysteine and glutathione did not change with the increasing dietary methionine concentrations (Figure 3.2B and C).

3.5.4 **Liver S-adenosylmethionine (SAM) and S-adenosylhomocysteine (SAH) responses to dietary methionine**

Liver SAM concentrations ($r^2 = 0.63$; $P < 0.0001$) linearly increased with increasing dietary methionine levels (Figure 3.3A), while SAH concentrations did not significantly respond to changing methionine levels in the diet (Figure 3.3B). SAM/SAH ratio ($r^2 = 0.74$; $P < 0.0001$) gradually increased with increasing dietary methionine concentrations (Figure 3.3C).

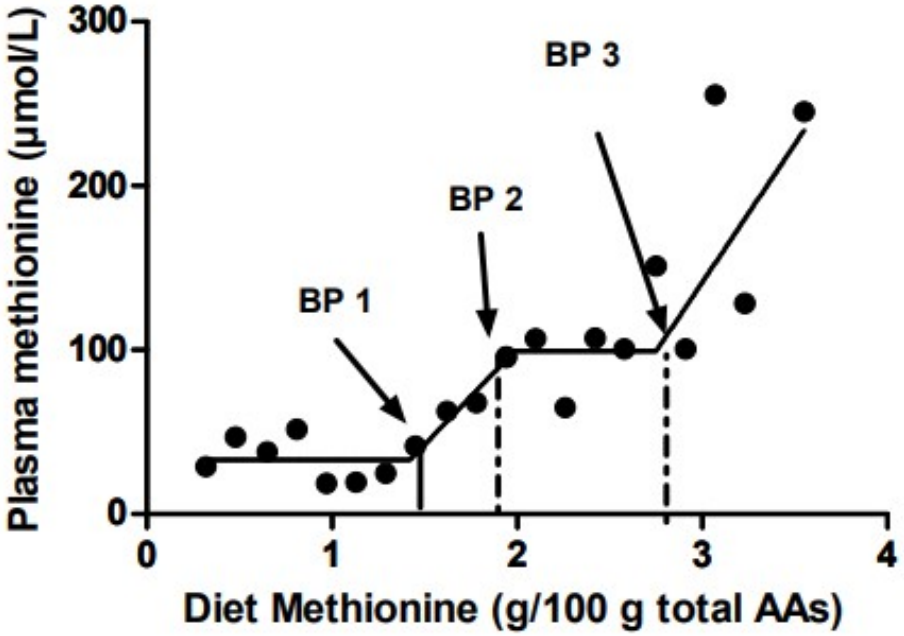

Figure 3.1 Plasma methionine concentration response curve in piglets fed a range of dietary methionine concentrations.

Points represent data from individual pigs; data were regressed by using dual linear regression with breakpoint analysis (indicated by the arrow). The solid vertical line reflects the breakpoint mean methionine requirement; dashed lines refer to breakpoints obtained from visual inspection. BP 1= 1.42 ± 0.24, BP 2= 1.94, BP 3= 2.75. BP, break point; AA, amino acid; diet, dietary.

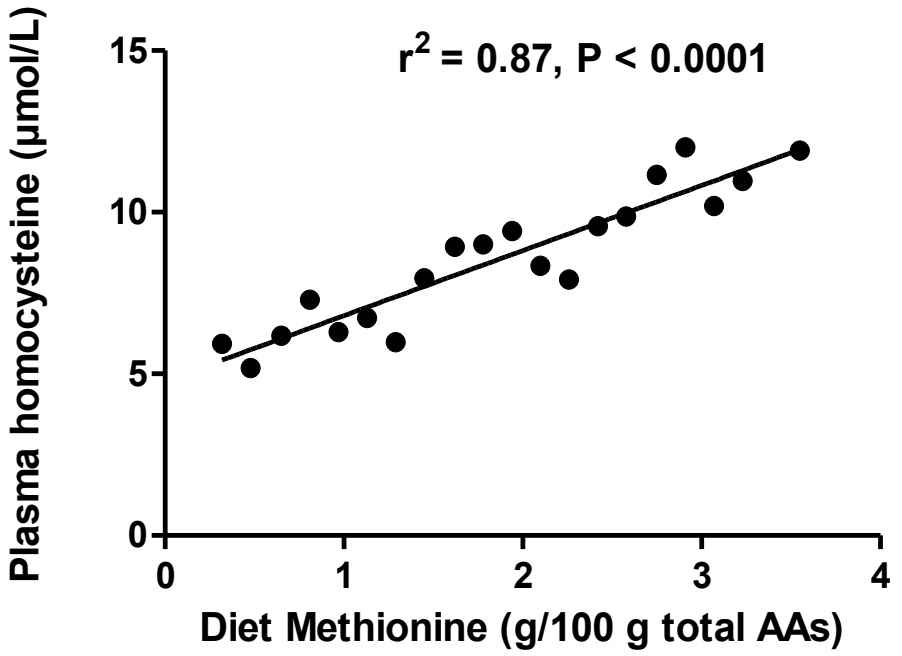

Figure 3.2A

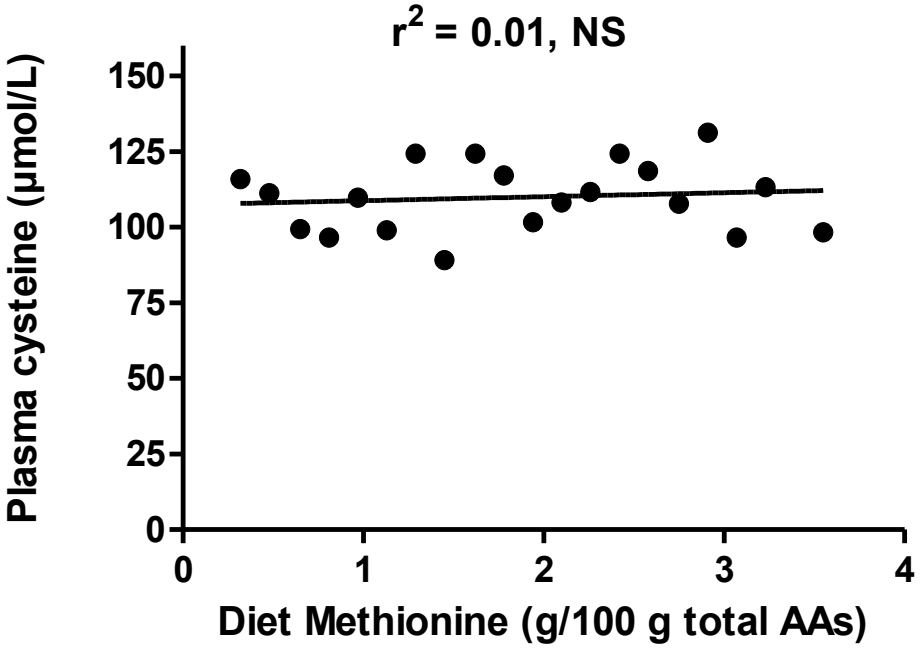

Figure 3.2B

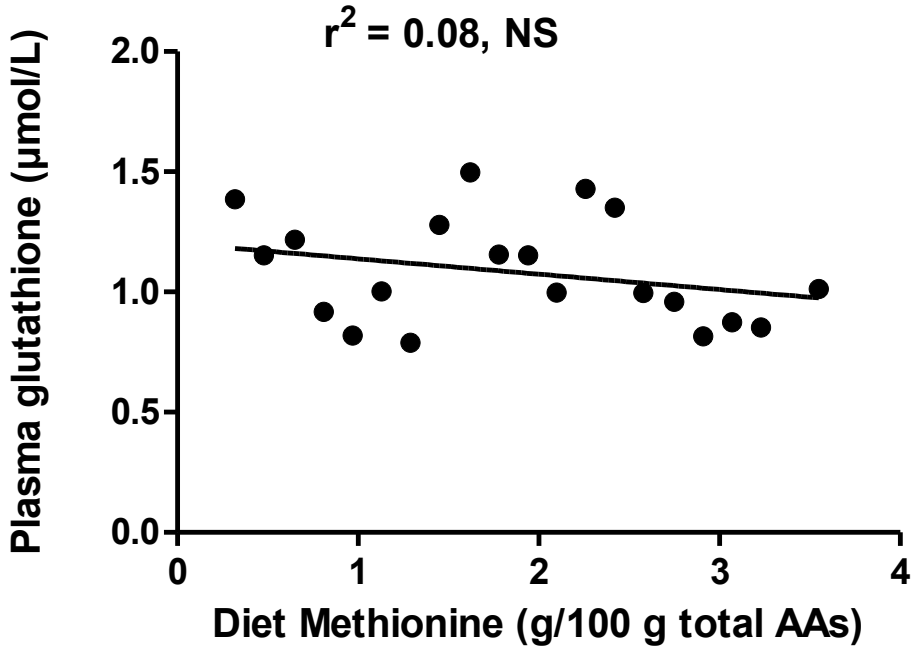

Figure 3.2C

Figure 3.2 Plasma concentrations of homocysteine (A), cysteine (B), glutathione (C) in piglets fed a range of dietary methionine concentrations. Each point represents data from individual pigs; data are regressed by using single linear regression. (NS, $P > 0.05$) AA, amino acid; diet, dietary.

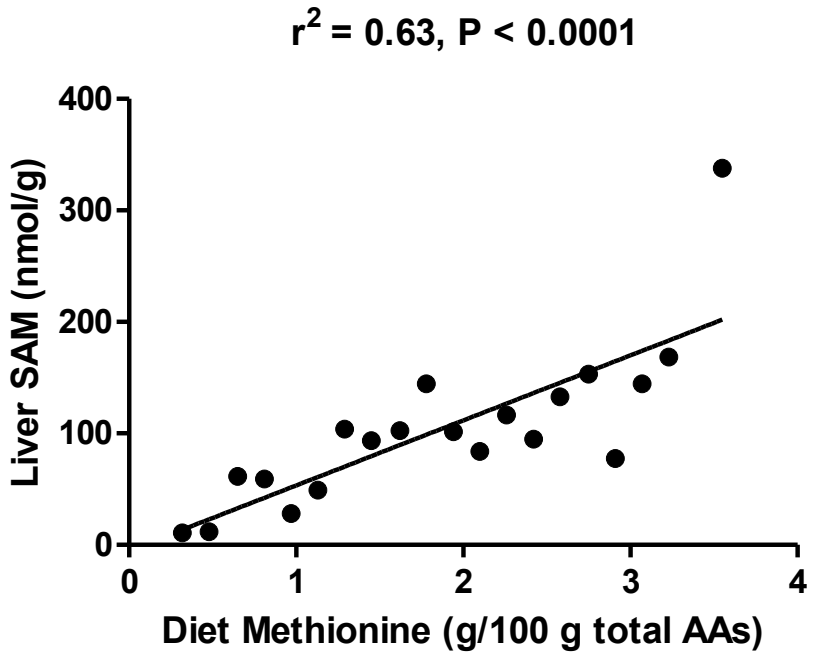

Figure 3.3A

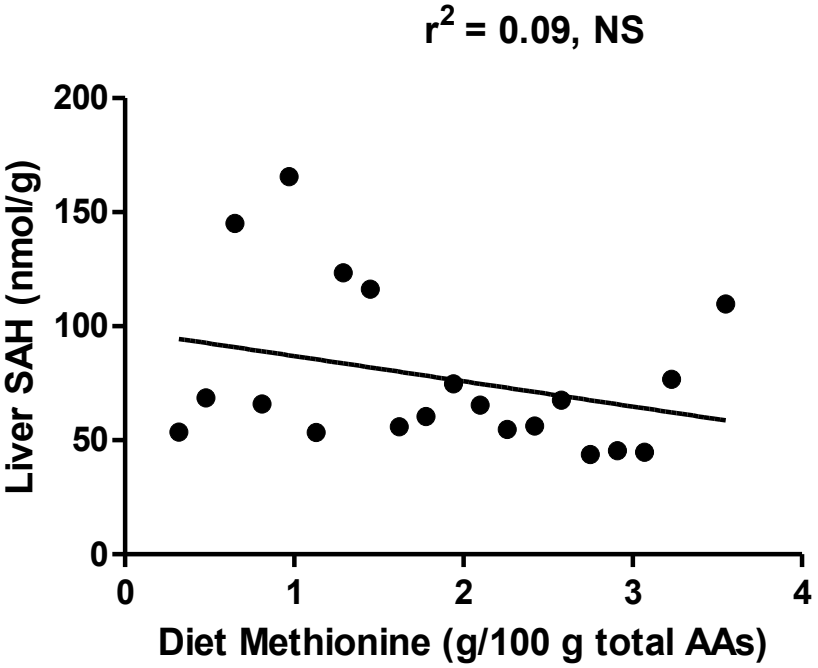

Figure 3.3B

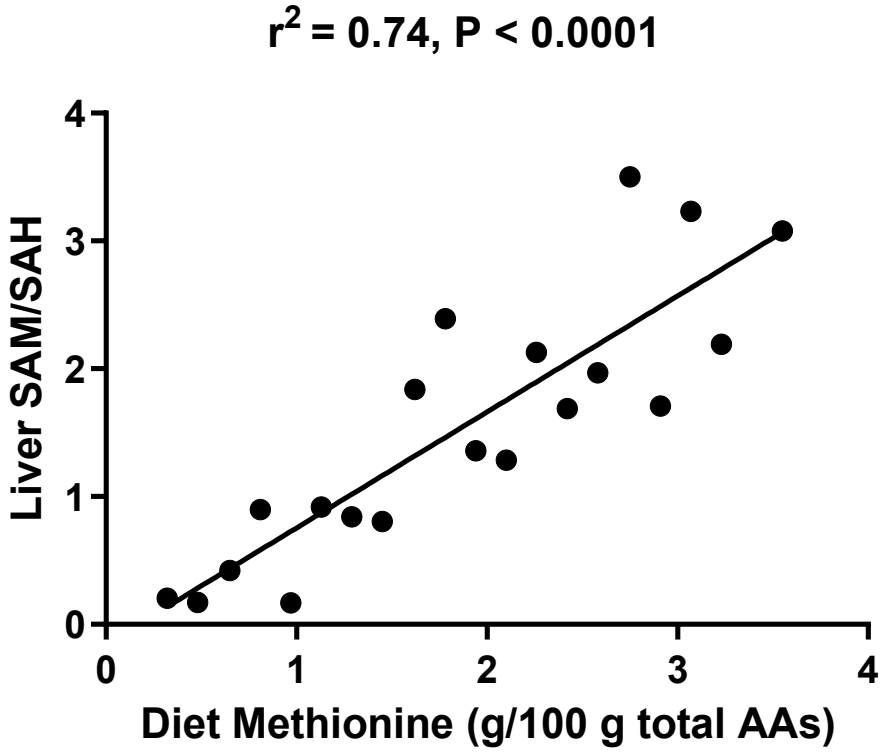

Figure 3.3C

Figure 3.3 Liver concentrations of SAM (A), SAH (B) and SAM/SAH ratio (C) in piglets fed a range of dietary methionine concentrations. Points represent data from individual pigs; data are regressed by using single linear regression. (NS, $P > 0.05$). AA, amino acid; diet, dietary.

3.5.5 **Responses of hepatic transmethylated products to dietary methionine**

The fraction of [^{3}H-methyl] incorporation to creatine and the rates of [^{3}H-methyl] incorporation to DNA and PC were calculated. [^{3}H-methyl] incorporation to creatine was gradually increased over low dietary methionine concentrations and reached a plateau at 1.84 ± 0.22 g methionine /100 g total AAs, which fit a dual linear regression model ($r^2 = 0.72$; $P < 0.0001$) (Figure 3.4A). Similarly, [^{3}H-methyl] incorporation to DNA was maximized at 1.52 ± 0.38 g methionine /100 g total AAs ($r^2 = 0.33$; $P = 0.03$, Figure 3.4B).

[^{3}H-methyl] incorporation to PC was increased linearly with increasing dietary methionine concentrations ($r^2 = 0.53$; $P < 0.0003$, Figure 3.4C) and did not reach a peak within the range of tested methionine concentrations.

3.5.6 **Whole-body protein responses to dietary methionine**

Whole-body protein synthesis increased as dietary methionine concentrations increased from 0.32 to 1.73 g methionine /100 g total AAs and remained constant at dietary methionine concentrations > 1.73 g/100 g total AAs. The breakpoint of 1.73 ± 0.27 g methionine /100 g total AAs ($r^2 = 0.53$; $P = 0.0014$, Figure 3.5) reflects the methionine requirement for whole-body protein synthesis.

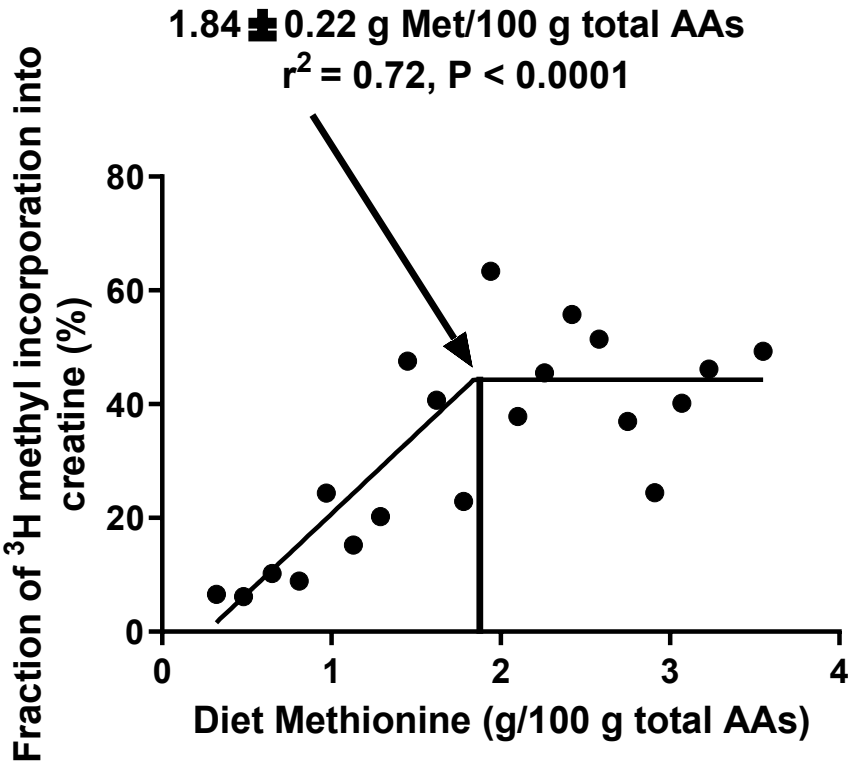

Figure 3.4A

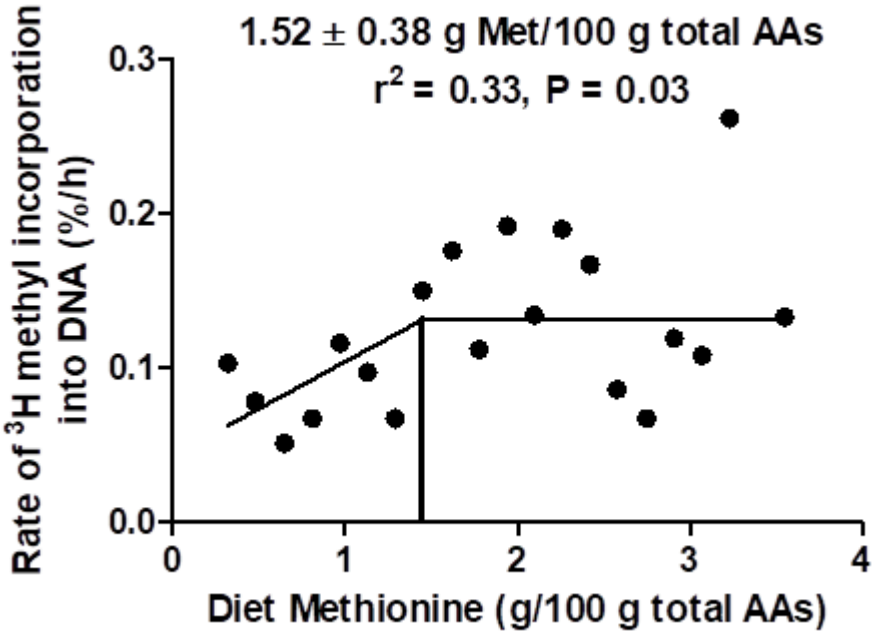

Figure 3.4B

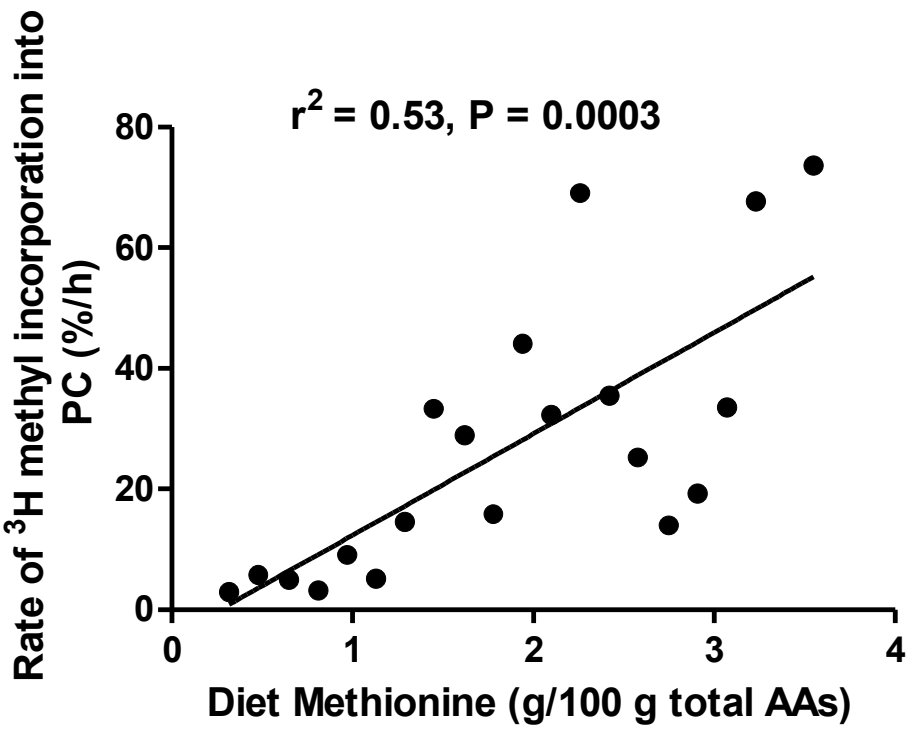

Figure 3.4C

Figure 3.4 Fraction of ^{3}H methyl incorporation into creatine (A), rate of ^{3}H methyl incorporation into DNA (B) and PC (C) in piglets fed a range of dietary methionine concentrations. Points represent data from individual pigs; data are regressed by using dual linear regression with breakpoint analysis (creatine, DNA) or single linear regression (PC). The solid vertical lines represent breakpoints from dual linear regression models. AA, amino acid; diet, dietary

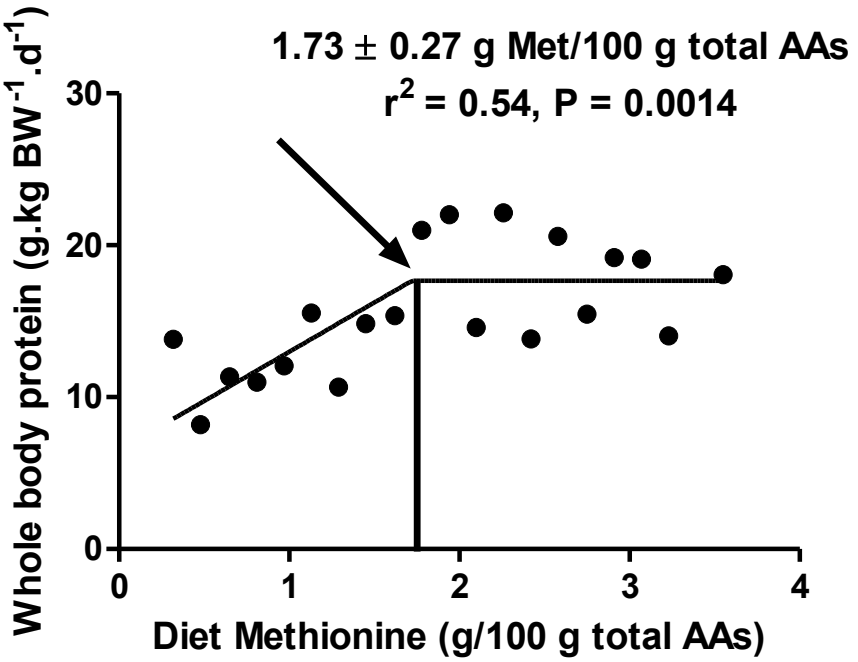

Figure 3.5 Whole body protein synthesis response curve in piglets fed a range of dietary methionine concentrations. Points represent data from individual pigs; data are regressed by using dual linear regression with breakpoint analysis (indicated by the arrow). The solid vertical line reflects the breakpoint mean methionine requirement. AA, amino acid; diet, dietary.

3.5.7 **Tissue-specific protein responses to dietary methionine**

The graphs in Figure 3.6 display the Ks values in relation to varying dietary methionine concentrations for the liver, biceps femoris muscle, kidney, and jejunum. The responses followed the one-slope/dual linear regression model significantly in all tissues except for the jejunum. Details about the breakpoints and the maximal rates during the plateau phase are outlined in Supplemental Table 2. No distinct breakpoint was observed in the jejunum, and the data remained consistent across different dietary methionine concentrations. The liver demonstrated the highest maximum fractional rate of protein synthesis at 104.4% per day, followed by the jejunum at 83.74%, the kidney at 63.52%, and the muscle, which exhibited the lowest rate at 11.84% (Supplemental Table 2). Among the tissues displaying breakpoints, only the liver exhibited a dietary methionine breakpoint below the methionine requirement for whole-body protein synthesis.

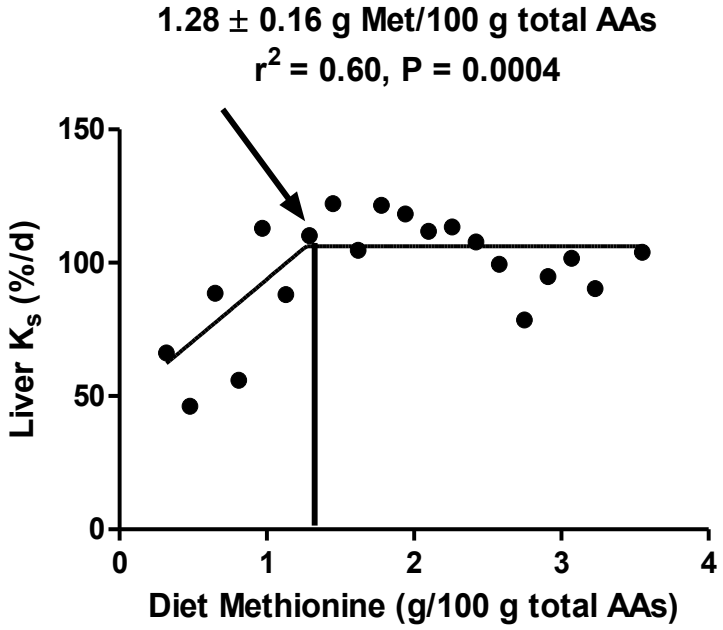

Figure 3.6A

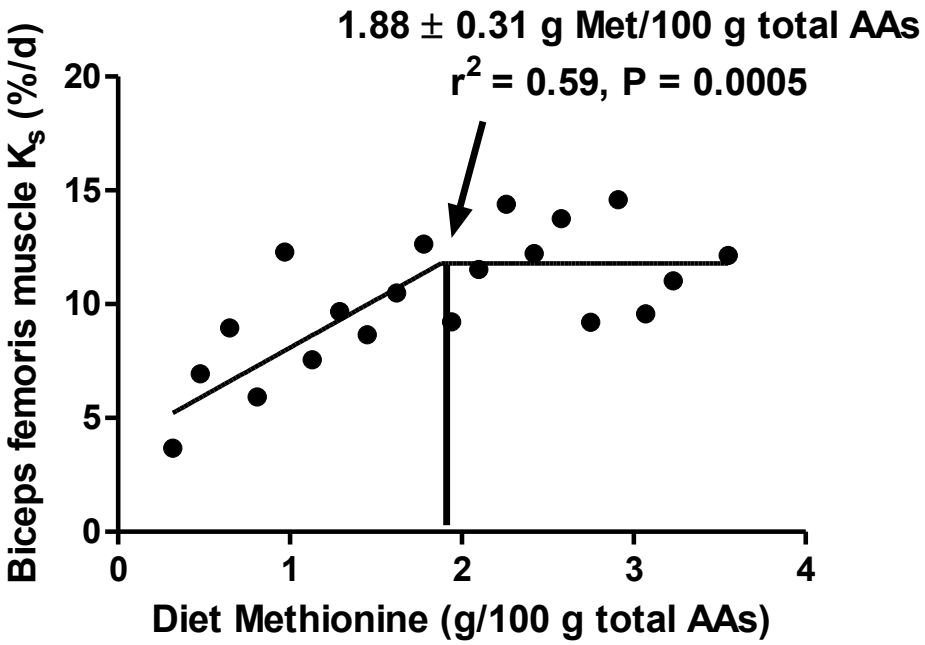

Figure 3.6B

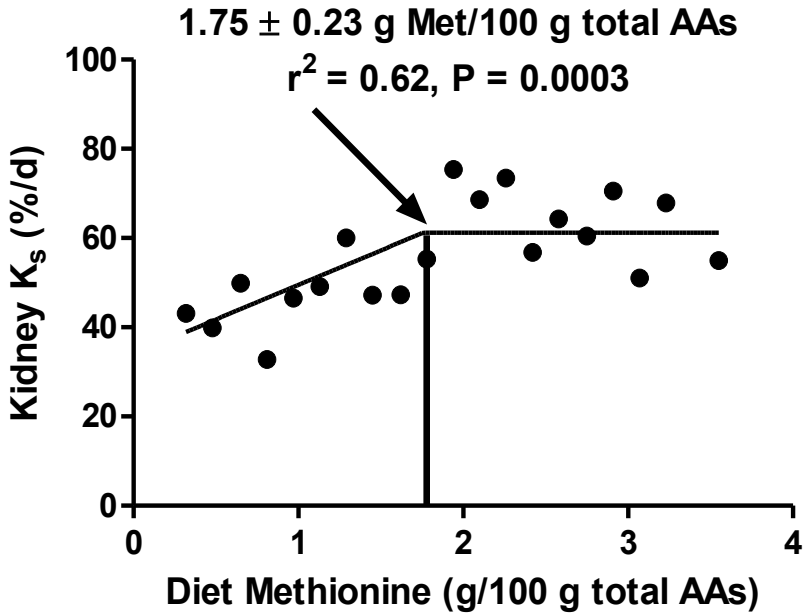

Figure 3.6C

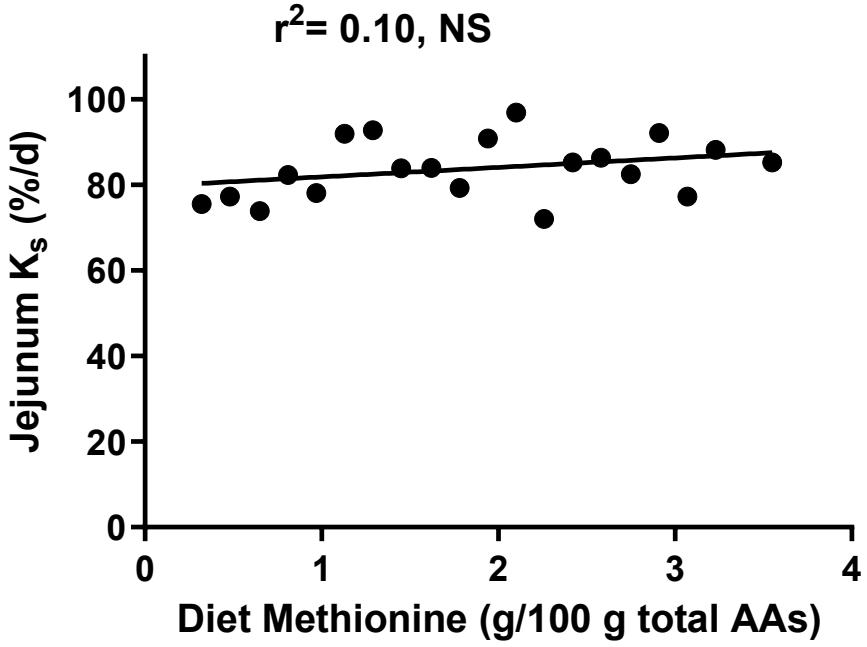

Figure 3.6D

Figure 3.6 Fractional protein synthesis (Ks) (%/d) in tissues [liver (A), Biceps femoris muscle (B), kidney (C), mid jejunum (D)] in piglets fed a range of dietary methionine concentrations. Each point represents data from individual pigs; data are regressed by using dual linear regression with breakpoint analysis (liver, biceps femoris muscle, kidney) or single linear regression (jejunum). The solid vertical lines represent breakpoints from dual linear regression models (Supplemental Table 2). NS, $P > 0.05$. AA, amino acid; diet, dietary; Ks, rate of fractional protein synthesis

Discussion

Methionine requirements are particularly high during early development, due to its increased need for protein synthesis to accommodate growth, and for transmethylation reactions to synthesize various essential metabolites. This enhanced demand requires efficient allocation of available methionine between these two essential functions. Our objective in the present study was to determine the methionine requirement for protein and non-protein pathways in a neonatal model under well-controlled conditions. By feeding diets with increasing levels of methionine, ranging from 0.32 g methionine /100 g total AAs (20% of estimated methionine requirement in piglets; Shoveller et al., 2003a) to 3.55 g methionine /100 g total AAs, we were able to identify breakpoints for these various functions. Because we were interested in the fate of dietary methionine, we employed enteral infusion of tracers to follow the label from dietary methionine to various products, including first pass metabolism, which is substantial in the gut and liver for methionine (Shoveller et al.,2003b; Riedijk et al., 2007; Bertolo et al., 2005). Shoveller et al. (2003) previously established the dietary methionine requirement in neonatal piglets, but only for whole-body protein synthesis measured using the indicator amino acid oxidation rate. However, it appears that transmethylation reactions may utilize a larger quantity of dietary methionine compared to protein synthesis, suggesting that the estimated dietary methionine requirement may be underestimated. We hypothesized that protein synthesis will be maximized at the lowest intakes of dietary methionine, while creatine synthesis will be maximized at a higher intake. A higher intake of methionine will be required to maximize the synthesis of PC, compared to the amounts needed for creatine and protein synthesis.

The findings of the present study indicate that the estimated methionine requirement for whole-body protein synthesis is approximately 1.73 methionine g/100 g total AAs (0.26 $g \cdot kg^{-1} \cdot d^{-1}$), which

aligns closely with the methionine requirement (0.25 $g \cdot kg^{-1} \cdot d^{-1}$) estimated by indicator amino acid oxidation in domestic piglets (Shoveller et al., 2003a). Furthermore, this estimation is approximately consistent with the first breakpoint in plasma methionine (1.42 ± 0.24 methionine g/100 g total AAs) shown in Figure 3.1.

One of the objectives of this study was to identify the hepatic transmethylation pathways that take priority when methionine is limiting. The liver is likely the primary site where methionine partitioning occurs (Walkey et al. 1998; Vance et al. 2007; Bertolo et al., 2013). During the suckling period, creatine accretion utilizes the equivalent of approximately 30% of dietary methionine (Brosnan et al., 2011; Brosnan et al., 2009). In our study, hepatic creatine synthesis was maximized at 1.84 methionine g/100 g total AAs, which was higher than that needed to maximize kidney and liver protein synthesis. These data demonstrate that if dietary methionine intake is deficient, then hepatic creatine synthesis is likely compromised at the expense of maintenance of hepatic protein synthesis. This observation suggests organ growth and function is prioritized over creatine synthesis.

Our previous studies utilizing [^{3}H- methyl] methionine indicated that a larger quantity of methionine is allocated for hepatic PC synthesis compared to creatine synthesis in neonatal piglets (McBreairty et al., 2013). According to Robinson et al (2016a), a higher fractional synthesis rate of PC corresponded to a lower fractional synthesis rate of creatine, when dietary methyl donors were limiting. Conversely, our findings indicate that when dietary methionine, the primary methyl donor, is limited (i.e. less than 2 g/100 g AA), the synthesis of creatine is similar to the synthesis of PC. However, it should be noted that Robinson et al. (2016a) also excluded choline in their methyl-deficient diets, which would limit PC synthesis via the CDP-choline pathway. At higher levels, PC synthesis was highly variable and did not reach a plateau within the tested range of

dietary methionine intakes, suggesting PC synthesis responds to dietary methionine at levels well above that for creatine synthesis. Several factors could have influenced why PC synthesis did not reach a plateau. Although we measured PC synthesis via the PEMT pathway, the CDP-choline pathway might be capable of satisfying PC synthesis, even when dietary methionine is limited. However, with increased methionine levels, PC synthesis might be promoted through the PEMT pathway, at the expense of the other pathway. As a result, a balance in PC synthesis may be achieved through the combined utilization of both pathways, although both pathway fluxes need to be measured to determine this interaction. Moreover, measurement of these hepatic pathways are further complicated by the independent complexity of hepatic release mechanisms for both PC and creatine (Brosnan et al., 2009; Vance et al., 2008).

DNA methylation rate was maximized at 1.52 methionine g/100 g total AAs, which appeared lower than the requirement for creatine and higher than that of liver protein synthesis. This result emphasizes the importance of DNA methylation, which is prioritized over creatine synthesis in neonatal piglets when dietary methionine intake is limiting. DNA methylation is a vital epigenetic alteration that plays an essential role in regulating gene expression, cellular differentiation, development, and the overall stability of the genome (Bird et al., 2002). Therefore, at low methionine intakes, it makes sense that DNA methylation gets prioritized over other trans-methylated products and some tissue-specific proteins.

In studies of sulfur amino acid deficiency (Bauchart-Thevret et al., 2009) and methyl donor deficiency (Robinson et al., 2016b), piglets prioritized methionine flux to whole body protein synthesis over transmethylation. However, our results demonstrate that the breakpoints for whole body protein (1.73 g/100 g AA), muscle (1.88 g/100 g AA) and creatine (1.84 g/100 g AA) were all similar, suggesting at lower dietary intakes, they all had the same priority. However,

transmethylation includes PC synthesis, which was not maximized in the range of intake tested but would have led to a higher breakpoint when combined with creatine (i.e. transmethylation) compared to that for whole body protein synthesis.

The liver seems to conserve protein synthesis at lower methionine intakes, similar to when methyl groups are restricted (Robinson et al., 2016d). The conservation of liver protein synthesis suggests that the liver regulates the distribution of methionine among various pathways. (Robinson et al., 2016d). Studies have shown that among all the measured products in the liver, protein accounted for only ~20-33% of [^{3}H-methyl] methionine incorporation (Robinson et al., 2016a. McBreairty et al., 2013). But it should be noted that a significant proportion of liver proteins are secreted (Anderson 1997). Indeed, the liver expresses the top 10 transcripts for secretory proteins and contributes to various physiological processes in the body (Uhlen et al., 2015). Consistent with this, the liver displayed the highest maximal rate of protein synthesis among all the measured tissues. Because the contributions of already secreted proteins are not captured in these tissue-specific fractional synthesis rate techniques, protein synthesis estimates are likely underestimated for secretory tissues like the liver. Given its significant role in protein metabolism and overall physiological functions, it is not unexpected that hepatic protein synthesis is responsive to dietary methionine supply.

Jejunum has higher protein synthesis rates than the kidney and muscle, which is consistent with its substantial protein requirements associated with high cell turnover rates. Absolutely, the gut undergoes a complete regeneration process approximately every 5-6 days (Lipkin 1985). Even though the gut represents only 4-6% of the total body mass, it contributes to approximately 35% of the overall protein turnover in the entire body (Stoll et al., 2006). In the current study, protein synthesis in jejunum remains unaffected even with lower methionine content, in contrast to all

other tissues measured. This suggests that jejunum tissues might prioritize dietary methionine for their protein synthesis needs, potentially at the expense of other tissues. Around 30% of the dietary methionine is extracted during the splanchnic first-pass metabolism (Shoveller et al., 2003b), and 20% of dietary methionine is metabolized by the gut in neonatal piglets (Riedijk et al., 2007). So the jejunum can extract sufficient methionine from dietary sources even when methionine availability is low; this further limits methionine for all other tissues and organs.

The methionine requirement to maximize protein synthesis in the kidney was 1.75 methionine g/100g total AAs, which was higher than that of the liver. These data demonstrate that the protein synthesis in these tissues is maintained when dietary methionine is deficient. Taken together, the results of this study illustrate that dietary methionine is preferentially used for protein synthesis in certain tissues, over trans-methylated products.

The dynamic nature of protein metabolism is particularly significant for piglets at this stage of development, as their tissues undergo rapid synthesis and degradation to grow and expand (Reeds et al., 2000; Dupont et al., 2003). The biceps femoris muscle protein reached its maximum synthesis rate at an intake of 1.88 methionine g/100g of AAs, which is higher than the methionine requirement for liver and kidney protein synthesis. Muscle is often considered a corollary of whole body growth; indeed, the SE for muscle's breakpoint overlapped the whole body methionine requirement (minus SE) and so were not considered different. Insufficient dietary methionine intake could lead to potential compromises in muscle growth and the synthesis of other transmethylated products, in order to protect liver and kidney protein synthesis and function. Indeed, these findings align with the results from our other studies conducted in pigs. In piglets that were provided with a diet lacking sufficient methyl donors, there was a notable 50% reduction in protein synthesis observed in both skeletal muscle and the jejunum, but not liver (Robinson

2016d). A consistent finding among these pig studies is that methionine deficiency consistently leads to lower muscle protein synthesis, emphasizing methionine's critical role in supporting muscle protein synthesis. Moreover, given that muscle tissue constitutes the largest reservoir of amino acids in the body, it is reasonable to expect that this tissue would lower its protein synthesis rate during methionine deficiency to spare amino acids for more important maintenance functions in other tissues. Even a slight decrease in the rate of protein synthesis in muscles would result in a significant conservation of methionine, which other metabolically important tissues in the body can then utilize.

Our study employed a brief adaptation period of 24-48 hours to the different dietary methionine concentrations, which has been validated in amino acid requirement studies given the sensitivity of protein synthesis and amino acid oxidation to dietary supply (Elango 2008). This short adaptation time is particularly sufficient due to the rapid growth rate of piglets, which increases sensitivity to dietary changes (Kim 1983). Several investigations have been conducted in young and adult pigs and humans, exploring adaptation times ranging from hours to weeks. These studies consistently found no significant differences in protein synthesis or oxidation measurements (Elango et al., 2008; Elango et al., 2009; Moehn et al., 2004). As amino acid requirement studies are primarily based on the partitioning of amino acids between protein synthesis and oxidation, only changes in acyl-transfer RNA levels are needed to influence the measured outcomes, and these pools can adapt within a short time frame of less than 4 hours (Crim 1994). Moreover, studies from our lab have also shown that transmethylated products can rapidly adjust to changes in methionine intakes (Robinson et al., 2016b). In this study, our aim was to determine partitioning among existing enzymes when methionine is deficient. While we can detect acute changes in

protein synthesis, these dietary methionine concentrations would likely need to be administered for a more extended period before measurable effects on tissue function become apparent.

The plasma methionine response to varying dietary methionine concentrations (Figure 3.1) is consistent with data from other experiments to determine amino acid requirements (Munasinghe et al., 2017; House et al., 1997). The deviation from a linear methionine concentration after 1.49 methionine g/100 g total AAs intake supports the existence of a sigmoidal response curve in plasma methionine in response to increasing intakes, or a series of linear breakpoints. Hence, with a sufficient range of dietary methionine intakes, it could be feasible to determine not only one breakpoint or mean methionine requirement, but also to identify second and third breakpoints. There were not enough data points available for a thorough statistical analysis of multiple breakpoints in our study, but a visual examination indicated a second breakpoint at 1.94 methionine g/100 g total AAs intake, and a third breakpoint at 2.75 methionine g/100 g total AAs intake. When dietary methionine is deficient, plasma methionine concentrations are low and constant because additional methionine is likely immediately incorporated into protein, especially in the liver. In excess of this 'organ' requirement (Breakpoint 1; Figure 3.1), methionine is available in the plasma to be taken up by muscle for protein synthesis, and by various tissues for other non-protein pathways such as synthesis of PC, creatine, and DNA. Following the saturation of muscle protein synthesis and non-protein pathways (Breakpoint 2; Figure 3.1), any surplus methionine is delivered to the liver for oxidation until the capacity of the oxidation enzymes becomes saturated (Breakpoint 3; Figure 3.1). Above dietary intakes of 2.75 g/100 g AAs, excess methionine cannot be oxidized in the liver and must be released into the bloodstream and transported to the kidneys for excretion. This process leads to the accumulation of methionine in the plasma pool.

The allosteric control of methionine metabolism is governed by the SAM molecule, with its concentration reflecting the availability of methyl groups (Robinson et al., 2016c, Finkelstein 1986). SAM and SAH concentrations serve as indicators of methyl group availability when expressed individually or as a ratio. Our findings revealed that the hepatic SAM concentration rises with an increase in methionine levels in the diet, which is consistent with numerous studies indicating that hepatic SAM concentrations also increase in the presence of an abundance of methyl groups (Finkelstein 1986; Zeisel 1989; Balaghi 1993). SAH acts as an inhibitor of transmethylation reactions (Finkelstein 2000). However, the current study demonstrated that there is no notable alteration in SAH levels as dietary methionine intake rises, aligning with the observed increase in transmethylation pathways such as PC synthesis.

The positive correlation of plasma homocysteine and dietary methionine was similar to that reported in several other studies in animals and humans (Courtney et al., 2008; Shoveller et al., 2004). Elevated plasma homocysteine levels were measured with higher intakes of methionine, so it is likely that SAM was readily converted to homocysteine via transmethylation, which increased with each increment of dietary methionine. As hyperhomocysteinemia is a potential risk factor for cardiovascular diseases (Hogeveen et al., 2002), any adjustments made to dietary formulations to increase methionine intake should be approached with caution to avoid the development of hyperhomocysteinemia. Alternatively, methyl donors such as betaine and choline can be used to rapidly remethylate newly synthesized homocysteine back to methionine, perhaps lowering the need for higher methionine intakes.

Our current data shows that plasma cysteine and glutathione did not significantly correspond to dietary methionine changes. Although accumulating homocysteine was converted into cysteine, it rapidly enters disposal pathways in the liver, reflecting a constant level of cysteine in the plasma.

When animals are adequately supplied with cysteine, the primary function of the transsulfuration pathway is the oxidation of methionine and homocysteine, rather than the synthesis of cysteine, which appears consistent with its robust control by SAM. This control mechanism aims to limit methionine/homocysteine degradation during periods of low methionine availability and to facilitate it when methionine levels are elevated. The synthesis of cysteine as an outcome of the transsulfuration pathway can be regarded as a component of the methionine/homocysteine degradation process, where cysteine serves as the channel for converting sulfur from methionine/homocysteine into end products that can be expelled through urine (Stipanuk et al., 2011).

3.6 Conclusions

Through the analysis of breakpoints of various metabolites, we evaluated how dietary methionine is distributed among hepatic transmethylated products and target organs for protein synthesis, especially as dietary methionine becomes limited. These findings also contribute to a better understanding of the dietary methionine required for optimal growth in neonates. Since not all tissues have the same methionine requirement, our data shed light on why growth is constrained before impacting intestinal function and the critical metabolic processes in other vital organs. By determining tissue-specific dietary methionine requirements through breakpoints, we can estimate the precise amount of dietary methionine needed to sustain protein synthesis and transmethylation patterns in each specific tissue. This information is valuable for designing nutritionally balanced diets that support optimal growth and development in neonates and other populations.

This study quantified dietary methionine requirements for hepatic transmethylation, tissue-specific protein synthesis, and whole-body protein synthesis in neonates. By analyzing these breakpoints, we were able to evaluate the partitioning of dietary methionine to methylated products and the

tissue-specific proteins when dietary methionine is limiting. We concluded that hepatic proteins and hepatic DNA methylation are prioritized over hepatic creatine synthesis while PC synthesis continuously increases with methionine intake. The demonstrated plasticity of critical transmethylation reactions in response to methionine availability highlights the importance of ensuring adequate methionine intake in infants. Our data also concluded that when methionine was limited, liver protein synthesis was prioritized, followed by kidney and muscle protein synthesis. Moreover, because remethylation accounts for 7 - 10% of the whole-body methionine flux (Robinson et al., 2016b; Riedijket al., 2007), a deficiency in methyl donors may also affect the methionine requirement. Hence, optimal nutrition, including sufficient methionine levels, is crucial for supporting essential cellular processes that rely on transmethylation reactions during early development and growth. The significance of transmethylation cannot be emphasized enough, as it serves as a major consumer of dietary methionine. The metabolic ability to support this critical process is vital for meeting the methionine requirements in pediatric nutrition and is essential for promoting healthy development in infants and children. These data help further define the dietary requirement of methionine to accommodate all fates of methionine in neonates. Since more methionine is required to meet non-protein demands, protein synthesis should not solely be used to determine whole-body requirements for methionine.

3.7 References

Anderson L, Seilhamer J. A comparison of selected mRNA and protein abundances in human liver. Electrophoresis. 1997;18:533–7.

Balaghi M, Horne DW, Wagner C. Hepatic one-carbon metabolism in early folate deficiency in rats. Biochem. J. 993;291(1):145-9.

Bartlett GR. Phosphorus assay in column chromatography. J Biol Chem. 1959;234: 466-468.

Bauchart-Thevret C, Stoll B, Burrin DG. Intestinal metabolism of sulfur amino acids. Nutr Res Rev. 2009;22(2):175-87.

Bertolo RF and McBreairty LE. The nutritional burden of methylation reactions. Curr Opin Clin Nutr Metab Care. 2013; 16(1): 102-108.

Bertolo RF, Chen CZ, Pencharz PB, Ball RO. Intestinal atrophy has a greater impact on nitrogen metabolism than liver by-pass in piglets fed identical diets via gastric, central venous or portal venous routes. J Nutr. 1999;129:1045–52.

Bertolo RF, Pencharz PB, Ball RO. Role of intestinal first-pass metabolism on whole-body amino acid requirements. InBiology of growing animals. 2005;3:127-156.

Bidlingmeyer BA, Cohen SA, Tarvin TL. Rapid analysis of amino acids using pre-column derivatization. J Chromatogr B Biomed Sci Appl. 1984;336:93-104.

Bird A. DNA methylation patterns and epigenetic memory. Genes Dev. 2002;16:6–21.

Brosnan JT, da Silva RP, Brosnan ME. The metabolic burden of creatine synthesis. Amino Acids 2011;40:1325–31.

Brosnan JT, Wijekoon EP, Warford-Woolgar L, Trottier NL, Brosnan ME, Brunton JA, Bertolo RF. Creatine synthesis is a major metabolic process in neonatal piglets and has important implications for amino acid metabolism and methyl balance. J Nutr. 2009;139: 1292–7

Cordero P, Gomez-Uriz AM, Campion J, Milagro FI, Martinez JA. Dietary supplementation with methyl donors reduces fatty liver and modifies the fatty acid synthase DNA methylation profile in rats fed an obesogenic diet. Genes Nutr. 2013;8:105–13

Courtney-Martin G, Chapman KP, Moore AM, Kim JH, Ball RO, Pencharz PB. Total sulfur AA requirement and metabolism in parenterally fed postsurgical human neonates. Am J Clin Nutr. 2008;88:115–24.

Crim MC, Munro HN. Proteins and amino acids. Modern nutrition in health and disease. 1994;1:16-8.

de Betue CT, Casal XCG, van Waardenburg DA, Schexnayder SM, Joosten KF, Deutz NE, Engelen MP. 24-Hour protein, arginine and citrulline metabolism in fed critically ill children–A stable isotope tracer study. Clin Nutr. 2017;36(3):876-887.

Dinesh OC, Kankayaliyan T, Rademacher M, Tomlinson C, Bertolo RF, Brunton JA. Neonatal piglets can synthesize adequate creatine, but only with sufficient dietary arginine and methionine, or with guanidinoacetate and excess methionine. J Nutr. 2021;151:531-539.

Dodge ME, Bertolo RF, Brunton JA. Enteral feeding induces early intestinal adaptation in a parenterally fed neonatal piglet model of short bowel syndrome. J Parenter Enteral Nutr. 2012;36:205–12.

Dupont C. Protein requirements during the first year of life. Am J Clin Nutr. 2003;77:1544S–9S.

Edison EE, Brosnan ME, Aziz K, Brosnan JT. Creatine and guanidinoacetate content of human milk and infant formulas: implications for creatine deficiency syndromes and amino acid metabolism. Br J Nutr. 2013;110:1075–8.

Elango R, Ball RO, Pencharz PB. Individual AA requirements in humans: an update. Curr Opin Clin Nutr Metab Care. 2008;11:34–9.

Elango R, Ball RO, Pencharz PB. Recent advances in determining protein and AA requirements in humans. Br J Nutr. 2012;108(2):S22–30.

Elango R, Humayun MA, Ball RO, Pencharz PB. Indicator AA oxidation is not affected by period of adaptation to a wide range of lysine intake in healthy young men. J Nutr. 2009;139:1082–7

Finkelstein JD, Martin JJ. Methionine metabolism in mammals. Adaptation to methionine excess. J Biol Chem. 1986;261:1582–7.

Finkelstein JD. Pathways and regulation of homocysteine metabolism in mammals. Semin Thromb Hemost. 2000;26:219–25.

Folch J, Lees M and Stanley GHS. A simple method for the isolation and purification of total lipids from animal tissues. J Biol Chem. 1957; 226: 497-509.

Garlic PJ, McNurlan MA, Preedy VR. A rapid and convenient technique for measuring the rate of protein synthesis in tissues by injection of [3H] phenylalanine. Biochem J. 1980;192(2):719-723.

Hogeveen M, Blom HJ, Van Amerongen M, Boogmans B, Van Beynum IM, Van De Bor M. Hyperhomocysteinemia as a risk factor for ischemic and hemorrhagic stroke in newborn infants. J Pediatr. 2002;141:429 –31

House JD, Pencharz PB, Ball RO. Phenylalanine requirements determined by using L-[1-14C] phenylalanine in neonatal piglets receiving total parenteral nutrition supplemented with tyrosine. Am J Clin Nutr. 1997;65(4):984-93.

Kim KI, McMillan I, Bayley HS. Determination of AA requirements of young pigs using an indicator AA. Br J Nutr. 1983;50:369–82.

Kotsopoulos J, Sohn K-J, Kim Y-I. Postweaning dietary folate deficiency provided through childhood to puberty permanently increases genomic DNA methylation in adult rat liver. J Nutr. 2008;138:703–9.

Lamarre SG, Edison EE, Wijekoon EP, Brosnan ME, Brosnan JT. Suckling rat pups accumulate creatine primarily via de novo synthesis rather than from dam milk. J Nutr. 2010;140:1570-1573.

Lamarre SG, MacMillan L, Morrow GP, Randell E, Pongnopparat T, Brosnan ME, Brosnan JT. An isotope-dilution, GC–MS assay for formate and its application to human and animal metabolism. Amino acids 2014;46(8):1885-1891

Lipkin M. Growth and development of gastrointestinal cells. Annu Rev Physiol. 1985;47:175–97.

McBreairty LE, McGowan RA, Brunton JA and Bertolo RF. Partitioning of [methyl-3H] methionine to methylated products and protein is altered during high methyl demand conditions in young Yucatan miniature pigs. J Nutr. 2013;143:804-809

McMillen IC, Robinson JS. Developmental origins of the metabolic syndrome: prediction, plasticity, and programming. Physiol Rev. 2005;85:571–633.

Moehn S, Bertolo RF, Pencharz PB, Ball RO. Indicator AA oxidation responds rapidly to changes in lysine or protein intake in growing and adult pigs. J Nutr. 2004;134:836–41.

Munasinghe LL, Robinson JL, Harding SV, Brunton JA, & Bertolo RF. Protein synthesis in mucin-producing tissues is conserved when dietary threonine is limiting in piglets. J Nutr. 2017;147(2):202-210.

National Research Council. Nutrient requirements of swine. Washington, DC: National Acadamies Press; 2012

Nichols NL, Bertolo RF. Luminal threonine concentration acutely affects intestinal mucosal protein and mucin synthesis in piglets. J Nutr. 2008;138:1298-303.

Niculescu MD, Zeisel SH. Diet, methyl donors and DNA methylation: interactions between dietary folate, methionine and choline. J Nutr. 2002;132:2333S–5S

Pfeiffer CM, Huff DL and Gunter EW. Rapid and accurate HPLC assay for plasma total homocysteine and cysteine in a clinical laboratory setting. Clin Chem. 1999;45:290-292.

Ratnam S, Wijekoon EP, Hall B, Garrow TA, Brosnan ME, Brosnan JT. Effects of diabetes and insulin on betaine-homocysteine S-methyltransferase expression in rat liver. Am J Physiol Endocrinol Metab. 2006;290:E933–9.

Reeds PJ, Burrin DG, Davis TA, Fiorotto ML, Stoll B, van Goudoever JB. Protein nutrition of the neonate. Proc Nutr Soc. 2000;59:87–97

Riedijk MA, Stoll B, Chacko S, Schierbeek H, Sunehag AL, van Goudoever JB, Burrin DG. Methionine transmethylation and transsulfuration in the piglet gastrointestinal tract. Proceedings of the National Academy of Sciences 2007;104(9):3408-13.

Robinson JL, Bartlett RK, Harding SV, Randell EW, Brunton JA, Bertolo RF. Dietary methyl donors affect in vivo methionine partitioning between transmethylation and protein synthesis in the neonatal piglet. Amino Acids. 2016b;48(12):2821-30.

Robinson JL, Bertolo RF. The pediatric methionine requirement should incorporate remethylation potential and transmethylation demands. Adv Nutr. 2016c;7(3):523-34.

Robinson JL, Harding SV, Brunton JA, Bertolo RF. Dietary methyl donors contribute to whole-body protein turnover and protein synthesis in skeletal muscle and the jejunum in neonatal piglets. J Nutr. 2016 d;146(10):2007-12.

Robinson JL, McBreairty LE, Randell EW, Brunton JA, Bertolo RF. Restriction of dietary methyl donors limits methionine availability and affects the partitioning of dietary methionine for creatine and phosphatidylcholine synthesis in the neonatal piglet. J Nutr Biochem. 2016 a;35:81-6.

Shoveller AK, Brunton JA, House JD, Pencharz PB and Ball RO. Dietary cysteine reduces the methionine requirement by an equal proportion in both parenterally and enterally fed piglets. J Nutr 2003a.;133:4215-4224.

Shoveller AK, Brunton JA, Pencharz PB, Ball RO. The methionine requirement is lower in neonatal piglets fed parenterally than in those fed enterally. J Nutr. 2003b;133:1390–1397.

Shoveller AK, House JD, Brunton JA, Pencharz PB, Ball RO. The balance of dietary sulfur amino acids and the route of feeding affect plasma homocysteine concentrations in neonatal piglets. J Nutr. 2004;134:609 –12

Stead LM, Au KP, Jacobs RL, Brosnan ME and Brosnan JT. Methylation demand and homocysteine metabolism: effects of dietary provision of creatine and guanidinoacetate. Am J Physiol Endocrinol Metab. 2001;281:E1095-E1100.

Stipanuk MH, Ueki I. Dealing with methionine/homocysteine sulphur: cysteine metabolism to taurine and inorganic sulphate. Biochem J. 2011;436(3):609–622.

Stoll B, Burrin DG. Measuring splanchnic amino acid metabolism in vivo using stable isotopic tracers. J Anim Sci. 2006;84(l):E60–72.

Thivierge MC, Bush JA, Suryawan A, Nguyen HV, Orellana RA, Burrin DG, Jahoor F, Davis TA. Whole body and hindlimb protein breakdown are differentially altered by feeding in neonatal piglets. J Nutr. 2005;135:1430–7.

Thompson GN, Pacy PJ, Merritt H, Ford GC, Read MA, Cheng KN, Halliday D. Rapid measurement of whole body and forearm protein turnover using a [2H5] phenylalanine model. Am J Physiol Endocrinol Metab. 1989;256(5):E631-9.

Tomlinson C, Rafii M, Sgro M, Ball RO, Pencharz P. Arginine is synthesized from proline, not glutamate, in enterally fed human preterm neonates. Pediatr Res. 2011;69(1):46-50

Uhlén M, Fagerberg L, Hallström BM, Lindskog C, Oksvold P, Mardinoglu A, Sivertsson Å, Kampf C, Sjöstedt E, Asplund A, Olsson I. Tissue-based map of the human proteome. Science. 2015;347(6220):1260419.

Vance DE. Role of phosphatidylcholine biosynthesis in the regulation of lipoprotein homeostasis. Curr Opin Lipidol. 2008;19:229–34.

Vester B, Rasmussen K. High-performance liquid chromatography method for rapid and accurate determination of homocysteine in plasma and serum. Clin Chem Lab Med. 1991;29:549-554.

Walkey CJ, Yu L, Agellon LB, Vance DE. Biochemical and evolutionary significance of phospholipid methylation. J Biol Chem. 1998;273(42):27043-6.

Zeisel SH, Zola T, daCosta KA, Pomfret EA. Effect of choline deficiency on S adenosylmethionine and methionine concentrations in rat liver. Biochem J. 1989;259:725–9.

Chapter 4: Dietary methionine enhances portal appearance of guanidinoacetate and synthesis of creatine in Yucatan miniature piglets

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Chapter 4 was designed by M. Asiriwardhana, O. Dinesh and R. Bertolo. M. Asiriwardhana and O. Dinesh conducted the animal experiments, and all other research and data analyses were conducted by M. Asiriwardhana. The manuscript was prepared by M. Asiriwardhana with guidance from R. Bertolo. The final manuscript was read and approved by all authors.

4.1 Abstract

Background: Creatine plays a significant role in energy metabolism and positively impacts anaerobic energy capacity, muscle mass, and physical performance. Endogenous creatine synthesis requires guanidinoacetic acid (GAA) and methionine. GAA can be an alternative to creatine supplements and has been tested as a beneficial feed additive in the animal industry. When pigs are fed GAA with excess methionine, creatine is synthesized without feedback regulation. In contrast, when dietary methionine is limiting, creatine synthesis is limited, yet GAA does not accumulate in plasma, urine, or liver.

Objective: We hypothesized that portal GAA appearance requires adequate dietary methionine.

Methods: Yucatan miniature piglets (17–21-day old; n=20) were given a 4 h duodenal infusion of complete elemental diets with supplemental GAA plus one of 4 methionine concentrations representing either 20%, 80%, 140%, or 200% of the dietary methionine requirement. Arterial and portal blood metabolites were measured along with blood flow to determine mass balance across the gut. [^{3}H-methyl] methionine was infused to measure methionine incorporation rate into creatine.

Results: GAA balance across the gut was highest in the 200% methionine group, indicating excess dietary methionine enhanced GAA absorption. Creatine synthesis in the liver and jejunum was higher with the higher levels of methionine, emphasizing that transmethylation of GAA to creatine depends on sufficient dietary methionine. Hepatic GAA concentration was higher in the 20% methionine group, suggesting low dietary methionine limited GAA conversion to creatine, which led to GAA accumulation in the liver.

Conclusions: GAA absorption and conversion to creatine require a sufficient amount of methionine, and the supplementation strategies should accommodate this interaction.

Keywords: Methionine; creatine; GAA supplementation; GAA absorption; piglets

4.2 Introduction

Guanidinoacetic acid (GAA) is an amino acid derivative and endogenous substance in body tissues (1,2). GAA acts as an immediate precursor for creatine, which plays a key role in energy metabolism, especially in muscle (3) and brain (4). Because of its role in muscle energetics, creatine supplementation has been popular as a performance-enhancing agent among athletes (5) as well in the animal production industry (6, 7). But creatine supplementation has significant drawbacks, including instability during the manufacturing process, high cost (8), and relatively low bioavailability (9). Hence, supplemental GAA has been proposed as a potential alternative approach to enhance creatine availability in the body. Moreover, GAA also seems to have several beneficial non-creatine roles in cellular metabolism. For example, GAA can directly or indirectly affect endocrine functions, neuromodulation, and oxidant-antioxidant processes (10, 11).

Endogenous creatine synthesis and dietary creatine intake are essential to replenish daily creatine losses, estimated to be 1.7% of the body pool per day in young humans (12). Moreover, plant-based diets may not contain sufficient amounts of creatine, which would then need to be synthesized entirely. Similarly, although neonatal piglets consume milk that is high in creatine, they still need to produce 80% of their own creatine because demands for their rapid growth exceed dietary supply (13). Creatine biosynthesis involves two enzymes and three amino acids (i.e., arginine, glycine, and methionine) in an inter-organ process. In the kidney, arginine transfers its amidino group to glycine to synthesize GAA and ornithine via arginine:glycine amidino transferase (AGAT). GAA produced by the kidney is transported to the liver, where it is methylated using a methyl group from S-adenosylmethionine (SAM), which is a direct metabolite of methionine and the primary methyl donor in the body. This reaction produces creatine and S-adenosylhomocysteine (SAH) and is catalyzed by guanidinoacetate methyltransferase (GAMT)

(12). This inter-organ pathway is responsible for most whole body creatine synthesis; however creatine biosynthesis is not restricted to these tissues and occurs at varying rates in other organs (13). In pigs, GAMT activity is highest in the liver, although it is somewhat ubiquitous, whereas AGAT occurs mainly in the kidney. The pancreas also has considerable AGAT activity in pigs (13), but more than 80% of total GAA is released from the kidney, suggesting the kidney is quantitatively the most important source of GAA for whole body creatine synthesis in piglets (14).

GAA can also be fed to provide a direct precursor for creatine synthesis. We have shown that supplemental GAA readily converts to creatine, but only when sufficient methionine is available to transmethylate GAA to creatine in the liver (via SAM) (15). Indeed, other studies have shown that supplemental GAA with methionine has beneficial effects on animal performance (16,17). However, the fate of unconverted GAA, when methionine is limiting, is unclear. In our recent study, when dietary methionine was limited (80% of requirement), creatine synthesis was limited, yet GAA did not appear to accumulate in plasma, intestinal mucosa, kidney, brain, muscle, or liver (18). One possibility is that absorption of supplemental GAA across the gut depends on sufficient dietary methionine; however, the effect of dietary methionine on GAA absorption across the gut is unknown. The role of the gut in creatine synthesis has not been extensively studied, and little is known about dietary methionine requirement for the de-novo creatine synthesis in the gut.

The neonatal piglet is widely regarded as the closest non-primate model for the human infant and is an ideal animal model for studying nutritional metabolism because it shares similarities with the infant in terms of gastrointestinal tract morphology, physiology, and metabolic changes during development (19,20,21). Moreover, the piglet has been extensively utilized in studies examining the requirements of amino acids, and their metabolism has been shown to respond to rapid dietary modifications, including changes in the composition of amino acids in the diet (22). Therefore, the

current study utilized Yucatan miniature piglets as an experimental model to investigate amino acid metabolism. We hypothesized that dietary GAA appearance in portal vein requires adequate dietary methionine. The main objective of this study was to determine the effects of dietary methionine on supplemental GAA absorption and creatine synthesis in neonatal piglets. Furthermore, we evaluated the distribution of GAA and creatine in Yucatan miniature pigs supplied with different methionine levels in the diet.

4.3 Methods and materials

4.3.1 Reagents

[^{3}H-Methyl]-methionine was obtained from American Radiochemicals, Inc. All other chemicals were of analytical grade and were from Sigma (St. Louis, MO, USA) or Fisher Scientific (Fair Lawn, NJ, USA).

4.3.2 Piglets and surgical procedures

All animal protocols were approved by the Institutional Animal Care Committee of the Memorial University of Newfoundland in accordance with the guidelines of the Canadian Council on Animal Care. Yucatan miniature piglets (n = 20; 11/9 M/F, 17–21 d old, weight, 2.46-3.38 kg) were removed from the sows at the Memorial University of Newfoundland breeding colony and transported to the Animal Care Facilities on campus. Piglets were feed-restricted for 3 h before surgery to ensure that the test nutrients to be administered had no interaction with the gut contents from suckling. Piglets were randomly assigned to 1 of the 4 different infusion treatments and balanced for sex (i.e., 2-3 males per group) and weight across treatments. Piglets were sedated, intubated, and anesthetized, as described previously (23). Under general anesthesia, piglets underwent a surgical procedure for implanting catheters and probes.

The carotid artery was isolated by blunt dissection, and a catheter was inserted to allow arterial blood sampling. A jugular vein catheter was advanced to the superior vena cava to hydrate the pig throughout the procedure and to allow venous blood sampling. A laparotomy was then performed, the portal vein was isolated, and an ultrasonic perivascular blood flow probe (6 mm) (Transonic Systems Inc.) was secured around the portal vein to measure the blood flow. A catheter was also implanted in the portal vein for repeated blood sampling. A duodenal catheter was placed to infuse experimental diet treatments (described below), and the exposed visceral organs were moistened with warmed saline and covered with wet gauze and plastic wrap during the surgery to prevent dehydration.

4.3.3 **Experimental diets**

During the intraduodenal infusion, following diet treatments were administered. Four treatments were: 1) GAA plus 20% methionine (20% Met); 2) GAA plus 80% methionine (80% Met); 3) GAA plus 140% methionine (140% Met); or 4) GAA plus 200% methionine (200% Met). For these test infusions, methionine was added to provide different levels of methionine ranging from 2.08-20.77 $mg \cdot kg^{-1}$ $BW \cdot h^{-1}$; this represents 20%-200% of the methionine requirement with excess cysteine determined by Shoveller et al. (24). GAA was added to the base dietary infusate to provide it at a rate of 3.75 $mg \cdot kg^{-1}$ $BW \cdot h^{-1}$. This amount of GAA, if completely converted to creatine, would fulfill the piglet's total creatine accretion rate at this age (13). Methionine was delivered at a rate of 2.08, 8.30, 14.53, 20.77 Met $mg \cdot kg^{-1}$ $BW \cdot h^{-1}$. [^{3}H-methyl]-Methionine (prime: 30 $\mu Ci \cdot kg^{-1}$ BW; constant: 30 $\mu Ci \cdot kg^{-1}$ $BW \cdot h^{-1}$) infusions (1 $mL \cdot kg^{-1}$ $BW \cdot h^{-1}$) were administered to trace the ^{3}H-methyl incorporation into creatine.

All of the experimental diet treatments were made as previously described (23, 25). The test diets were completely elemental and were identical to each other except for methionine (test amino acid)

and alanine (to balance nitrogen) (Supplemental Table 3). The complete diet, which was designed to supply all nutrients required by piglets, was delivered at 10 $mL \cdot kg^{-1}$ $BW \cdot h^{-1}$ to provide 13 g of amino $acids \cdot kg^{-1}$ $BW \cdot d^{-1}$ (Ajinomoto, Evonik Industries AG, Sigma Aldrich, or Bachem) and non-protein energy of 0.55 $MJ \cdot kg^{-1}$ $BW \cdot d^{-1}$ from dextrose (Sigma Aldrich, Canada). The solutions were sterile filtered via 0.22 µm filters (PALL Life Science) and stored in a cooler until used. All vitamins and minerals were supplied at >120% of the requirement for neonatal piglets (26). Just prior to use, multivitamins, iron dextran (Bimeda-MTC Animal Health), and trace elements (Sigma-Aldrich Canada) were added to each diet, as previously described (27). The experimental treatments were infused for 4 h. To achieve a postprandial state more quickly, a bolus priming dose of diet equal to the hourly rate was first infused, followed by continuous infusion at a rate of 11 $mL \cdot kg^{-1}$ $BW \cdot h^{-1}$via a syringe pump.

4.3.4 **Blood flow measurement and blood sampling**

Blood samples from the portal vein and the carotid artery and portal blood flow rate measurements ($mL \cdot min^{-1}$) were obtained every 15 min for a total of 4 h. For the gut, blood flow from the portal vein was first measured 30 min after initiating the intraduodenal infusion. The blood flow was continuously recorded over a minimum of 2 min when the flow measurements' variability was <5%, and those data were used to calculate the mean for that timepoint. During the treatment period (4 h), blood was sampled from the carotid artery (representing arterial metabolite concentrations available to the gut) and from portal vein every 15 min. At the end of the experiment, tissues were removed (liver, kidneys, small intestine; section of mid jejunum, biceps femoris muscle) and weighed (liver and kidneys). The piglets died by exsanguination after removal of the organs and tissue samples were immediately frozen in liquid nitrogen and stored at −80°C until analyzed.

4.3.5 **Metabolite analyses**

4.3.5.1 Tissue and plasma GAA determination

Plasma and tissue GAA concentrations were measured by HPLC with ninhydrin derivatization and fluorescence detection (28). The GAA concentration in plasma was measured using samples taken at three different time points: 3 h, 3.5 h, and 4 h. The mean value of these measurements was then utilized to determine the arteriovenous balance.

4.3.5.2 Tissue creatine concentration and specific radioactivity (SRA) determination

Tissue creatine concentrations were measured by HPLC using a modified method by Lamarre et al. 2012 (29). Tissue homogenates were deproteinized with trifluoroacetic acid after being homogenized in a 50 $mmol \cdot L^{-1}$Tris buffer (pH 7.4) and left at room temperature for 20 min to allow for complete conversion of phosphocreatine to creatine. Creatine was separated out with an isocratic mobile phase of 0.1% trifluoroacetic acid and 3% methanol. Then, creatine fractions were collected, and the radioactivity associated with these fractions was determined by liquid scintillation counting using Scintiverse (Fisher Chemical).

4.3.5.3 Tissue and plasma methionine determination

Plasma and tissue methionine concentrations were measured by HPLC following derivatization with phenylisothiocyanate with norleucine as the internal standard (30). Plasma samples were first deproteinized with 0.5% trifluoroacetic acid in methanol. Tissue samples were homogenized in perchloric acid and the supernatant was used to determine free methionine concentration. Methionine fractions were collected during HPLC analysis, and the radioactivity associated with these fractions was determined by liquid scintillation counting.

4.3.6 **Arteriovenous balance**

Net arteriovenous balance across the gut was determined by multiplying the concentration difference between venous and arterial blood (portal vein concentration minus arterial concentration) by the blood flow and correcting for body weight. The balance data were converted to percent change, using the lowest methionine group (20% Met) as a reference.

4.3.7 **Calculations**

The percent of dietary GAA that appeared in portal circulation was determined by dividing arteriovenous balance by dietary GAA infusion rate:

Portal GAA appearance (%) = Arteriovenous GAA balance / GAA infusion rate * 100

The fractional ^{3}H-methyl incorporation (%) into creatine was determined using the following equation:

Fractional ^{3}H-methyl incorporation (%) = (SRA Product/SRA Precursor) × 100,

where the SRA Product was DPM·μmol^{-1}for creatine, and the SRA Precursor was DPM·μmol^{-1}of tissue methionine.

4.4 **Statistical analyses**

Data were analyzed using one-way ANOVA followed by Duncan's multiple range test to detect the differences between treatments. Polynomial contrasts were carried out to determine the linear and quadratic effects of increasing methionine levels (IBM SPSS Statistics; Version 27). Significant differences were recognized when the P-value was less than 0.05. In addition, data were tested to verify whether they were significantly different from zero by one-sample t-test (GraphPad Prism; Graph Pad Software Inc.).

4.5 Results

4.5.1 Jejunum GAA and creatine parameters

The ANOVA showed that dietary methionine had a significant effect on GAA and creatine concentrations and the fractional ^{3}H-methyl incorporation into creatine in jejunum. Moreover, polynomial contrasts showed, with increasing methionine levels, jejunum GAA and creatine concentrations, and ^{3}H -methyl incorporation into creatine increased linearly ($P < 0.001$; Table 4.1,Supplemental Table 4,Supplemental Table 5). GAA concentration in jejunal mucosa was significantly higher in piglets infused with 200% Met treatment ($P < 0.001$; Figure 4.1A). The jejunum creatine concentration ($P < 0.001$; Figure 4.1B) and the fractional ^{3}H-methyl incorporation into creatine ($P < 0.001$; Table 4.1) were also significantly higher in the 200%, and 140% Met groups.

4.5.2 Plasma GAA and creatine concentrations

Plasma creatine and portal GAA concentrations were significantly different across various methionine levels. But carotid GAA concentration was not significantly different among the groups ($P > 0.05$); Figure 4.3A). According to polynomial contrasts, portal GAA and creatine concentrations ($P < 0.001$) and carotid creatine concentration ($P = 0.002$) increased linearly with increasing methionine levels (Supplemental Table 4,Supplemental Table 5). GAA and creatine concentrations in the portal vein samples were significantly higher in the 200% Met group ($P < 0.001$; Figure 4.2). Carotid creatine concentration was significantly higher in the 80%-200% Met groups compared to 20% Met group ($P = 0.017$; Figure 4.3B).

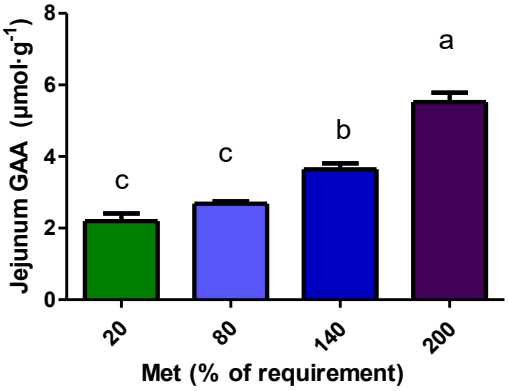

Figure 4.1 (A)

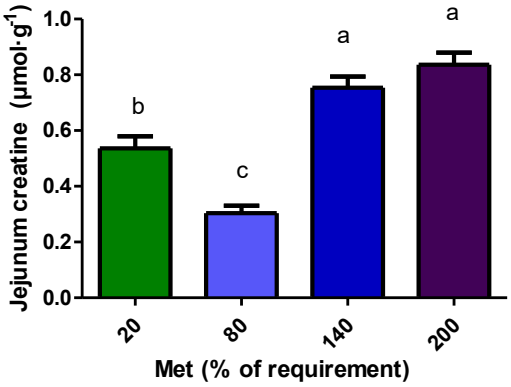

Figure 4.1 (B)

Figure 4.1 GAA (A) and creatine (B) concentration in jejunum following 4 h duodenal infusion with diets containing 20% Met, 80% Met, 140% Met, or 200% Met in piglets. Values are means ± SEM; P values were calculated by ANOVA with polynomial contrasts for linear trend. Bars with different letters are significantly different ($P < 0.05$).

Figure 4.1A: P value for Trt < 0.001; P value for L < 0.001, Figure 4.1B: P value for Trt < 0.001; P value for L < 0.001 (L, linear effect; Trt, treatment effect)

4.5.3 **Gut GAA and creatine balance**

The ANOVA results revealed significant differences in change in GAA portal balance and GAA appearance with increasing methionine levels. Furthermore, there was a clear linear increase in GAA appearance as methionine levels increased ($P < 0.001$; Supplemental Table 4). 200% Met group had significantly greater change in GAA balance compared to 20%- 140% Met groups ($P < 0.001$; Figure 4.4A). GAA appearance in the portal vein was significantly higher in the 200% Met group (104.9%) compared to 20%, 80% and 140% Met groups (31.1%, 27.4%, 26.3%, respectively) ($P < 0.001$; Figure 4.4C, Supplemental Table 4).

The change in creatine portal balance also exhibited significant differences among the various groups with different levels of methionine. 200% Met group demonstrated a significantly greater change in creatine balance across the gut, compared to 20% Met group ($P < 0.001$; Figure 4.4B)

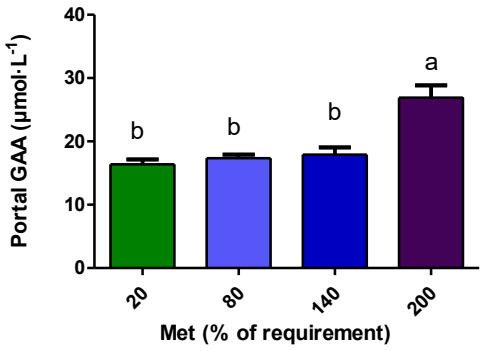

Figure 4.2 (A)

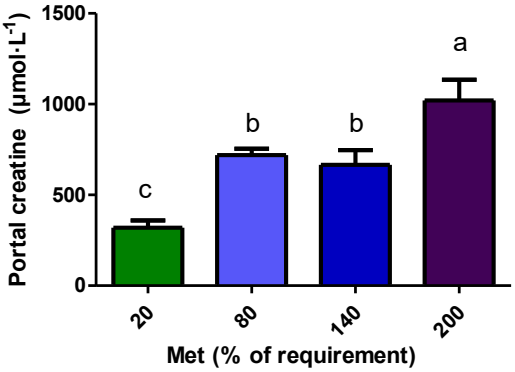

Figure 4.2 (B)

Figure 4.2 GAA (A) and creatine (B) concentration in portal vein following 4 h duodenal infusion with diets containing 20% Met, 80% Met, 140% Met, or 200% Met in piglets. Values are means ± SEM; P values were calculated by ANOVA with polynomial contrasts for linear trend. Bars with different letters are significantly different ($P < 0.05$)

Figure 4.2A: P value for Trt < 0.001; P value for L < 0.001, Figure 4.2B: P value for Trt < 0.001; P value for L < 0.001 (L, linear effect; Trt, treatment effect)

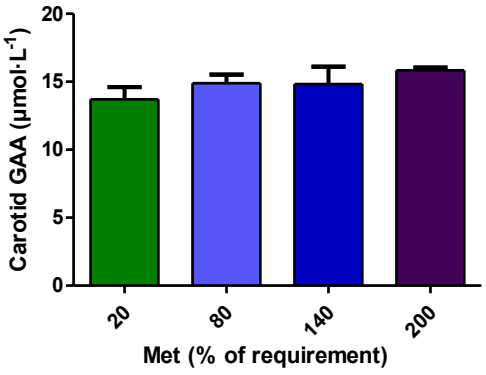

Figure 4.3 (A)

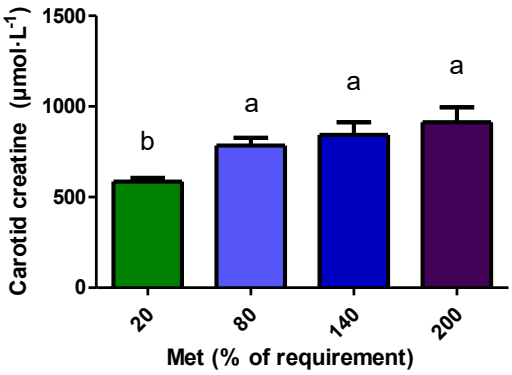

Figure 4.3 (B)

Figure 4.3 GAA (A) and creatine (B) concentration in carotid artery following 4 h duodenal infusion with diets containing 20% Met, 80% Met 140% Met, or 200% Met in piglets. Values are means ± SEM; P values were calculated by ANOVA with polynomial contrasts for linear trend. ANOVA. Bars with different letters are significantly different ($P < 0.05$)

Figure 4.3A: P value for Trt = 0.398; P value for L = 0.116, Figure 4.3B: P value for Trt = 0.017; P value for L = 0.002 (L, linear effect; Trt, treatment effect)

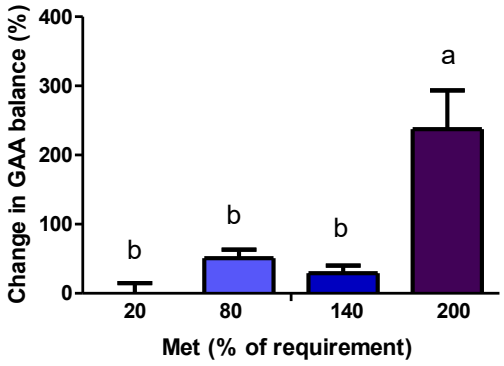

Figure 4.4 (A)

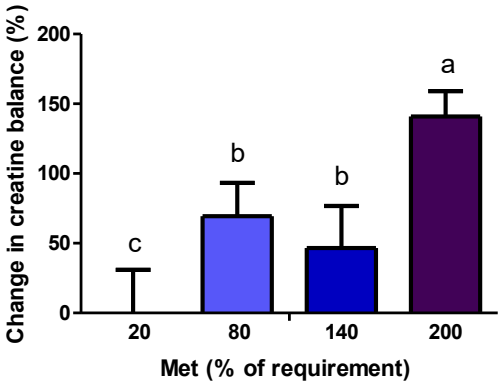

Figure 4.4 (B)

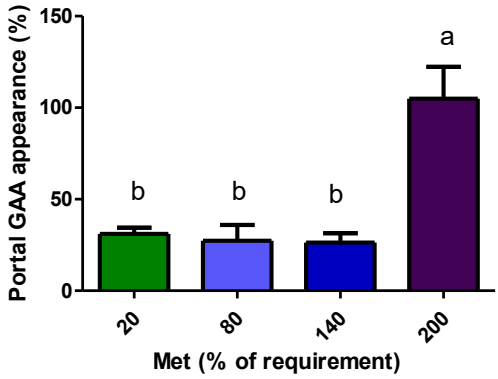

Figure 4.4 (C)

Figure 4.4 Change in portal GAA balance percentage (A), Change in creatine balance percentage (B) and portal GAA appearance (C) during 4 h duodenal infusion with diets containing 20% Met, 80% Met 140%, Met, or 200% Met in piglets. Values are means ± SEM; P values were calculated by ANOVA with polynomial contrasts for linear trend. Bars with different letters are significantly different ($P < 0.05$)

Figure 4.4A: P value for Trt < 0.001; P value for L < 0.001, Figure 4.4B: P value for Trt < 0.001; P value for L < 0.001, Figure 4.4C: P value for Trt < 0.001; P value for L < 0.001 (L, linear effect; Trt, treatment effect)

4.5.4 **Liver GAA and creatine parameters**

According to the ANOVA analysis, there were significant changes in liver GAA and creatine concentrations in response to varying levels of methionine. Liver GAA concentration decreased linearly with increasing methionine levels ($P < 0.001$; Supplemental Table 4). Creatine concentration ($P < 0.001$; Supplemental Table 5) and fractional ^{3}H-methyl incorporation into creatine ($P = 0.018$; Table 4.1) increased with the methionine level. Hepatic GAA concentration was significantly higher after the 20% Met treatment; but it was not significantly different among the 80%- 200% Met groups ($P = 0.004$; Figure 4.5A). Hepatic creatine concentration ($P < 0.001$; Figure 4.5B) and fractional ^{3}H-methyl incorporation into creatine ($P = 0.002$; Table 4.1) were significantly greater in 200% and 140% Met groups, respectively, compared to other treatment groups.

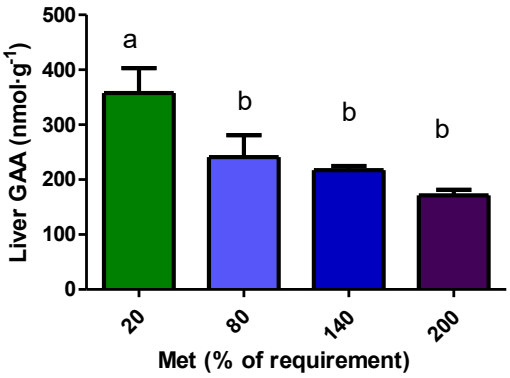

Figure 4.5 (A)

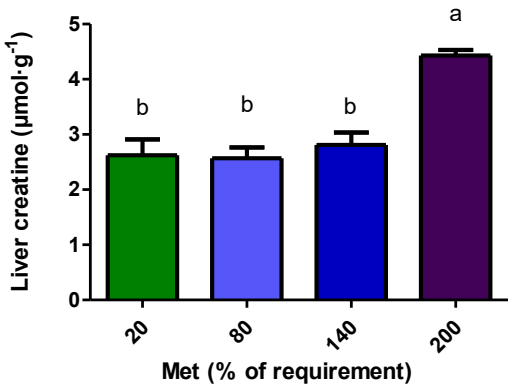

Figure 4.5 (B)

Figure 4.5 GAA (A) and creatine (B) concentration in the liver following 4 h duodenal infusion with diets containing 20% Met, 80% Met 140% Met, or 200% Met in piglets. Values are means ± SEM; P values were calculated by ANOVA with polynomial contrasts for linear trend. . Bars with different letters are significantly different ($P < 0.05$)

Figure 4.5A: P value for Trt = 0.004; P value for L < 0.001, Figure 4.5B: P value for Trt < 0.001; P value for L < 0.001 (L, linear effect; Trt, treatment effect)

Table 4.1 Fractional ^{3}H methyl incorporation (%) into creatine in piglets given 4 h duodenal infusion with diets containing 20% Met, 80% Met 140% Met, or 200% Met

	Treatments					P-value[1]		
Fractional ^{3}H methyl incorporation (%) into creatine	20% Met	80% Met	140% Met	200% Met	SEM	Trt	L	Q
Jejunum (%)	14.94[b]	22.04[b]	90.00[a]	114.14[a]	7.08	<0.001	<0.001	0.556
Liver (%)	17.22[b]	31.70[b]	81.76[a]	41.05[b]	5.00	0.002	0.018	0.015
Kidney (%)	0.90	0.81	1.39	1.36	0.15	0.386	0.167	0.294
Muscle (%)	1.95 [a]	0.52 [b]	0.37 [b]	0.69 [b]	0.08	<0.001	<0.001	<0.001

[1] L, linear effect; Q, quadratic effect; Trt, treatment effect.

[a–c] Differences between treatments ($P < 0.05$).

SEM, standard error of the mean

Met, methionine; 20% Met = 2.08 $mg \cdot kg^{-1}$ $BW \cdot h^{-1}$, 80% Met= 8.3 $mg \cdot kg^{-1}$ $BW \cdot h^{-1}$, 120% Met=14.53 $mg \cdot kg^{-1}$ $BW \cdot h^{-1}$, 200% Met=20.77 $mg \cdot kg^{-1}$ $BW \cdot h^{-1}$

4.5.5 **Kidney GAA and creatine parameters**

Dietary methionine levels did not impact the GAA and creatine concentrations in the kidney ($P > 0.05$; Figure 4.6). Creatine SRA was significantly higher in the 200% Met group than in other groups ($P < 0.001$; Table 4.3); however, the fractional ^{3}H-methyl incorporation into creatine was not significantly different among the groups ($P > 0.05$; Table 4.1).

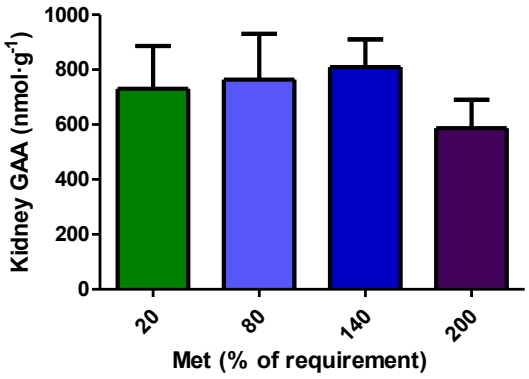

Figure 4.6 (A)

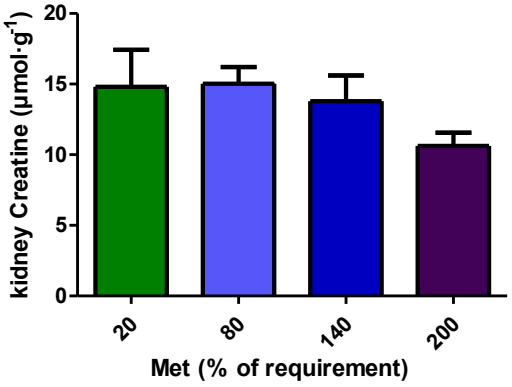

Figure 4.6 (B)

Figure 4.6 GAA (A) and creatine (B) concentration in the kidney following 4 h duodenal infusion with diets containing 20% Met, 80% Met 140% Met, or 200% Met in piglets.Values are means ± SEM; P values were calculated by ANOVA with polynomial contrasts for linear trend.

Figure 4.6A: P value for Trt = 0.824; P value for L = 0.515, Figure 4.6B: P value for Trt = 0.069; P value for L = 0.032 (L, linear effect; Trt, treatment effect)

4.5.6 **Muscle creatine parameters**

We were unable to observe any increase in muscle creatine during the 4 h infusion; as shown in Figure 4.7, muscle creatine concentration was not significantly different among the groups ($P > 0.05$). However, with increasing methionine level, fractional ^{3}H-methyl incorporation into creatine increased linearly ($P < 0.001$; Table 4.1). The fractional ^{3}H-methyl incorporation into creatine in muscle was significantly higher in 20% Met group ($P < 0.001$; Table 4.1) compared to the other groups.

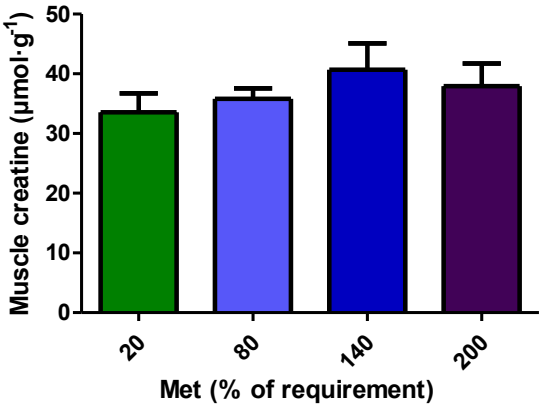

Figure 4.7

Figure 4.7 Creatine concentration in muscle following 4 h duodenal infusion with diets containing 20% Met, 80% Met 140% Met, or 200% Met in piglets. Values are means ± SEM; P values were calculated by ANOVA with polynomial contrasts for linear trend Figure 7: P value for Trt = 0.522; P value for L = 0.258 (L, linear effect; Trt, treatment effect)

4.5.7 **Carotid creatine SRA**

Carotid creatine SRA was not significantly different among the Met groups ($P > 0.05$; Table 4.3).

4.5.8 **Methionine concentrations in plasma and tissues**

The concentrations of methionine in both plasma and tissues displayed significant changes in response to varying levels of methionine intake. Polynomial contrasts showed, with increasing methionine level, tissue and plasma methionine concentrations increased linearly ($P < 0.05$; Table 4.2). At the end of the 4 h infusion, methionine concentrations in the portal vein, carotid artery, kidney, liver, jejunum, and muscle were significantly higher in the 200% Met group; for jejunum, kidney, liver, and portal vein, 140% Met group was also significantly higher than the lower Met groups ($P < 0.05$; Table 4.2).

Table 4.2 Plasma and tissue methionine concentrations in piglets given 4 h duodenal infusion with diets containing 20% Met, 80% Met, 140% Met, or 200% Met

	Treatments					P-value[1]		
Methionine concentration	20% Met	80% Met	140% Met	200% Met	SEM	Trt	L	Q
Jejunum methionine ($nmol \cdot g^{-1}$)	580^{b}	669^{b}	1115^{a}	1434^{a}	54	<0.001	<0.001	0.302
Kidney methionine ($nmol \cdot g^{-1}$)	144^{b}	179^{b}	275^{a}	274^{a}	11	<0.001	<0.001	0.417
Liver methionine ($nmol \cdot g^{-1}$)	88^{b}	192^{a}	233^{a}	182^{a}	12	<0.001	0.011	0.007
Muscle methionine ($nmol \cdot g^{-1}$)	258^{b}	209^{b}	285^{b}	615^{a}	33	0.002	0.001	0.011
Portal methionine ($\mu mol \cdot L^{-1}$)	151^{b}	313^{b}	447^{a}	520^{a}	31	0.003	<0.001	0.483
Carotid methionine ($\mu mol \cdot L^{-1}$)	122^{b}	153^{b}	221^{b}	326^{a}	17	0.004	<0.001	0.3

[1] L, linear effect; Q, quadratic effect; Trt, treatment effect.

[a–c] Differences between treatments ($P < 0.05$).

SEM, standard error of the mean

Met, methionine; 20% Met = 2.08 $mg \cdot kg^{-1}$ $BW \cdot h^{-1}$, 80% Met= 8.3 $mg \cdot kg^{-1}$ $BW \cdot h^{-1}$, 120% Met=14.53 $mg \cdot kg^{-1}$ $BW \cdot h^{-1}$, 200% Met=20.77 $mg \cdot kg^{-1}$ $BW \cdot h^{-1}$

Table 4.3 Creatine SRA in tissues after 4 h duodenal infusion with diets containing 20% Met, 80% Met, 140% Met, or 200% Met in piglets

	Treatments					P-value[1]		
Creatine SRA	20% Met	80% Met	140% Met	200% Met	SEM	Trt	L	Q
Jejunum creatine SRA (DPM·µmol^{-1})	11416^{c}	23307^{b}	35978^{a}	32078^{a}	1774	<0.001	<0.001	0.041
Liver creatine SRA (DPM·µmol^{-1})	7201^{c}	5610^{c}	25394^{a}	15348^{b}	1034	<0.001	<0.001	0.058
Kidney creatine SRA (DPM·µmol^{-1})	559^{b}	514^{b}	555^{b}	866^{a}	19	<0.001	<0.001	<0.001
Muscle creatine SRA (DPM·µmol^{-1})	271	249	231	203	25	0.795	0.328	0.945
Carotid creatine SRA (DPM·µmol^{-1})	12803	12613	10378	11756	886	0.763	0.507	0.664

[1] L, linear effect; Q, quadratic effect; Trt, treatment effect.

$^{a–c}$ Differences between treatments ($P < 0.05$).

SEM, standard error of the mean

Met, methionine; 20% Met = 2.08 mg·kg^{-1} BW·h^{-1}, 80% Met= 8.3 mg·kg^{-1} BW·h^{-1}, 120% Met=14.53 mg·kg^{-1} BW·h^{-1}, 200% Met=20.77 mg·kg^{-1} BW·h^{-1}

4.6 Discussion

GAA is considered a novel supplement to restore creatine and enhance growth performance in the commercial animal industry (31, 32). The supplement is also being investigated in humans, and studies have evaluated the effects of dietary GAA as an alternate to creatine supplements (1). To achieve the optimum efficacy of exogenous GAA, it must be absorbed by the small intestine and converted into creatine in various target tissues, using methionine as a methyl donor. In this study in neonatal piglets, we demonstrated that the efficacy of GAA supplements is dependent on the amount of dietary methionine present with GAA.

This research question arose from a previous study in our lab in which piglets fed a diet supplemented with GAA, but with limited dietary methionine (80% of requirement), had limited creatine synthesis, compared to when sufficient methionine was fed (18). Yet, in spite of this limited GAA conversion to creatine, GAA did not appear to accumulate in plasma, liver, muscle, kidney, jejunum, or brain (18). If dietary GAA was not utilized for creatine synthesis and did not sequester in key organs, then presumably it must have been either rapidly excreted via the kidney, reversibly metabolized to arginine by AGAT, or was not absorbed when methionine was deficient. In those previous studies, we did not observe higher urinary GAA concentrations, nor did we observe an increase in arginine concentrations; so, we hypothesized that GAA absorption was dependent on dietary methionine availability. Therefore, this study aimed to describe the fate of supplemental GAA in neonatal piglets over a range of dietary methionine intakes. In the current study, we clearly demonstrated that insufficient dietary methionine limits dietary GAA absorption, lowers creatine synthesis in the liver, kidney, and jejunal mucosa, and leads to accumulation of GAA in the liver.

According to the current study, dietary methionine significantly impacted GAA absorption in piglets. But the mechanistic relationship between dietary methionine and GAA absorption is unknown. The present study showed that 140% Met and 200% Met groups resulted in the highest jejunum concentration of GAA. These data suggest that GAA absorption was enhanced by dietary methionine. In spite of this indirect evidence, there is not much known regarding GAA absorption across the gut in animals and humans. GAA supplements in chickens have 98-99% fecal digestibility, which is not affected by the level of supplemental GAA (33). However, the role of methionine in this digestibility assessment was not addressed. The current findings in piglets underscore the significance of having a sufficient supply of methionine to enhance complete GAA absorption across the gut. Notably, the group with 200% methionine exhibited a 105% portal GAA appearance, indicating the crucial role of an adequate methionine supply in promoting optimal GAA absorption; these data likely represent GAA absorption, since negligible amounts of GAA are synthesized de novo and released by the gut to the circulation (14). Surprisingly, the pigs administered 140% methionine, which is still above the estimated methionine requirement, had only ~26% of portal GAA appearance, similar to that in pigs fed deficient levels of methionine. We speculate that the remaining ~74% might either not be completely absorbed by the gut, utilized within the gut for creatine synthesis, or metabolized by gut microbes. Furthermore, the current study provides some of the first evidence on GAA absorption and its utilization towards its primary product, creatine, and how dietary methionine can alter these processes in piglets.

GAA transport is thought to occur through the creatine transporter (CRT/Solute carrier SLC6A8), taurine transporters (TauT/SLC6A6), γ-aminobutyric acid (GABA) transporter (SLC6 A13), and passive diffusion through plasmalemma (34). Supplementing GAA with creatine may lead to saturation of creatine transporters, increasing GAA transport via other transporters, resulting in

greater total GAA and creatine levels in cells (35). Intestinal transport of methionine mainly occurs through Na^+-independent or -dependent transporters. The apical transport systems are Na^+-dependent, and on the basolateral side, methionine efflux is facilitated by the Na^+-independent system (36). Because methionine and GAA use different transporters, there is no obvious competition or synergic mechanism at the transporter site. A more likely explanation is that when dietary methionine is abundant, GAA is transported and rapidly converted to creatine. This synthesis of creatine lowers GAA concentrations, maintaining a concentration gradient for GAA transport. Indeed, creatine concentrations in most tissues and blood increased in parallel with methionine levels. Therefore, when GAA is supplemented with excess methionine, it may increase GAA absorption and subsequently promote its conversion into creatine.

Although GAA transport from the lumen into the jejunum was higher in both 140% Met and 200% Met groups, GAA release to the portal vein was significantly higher only in the 200% Met group. Indeed, although the net absorption of GAA was over 100% in the 200% Met group, only 26-31% of dietary GAA appeared in the portal vein for the three lower Met groups. This finding aligns with the study by Dinesh et al. (2020), which suggested that GAA is released from the gut only when there is a sufficient amount of circulating creatine in the body. In the current study, it is also notable that the portal vein creatine concentrations were lower than the carotid artery concentrations for all groups except 200% Met. These data suggest that creatine is extracted from the circulation by the gut in the 20%, 80% and 140% Met groups; but in the 200% Met group, creatine and GAA are released by the gut to the circulation. In other words, when dietary methionine is low, the gut retains most dietary GAA, but also extracts creatine from the circulation, presumably because methionine is limiting GAA conversion to creatine in the gut. With excess dietary methionine (200% Met group), the gut appears to transport dietary GAA to the portal circulation. Overall,

excess dietary methionine was needed for GAA absorption from lumen to portal vein, which has the additional effect of increasing creatine release to the circulation.

Alternatively, there may be another reason behind the finding of the high amount of GAA in the jejunum with the presence of 200% methionine. The gut releases a significant amount of endogenously synthesized GAA, when blood creatine is abundant (14). This appears contradictory to our previous finding of undetectable levels (<10 $nmol \cdot min^{-1} \cdot g^{-1}$) of intestinal AGAT; however, the total small intestine (~120 g) may still have AGAT capacity to produce up to 1200 nmol/min of GAA, which can be significant to whole body creatine homeostasis. This capacity is ~32% of kidney AGAT capacity (211 $nmol \cdot min^{-1} \cdot g^{-1}$ x 18 g = ~3800 nmol/min) (13), so more work needs to be done on small intestinal creatine metabolism. We previously found that there is a net release of GAA from the gut, but only when there is excess circulating creatine in the body (14).

As methionine is increased in the diet, creatine balance across the gut increases, such that in the highest Met group, the increase reached approximately 140%. This underscores how elevated methionine levels promote the conversion of GAA to creatine, resulting in an increased creatine portal balance in piglets. Likewise, there was a noteworthy increase in the percentage change of GAA portal balance in the 200% Met group. This highlights the influence of methionine on GAA portal balance, even though the underlying mechanism remains incompletely understood.

We hypothesized that when circulating creatine is low, the gut synthesizes creatine from GAA to be used locally; but when creatine is readily available, GAA synthesized by the gut is released to the circulation for creatine biosynthesis elsewhere. In the current study, when excess dietary methionine was available, creatine biosynthesis was induced and abundant, which increased circulating creatine concentrations. Consistent with our previous study, any unused GAA accumulated in the gut and was released to the circulation. In contrast, when circulating creatine

is limited, as in the lower methionine groups, GAA synthesized by the gut was used locally for creatine biosynthesis.

Consistent with this hypothesis, higher dietary methionine with supplemental GAA increased jejunum creatine synthesis, supporting our previous data suggesting the gut is an important site of creatine synthesis, but only with sufficient dietary methionine (14). This is further supported by our finding of intermediate levels of GAMT activity in the piglet jejunum (13). Consistent with this, fractional ^{3}H-methyl incorporation into creatine in jejunum was highest in high methionine groups emphasizing that sufficient amount of methionine is required to synthesize creatine and improve creatine pools in the body. The fate of this locally synthesized creatine in the jejunum is unknown, as it can be used by the gut or transported to other tissues. But the creatine balance data suggest that there is net release of creatine from the gut only when methionine is in excess.

Creatine synthesis in the liver was also enhanced by the higher levels of methionine, underlining that transmethylation of GAA to creatine was active in the liver and dependent on adequate levels of dietary methionine. Current study results show higher creatine concentration in 140% Met group compared to 200% Met group. Within the 200% Met group, an increased synthesis of creatine occurs due to the surplus methionine, leading to an abundance of creatine available for liver uptake and utilization. However, this surplus of available creatine might also slow down further creatine production (via renal AGAT) and/or facilitate greater transport to other tissues compared to the 140% group. Consequently, the liver may end up retaining a lower amount of creatine when methionine is in excess (200% Met group). The portal vein transports the absorbed GAA to the liver, where hepatic GAMT activity is highest (13), and converts GAA to creatine using methyl groups from methionine. Therefore, the higher creatine concentration and fractional ^{3}H-methyl incorporation into creatine in 200% and 140% Met groups demonstrate that more dietary

methionine facilitates GAA conversion to creatine in the liver. Interestingly, this effect was stimulated acutely by luminal methionine, suggesting intrahepatic sources of methionine are not sufficient to maintain this conversion to creatine. Consequently, hepatic GAA concentrations decreased with increasing dietary methionine levels, as GAA is increasingly consumed for creatine synthesis.

In contrast, creatine synthesis was lowest in the 20% Met group, suggesting methyl groups were limiting for transmethylation of GAA to creatine. Therefore, GAA accumulated in the liver of 20% Met piglets, as it was not used for creatine synthesis. Of the tissues measured, the most profound difference in GAA accumulation was observed in the liver, reflecting its central role in whole body creatine synthesis. We have previously determined that there is no measurable AGAT activity in the piglet liver (13). This means that the accumulated GAA in the liver originated from either the absorbed supplemental GAA or endogenous GAA transported via circulation. In either case, the accumulation of unused GAA in some tissues could be dangerous (37). But so far, no studies have reported an incidence of neurotoxicity after dietary GAA supplementation in humans or pigs.

High dietary methionine also enhanced renal creatine synthesis with GAA supplementation. Although GAA and creatine concentrations in the kidney were not significantly different among the groups, creatine SRA was highest in the 200% Met group. These data suggest that newly synthesized creatine was transported to the kidney or was produced by the kidney as more methyl groups are available in the highest methionine group. Renal GAMT activity is significant in the piglet so the kidney is capable of complete biosynthesis of GAA and creatine (13). The rapid conversion of GAA to creatine in the kidney is supported by the fact that GAA did not accumulate in the kidney. Alternatively, newly synthesized GAA was rapidly transported to the plasma for other tissues to utilize for extra-renal creatine synthesis. Moreover, arterial (carotid) creatine SRA

was not significantly different among groups, emphasizing that the differences of tissue fractional ^{3}H methyl incorporation were more likely due to the new creatine synthesis in individual tissues.

Our short infusion time was not sufficient to detect any changes in muscle creatine concentrations. Since muscle has no detectable AGAT activity and very low GAMT activity, newly synthesized creatine may have been transported from the liver. Although we expected a higher level of creatine in the 200% Met group, our short experimental period likely did not allow us to detect changes in this very large pool of creatine (12). In a long-term feeding study in ducks, supplemental GAA with methionine enhanced muscle creatine loading capacity (6). So, it is possible that with a longer infusion time, muscle creatine concentrations could be increased.

In summary, the results from this study demonstrated that when piglets were supplemented with GAA and excess methionine, enhanced creatine synthesis in various tissues occurred. When dietary methionine was low, supplemented GAA was not absorbed, and what was absorbed accumulated in the liver. Although the gut is known to synthesize creatine, the fate and role of this creatine is not well investigated. Some studies have shown beneficial effects of creatine in the gut. For example, a few human case reports have demonstrated that dietary creatine attenuates inflammatory bowel disease (IBD) by maintaining gut barrier function (38). The current study indicates that GAA with sufficient methionine enhances creatine pools in jejunum, which may be a beneficial alternative to creatine supplements in maintaining gut functions. Besides its role in the gut, it has been demonstrated that GAA is a beneficial and safe dietary supplement in humans and animals. Amongst its many demonstrated benefits, GAA, likely via conversion to creatine, promotes growth, physical performance, reproductive parameters, and meat quality in commercial animals (10,11).

In conclusion, the findings from this study demonstrated that in neonatal piglets, excess methionine is required to achieve the 100% GAA absorption. However, the results of this study cannot define the methionine requirement for GAA absorption across the gut. Nevertheless, it is apparent that creatine stores were superior in piglets that received methionine at approximately twice the estimated whole body methionine requirement. However, caution is warranted before recommending doubling of methionine requirements, given its potential role in increasing homocysteine concentrations, a well-known cardiovascular disease risk factor (Boushey et al., 1995). In conclusion, it is clear that when using GAA supplements, sufficient dietary methionine should also be administered to enhance complete GAA absorption and creatine synthesis in neonatal piglets.

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4.7 References

1. Ostojic SM. Guanidinoacetic acid as a performance-enhancing agent. Amino Acids 2016;48(8):1867-75.
2. Ostojic SM, Jorga J. Guanidinoacetic acid in human nutrition: Beyond creatine synthesis. Food Sci Nutr 2023;11(4):1606-11.
3. Yan Z, Yan Z, Liu S, Yin Y, Yang T, Chen Q. Regulative mechanism of guanidinoacetic acid on skeletal muscle development and its application prospects in animal husbandry: A review. Front Nutr 2021;8:714567.
4. Ostojic SM. Guanidinoacetic acid loading for improved location-specific brain creatine. Clin Nutr 2021;40(1):324-6.
5. Jagim AR, Kerksick CM. Creatine supplementation in children and adolescents. Nutrients 2021;13(2):664.
6. James BW, Goodband RD, Unruh JA, Tokach MD, Nelssen JL, Dritz SS. A review of creatine supplementation and its potential to improve pork quality. J Appl Anim Res 2002;21(1):1-16.
7. Stahl CA, Greenwood MW, Berg EP. Growth parameters and carcass quality of broilers fed a corn-soybean diet supplemented with creatine monohydrate. Int J Poult Sci 2003;2(6):404-8.
8. Baker DH. Advances in protein-amino acid nutrition of poultry. Amino Acids 2009; 37:29–41.
9. Alraddadi EA, Lillico R, Vennerstrom JL, Lakowski TM, Miller DW. Absolute oral bioavailability of creatine monohydrate in rats: debunking a myth. Pharmaceutics 2018;10(1):31.

10. Ostojic SM. Advanced physiological roles of guanidinoacetic acid. Eur J Nutr 2015;54:1211–15.
11. Asiriwardhana M, Bertolo RF. Guanidinoacetic acid supplementation: A narrative review of its metabolism and effects in swine and poultry. Front Anim Sci 2022;3: 117.
12. Wyss M, Kaddurah-Daouk R. Creatine and creatinine metabolism. Physiol Rev 2000;80(3):1107–213.
13. Brosnan JT, Wijekoon EP, Warford-Woolgar L, Trottier NL, Brosnan ME, Brunton JA, Bertolo RFP. Creatine synthesis is a major metabolic process in neonatal piglets and has important implications for amino acid metabolism and methyl balance. J Nutr 2009;139:1292-7.
14. Dinesh OC, Brunton JA, Bertolo RF. The kidneys are quantitatively more important than pancreas and gut as a source of guanidinoacetic acid for hepatic creatine synthesis in sow-reared Yucatan miniature piglets. J Nutr 2020;150: 443-9.
15. McBreairty LE, McGowan RA, Brunton JA, Bertolo RF. Partitioning of [methyl-3H] methionine to methylated products and protein is altered during high methyl demand conditions in young Yucatan miniature pigs. J Nutr 2013;143(6):804-9.
16. Ibrahim D, El Sayed R, Abdelfattah-Hassan A, Morshedy AM. Creatine or guanidinoacetic acid? Which is more effective at enhancing growth, tissue creatine stores, quality of meat, and genes controlling growth/myogenesis in Mulard ducks. J Appl Anim Res 2019;47(1):159-66.
17. Majdeddin M, Golian A, Kermanshahi H, Michiels J, De Smet S. Effects of methionine and guanidinoacetic acid supplementation on performance and energy metabolites in

breast muscle of male broiler chickens fed corn-soybean diets. Br Poult Sci 2019;60(5):554-63.

18. Kankayaliyan T, Dinesh OC, Rademacher M, Brunton JA, Bertolo RF. Creatine de novo synthesis and methionine metabolism in neonatal piglets. Canadian Nutrition Society Annual Conference; 2014: Appl Physiol Nutr Metab, Vol. 39.
19. Puiman P, Stoll B. Animal models to study neonatal nutrition in humans. Curr Opin Clin Nutr Metab Care 2008;11:601–6.
20. Miller ER, Ullrey DE. The pig as a model for human nutrition. Annu Rev Nutr 1987;7:361–82.
21. Pond WG, Houpt KA. The Biology of the Pig. Cornell University Press; 1978.
22. Brunton JA, Shoveller AK, Pencharz PB, Ball RO. The indicator amino acid oxidation method identified limiting amino acids in two parenteral nutrition solutions in neonatal piglets. J Nutr 2007;137:1253–9.
23. Dodge ME, Bertolo RF, Brunton JA. Enteral feeding induces early intestinal adaptation in a parenterally fed neonatal piglet model of short bowel syndrome. J Parenter Enteral Nutr 2012;36:205-12.
24. Shoveller AK, Brunton JA, House JD, Pencharz PB, Ball RO. Dietary cysteine reduces the methionine requirement by an equal proportion in both parenterally and enterally fed piglets. J Nutr 2003;133(12):4215-24.
25. Brunton JA, Baldwin MP, Hanna RA, Bertolo RF. Proline supplementation to parenteral nutrition results in greater rates of protein synthesis in the muscle, skin, and small intestine in neonatal Yucatan miniature piglets. J Nutr 2012;142(6):1004-8.

26. National Research Council. Nutrient requirements of swine. 10th ed. Washington, DC: National Academic Press;1998.
27. Dinesh OC, Kankayaliyan T, Rademacher M, Tomlinson C, Bertolo RF, Brunton JA. neonatal piglets can synthesize adequate creatine, but only with sufficient dietary arginine and methionine, or with guanidinoacetate and excess methionine. J Nutr 2021;151(3):531-9.
28. Buchberger W, Ferdig M. Improved high-performance liquid chromatographic determination of guanidino compounds by precolumn derivatization with ninhydrin and fluorescence detection. J Sep Sci 2004;27:1309–12.
29. Lamarre SG, Edison EE, Wijekoon EP, Brosnan ME, Brosnan JT. Suckling rat pups accumulate creatine primarily via de novo synthesis rather than from dam milk. J Nutr 2012;140:1570–3.
30. Bidlingmeyer BA, Cohen SA, Tarvin TL. Rapid analysis of amino acids using pre-column derivatization. J Chromatogr 1984;336:93–104.
31. DeGroot AA, Braun U, Dilger RN. Efficacy of guanidinoacetic acid on growth and muscle energy metabolism in broiler chicks receiving arginine-deficient diets. Poult Sci 2018;97(3):890-900.
32. Khajali F, Lemme A, Rademacher-Heilshorn M. Guanidinoacetic acid as a feed supplement for poultry. Worlds Poult Sci J 2020;76(2):270-91.
33. Tossenberger J, Rademacher M, Németh K, Halas V, Lemme A. Digestibility and metabolism of dietary guanidino acetic acid fed to broilers. Poult Sci 2016;95(9):2058-67.

34. Tachikawa M, Kasai Y, Yokoyama R, Fujinawa J, Ganapathy V, Terasaki T, Hosoya KI. The blood–brain barrier transport and cerebral distribution of guanidinoacetate in rats: involvement of creatine and taurine transporters. J Neurochem 2009;111(2):499-509.
35. Ostojic SM. Co-administration of creatine and guanidinoacetic acid for augmented tissue bioenergetics: A novel approach? Biomed Pharmacother 2017;91:238–40.
36. Romanet S, Aschenbach JR, Pieper R, Zentek J, Htoo JK, Whelan RA, Mastrototaro L. Expression of proposed methionine transporters along the gastrointestinal tract of pigs and their regulation by dietary methionine sources. Genes Nutr 2021;16:1-15.
37. Adriano E, Salis A, Damonte G, Millo E, Balestrino M. Effects of delivering guanidinoacetic acid or its prodrug to the neural tissue: possible relevance for creatine transporter deficiency. Brain Sci 2022;12(1):85.
38. Roy A, Lee D. Dietary creatine as a possible novel treatment for Crohn's ileitis. ACG Case Rep J 2016;3(1):146.

Chapter 5: Guanidinoacetic acid transport across the small intestine is enhanced by dietary creatine in neonatal piglets.

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Chapter 5 was primarily designed by M. Asiriwardhana and R. Bertolo. M. Asiriwardhana, Z. Clancy and J. Edwards performed the animal experiments and metabolite analyses. M. Asiriwardhana analyzed the data and prepared the manuscript with guidance from R. Bertolo. The final manuscript was read and approved by all authors.

5.1 Abstract

Creatine and guanidinoacetic acid (GAA) supplements are popular to increase muscle mass and physical performance. GAA is an amino acid derivative and precursor for creatine, which plays a significant role in energy metabolism. Studies have shown that GAA readily converts to creatine, but only when sufficient methionine is available to transmethylate GAA to creatine. Recent studies have shown that oral GAA and creatine co-administration for four weeks enhanced muscle and brain creatine levels in healthy individuals. This combination was superior to creatine alone in enhancing exercise performance. Moreover, administering GAA with creatine may reduce the side effects of GAA supplements, such as hyperhomocysteinemia. There is no research findings regarding how the GAA + creatine mixture affects GAA absorption, transport, and utilization in pigs. The first objective was to determine the efficient supplement combination to increase the creatine stores in the body. The second objective was to determine the optimal ratio of creatine and GAA to enhance GAA absorption across the gut. Yucatan miniature piglets (17.1 ± 2.0 d old, 3.1 ± 0.3 kg, n=24; 12 M, 12 F;) were given a 3-hour duodenal infusion of complete elemental diets with no GAA; GAA alone; GAA+ creatine; or GAA+ methionine. At necropsy, tissue samples were collected for creatine analyses, and a section of mid-jejunum from control piglets was mounted in Ussing chambers. The buffer on the luminal side contained 3.2 mM GAA alone or with 0, 1, 1.6, 3.2, 6.4, or 9.6 mM creatine. ^{14}C GAA was used to determine GAA transport over 2 h across the small intestine. Our findings demonstrated that both GAA + creatine and GAA + methionine groups showed increased brain creatine levels, and creatine concentration in the liver was highest in GAA + creatine group, suggesting GAA + creatine is the best combination to improve hepatic creatine stores. In the Ussing chamber model, ^{14}C-GAA appearance rate in serosal buffer was highest with 1:3 (9.6 mM) GAA:creatine, emphasizing that a higher level of creatine

enhances GAA absorption across the jejunum. The findings of this study are significant in elucidating the transport mechanism of GAA in the intestine and determining the most efficient supplement combination to enhance creatine synthesis, muscle energy capacity, and performance.

5.2 Introduction

5.2.1 Experiment 1

Creatine is known as a safe and effective agent for enhancing energy levels, suitable for use in athletics (Kreider et al., 2017), clinical settings and agricultural animals. However, there are notable disadvantages associated with creatine supplementation, such as its instability during production and storage, high cost (Baker et al., 2009), and its comparatively limited capacity for absorption in the body (Alraddadi et al., 2018). Hence, as a substitute for creatine, its precursor, guanidinoacetic acid (GAA), has gained popularity as a supplement for enhancing muscle mass and physical performance (Ostojic et al., 2015, Asiriwardhana et al., 2022).

GAA demonstrates a greater ability than creatine to enhance creatine levels in both the human brain and skeletal muscles (Ostojic et al., 2016) as well as in pig muscle and other tissues (McBreairty et al., 2015). However, certain studies have suggested possible detrimental consequences of GAA loading, including the risk of hyperhomocysteinemia (Setoue et al., 2008) and adverse effects on the nervous system (Stockler-Ipsiroglu et al., 2014). A more recent study has proposed that combining creatine and GAA in a co-administration approach might be a superior strategy compared to supplementing each compound separately (Ostojic et al., 2017). For example, Semeredi et al. (2019) demonstrated that the oral administration of a GAA and creatine (1:3) combination over four weeks proved to be more effective than using creatine alone. This combination showed notable improvements in tissue creatine levels and upper body strength in healthy, active men. However, the mechanism of this synergistic effect of creatine and GAA on creatine stores has not been explored. It is possible GAA enhances creatine transport, or creatine enhances GAA absorption and conversion to creatine.

Creatine synthesis involves the transfer of a methyl group from SAM to GAA to form creatine. Methionine serves as a supplier of methyl groups necessary for the synthesis of creatine (McBreairty et al., 2015). Adding GAA to the diet in the absence of methionine does not significantly boost creatine synthesis. For example, when piglets were given GAA with 80% of the dietary methionine requirement, creatine synthesis was not improved; but when methionine was increased to 200% of requirement, creatine synthesis was increased (Dinesh et al., 2021). This suggests that GAA supplementation is likely to promote creatine synthesis only when there is an ample supply of methionine available to furnish the required methyl groups. Our previous research in Chapter 4 has demonstrated that combining GAA with excess methionine can boost creatine levels in the body.

Consequently, our primary objective was to compare the effectiveness of three supplementation options: GAA alone, GAA + methionine, and GAA + creatine, in enhancing creatine stores and GAA absorption in neonatal piglets. We hypothesized that GAA absorption will be more effective when combined with either creatine or methionine compared to GAA alone, and that the GAA + creatine combination will result in the largest creatine stores.

5.2.2 **Experiment 2**

As we are investigating the impact of a GAA and creatine combination on augmenting body creatine levels in piglets, this second experiment was consequently developed to assess the absorption kinetics of the GAA and creatine mixture at the gut level. The transport of GAA relies on several types of transporters, and their distribution varies throughout the body. Although the complete description of GAA transport mechanisms is not yet available, there are some potential candidates that have been proposed. The transport of GAA has been proposed to be facilitated by several transporters, including creatine transporters (CRT/Solute carrier SLC6A8), taurine

transporters (TauT/SLC6A6), the gamma-aminobutyric acid (GABA) transporter Q9 (SLC6A13), and through passive diffusion across the cell membrane (Tachikawa et al., 2009). For example, in the rat liver, it appears that GAA uptake is primarily mediated by the GABA transporter (Tachikawa et al., 2012). The SLC6A8 gene is expressed at significantly higher levels in the small intestine compared to the large intestine, and it exerts an inhibitory influence when substrate concentrations are elevated (Garcia-Miranda et al., 2009; Loike et al., 1986, Dai et al., 1999). This is particularly noteworthy because a crucial creatine transporter (SLC6A8) has been demonstrated to be influenced by the extracellular concentration of creatine (Loike et al., 1988; Wyss and Kaddurah-Daouk, 2000). While GAA can also be transported through creatine transporters, it seems to have a lower affinity for these transporters compared to creatine (Tachikawa et al., 2009). However, when GAA is supplemented alongside creatine, creatine can potentially saturate the creatine transporters, leading to increased GAA transport through other transporter systems. This dynamic may result in a synergistic effect, ultimately enhancing cellular concentrations of both GAA and creatine, when fed together (Ostojic et al., 2017).

It is important to investigate the absorption and transport of GAA through the gastrointestinal tract. Investigating the in vivo process of absorption kinetics poses challenges due to its complexity and cost, whereas employing Ussing chambers could provide a straightforward and feasible ex vivo substitute (Awati 2009). Ussing chambers have been widely utilized to examine the absorption of nutrients through the epithelial tissues of the gastrointestinal tract in various animal species, such as rats (Moazed 2007) and pigs (Boudry 2005). Ussing chambers offer a valuable and well-established technique for measuring the movement of electrolytes, nutrients, and medications across epithelial tissues (Clarke, 2009). Moreover, Ussing chambers have been used for investigating the absorption patterns of various substances including glucose (Ducroc 2007),

heparin (Moazed 2007), oligonucleotides (Wu-Pong 1999), antibiotics (Boudry 2005), and amino acids (Grøndahl 1997).

The Ussing chamber represents an ex vivo method where intestinal tissue is excised and positioned between two reservoirs filled with buffer solution (the luminal and serosal chambers). This arrangement enables the examination of the absorption of compounds across both the brush border and basolateral membranes (Awati 2009). Research has indicated that by supplying extra oxygen and necessary nutrients, the intestinal tissue can maintain its viability for more than 120 minutes within the Ussing chamber system (Soderholm et al., 2002). Several studies have demonstrated successful transport investigations employing the Ussing for pig intestinal mucosal tissues (Awati 2009; Tennakoon 2013). Consequently, we chose the Ussing chamber system as our in vitro model to assess the transport activity of GAA, employing pig jejunal tissue as a model system. The research involved comparing the absorption of GAA in six different GAA to creatine ratios within a complete amino acid mixture.

In this second experiment, our primary objective was to determine the effectiveness of creatine and GAA combinations in enhancing GAA absorption in neonatal piglets. We hypothesized that GAA absorption across the gut will be highest in a 1: 3 GAA to creatine ratio.

5.3 Methods

5.3.1 Reagents

^{14}C-GAA was obtained from Moravek Biochemicals and all other analytical grade chemicals were from Fisher Scientific or Sigma.

5.3.2 **Experiment 1**

5.3.2.1 Piglets and surgical procedures

Yucatan miniature piglets (17.1± 2.0 d old, 3.1±0.3 kg, n=24; 12 M, 12 F) were obtained from Animal Care Services at Memorial University of Newfoundland. All procedures were approved by the institutional Animal Care Committee at Memorial University of Newfoundland and complied with the guidelines of the Canadian Council on Animal Care. The piglets underwent a 3-hour fasting period and the piglets were moved to the animal care facilities located on campus. The piglets were then randomly allocated to one of the four distinct infusion treatments, while ensuring balance of sexes and mean weights among the treatment groups. The piglets were sedated, intubated, and anesthetized using previously established methods (Dodge et al., 2012). While under general anesthesia, the piglets underwent a surgical procedure to implant catheters and probes. The carotid artery was carefully separated through blunt dissection, and a catheter was inserted to facilitate arterial blood sampling. Additionally, a catheter was introduced into the jugular vein and advanced to the superior vena cava, for infusion and venous blood sampling. Subsequently, a laparotomy was carried out, during which the portal vein was isolated. An ultrasonic perivascular blood flow probe from Transonic Systems Inc. was securely positioned around the portal vein to monitor blood flow and a catheter was inserted for blood sampling. To administer the experimental diet treatments (described later), a catheter was also placed in the duodenum. Throughout the surgical procedure, the exposed visceral organs were kept moist with warmed saline and covered with wet gauze and plastic wrap to prevent dehydration.

5.3.2.2 Experimental diets

All experimental treatments were prepared according to the methods previously outlined in Chapter 4 and in previous studies (Brunton et al., 2012, Dodge et al., 2012). Experimental diets

were elemental (amino acids, glucose, minerals and vitamins) with GAA, creatine or methionine modified. To expedite the transition to a postprandial state, an initial prime dose of the diet, equivalent to the hourly rate, was first administered in a bolus. This was followed by continuous infusion at a rate of 11 $mL \cdot kg^{-1} \cdot h^{-1}$ using a syringe pump.

The following treatments were administered for 3 h into the duodenal catheter: 1) Control; 2) GAA; 3) GAA plus methionine; or 4) GAA plus creatine. The amino acid concentrations for both the control and experimental diets are outlined in Supplemental Table 6.

Control diet (CON): The control diet was formulated to compare the impact of GAA supplementation on creatine synthesis, either when GAA is given alone or in combination with creatine or methionine. Methionine was supplemented to meet 100% of the requirement determined by Shoveller et al. (2003b), while GAA was omitted from this diet.

GAA diet (GAA): The purpose of this diet was to determine optimal GAA absorption and conversion to creatine when the diet was supplemented with GAA. Control diet was supplemented with GAA at a rate of 3.75 $mg \cdot kg^{-1}$ $BW \cdot h^{-1}$. This amount of GAA, if fully converted into creatine, would fulfill the total creatine accretion rate of the piglet at this developmental age (Brosnan et al., 2009).

GAA plus methionine diet (GAA + Met): The purpose of this diet was to determine if methionine is the limiting factor for creatine synthesis from supplemented GAA. If methionine is limiting in the GAA group, this excess methionine group will increase utilization of GAA for creatine synthesis. Thus, this group received supplemented GAA along with excess methionine to ensure that GAA undergoes methylation to form creatine. Methionine was given at a rate of 20.77 $mg \cdot kg^{-1}$

$BW \cdot h^{-1}$, which represents 200% of the methionine requirement (with excess cysteine) determined by Shoveller et al., 2003.

GAA plus creatine diet (GAA + Cre): The purpose of this diet was to determine the biological maximum creatine accretion and the sparing effect of creatine on GAA absorption and its conversion to creatine. Half of the piglet's creatine accretion rate was provided by supplementing with GAA, while the other half was provided by supplementing with creatine. GAA was supplemented at a rate of 1.88 $mg \cdot kg^{-1}$ $BW \cdot h^{-1}$, which corresponds to half (on a molar basis) of the piglet's total creatine accretion rate at this age (Brosnan et al., 2009). Creatine (as creatine monohydrate) was given at a rate of 2.5 $mg \cdot kg^{-1}$ $BW \cdot h^{-1}$, which is equal to the half of the total creatine accretion rate of the piglet.

5.3.2.3 Blood flow measurement and blood sampling

During the course of the experiment, blood samples were taken from the carotid artery (representing arterial metabolite concentrations available to the gut) as well as from the portal and jugular veins.

Following the duodenal infusion, a segment of the small intestine from only the control group piglets was collected for use in the Ussing chamber experiment. This segment of the small intestine was surgically extracted from the mid jejunum, which is located approximately 100 cm distal to the ligament of Treitz. The isolated portion of the intestine was then detached from its connection to the mesentery, and the serous membrane beneath it was also disconnected. For all piglets in all groups, samples of the liver, kidneys, brain, jejunum, and muscle were collected, weighed, and rapidly frozen using liquid nitrogen. The samples were stored at -80°C until further analysis. The piglets were euthanized by exsanguination after the organs had been removed.

5.3.2.4 Tissue and plasma creatine concentration determination

Tissue and plasma creatine concentrations were determined through HPLC using a modified method by Lamarre et al., 2012. Carotid and portal plasma samples at the end of 3 h infusion were used for the analyses. Tissue homogenates were first deproteinized by treating them with trifluoroacetic acid. These homogenates were prepared in a 50 mM Tris buffer at pH 7.4 and were left at room temperature for 20 minutes to ensure the complete conversion of phosphocreatine to creatine. The separation of creatine was achieved using an isocratic mobile phase composed of 0.1% trifluoroacetic acid and 3% methanol.

5.3.2.5 Tissue and plasma amino acid determination

Plasma and tissue amino acid concentrations were measured by HPLC following derivatization with phenylisothiocyanate with norleucine as the internal standard (Bidlingmeyer et al., 1984). Carotid and portal plasma samples at the end of 3 h infusion were used for the analyses.

NOTE: This study was conducted in collaboration with another graduate student (Zack Clancy) who administered GAA stable isotopes to the same group of piglets simultaneously. The purpose of this other study was to determine GAA metabolism across different tissues and the conversion of GAA to creatine using tracers. However, the methods and results of the other graduate student's work are not presented in this chapter.

5.3.3 **Experiment 2**

The segment of jejunum from the control piglets was flushed with cold physiological saline solution to eliminate any remaining chyme, and then placed into a chilled, oxygenated incubation buffer for transportation to the laboratory. The composition of the incubation buffer solution, as outlined in Table 5.1, closely resembles that utilized on the "serosal" side of the Ussing chamber.

Table 5.1 Composition of modified Kreb 's buffer used in Ussing chamber experiment and as the incubation buffer

Ingredient	Concentration
NaCl	137 mM
KCl	5.4 mM
$CaCl_2 \cdot 2H_20$	2.8 mM
MgS04	1 mM
K_2HP04	0.3 mM
NaH_2P04	0.3 mM

5.3.3.1 Mounting of intestinal tissues on Ussing chamber

Upon arrival at the laboratory, the intestinal segments were dissected longitudinally and then washed again with cold saline solution to eliminate any residual contents present in the lumen. A section of the intestine measuring 2-3 cm was placed in an oxygenated and modified Krebs's buffer solution. It was left to float in the solution until it extended fully. Afterward, the section was affixed to a Styrofoam plate and then carefully positioned onto the pins located at the Ussing chamber port (Figure 5.1A). The complementary half of the chamber was appropriately aligned with the pins and linked to the gas lift mechanism. For this study, Ussing chambers from World Precision Instruments (CHM6 model, with a 1 cm^2 aperture) was used (Figure 5.1B).

Figure 5.1A

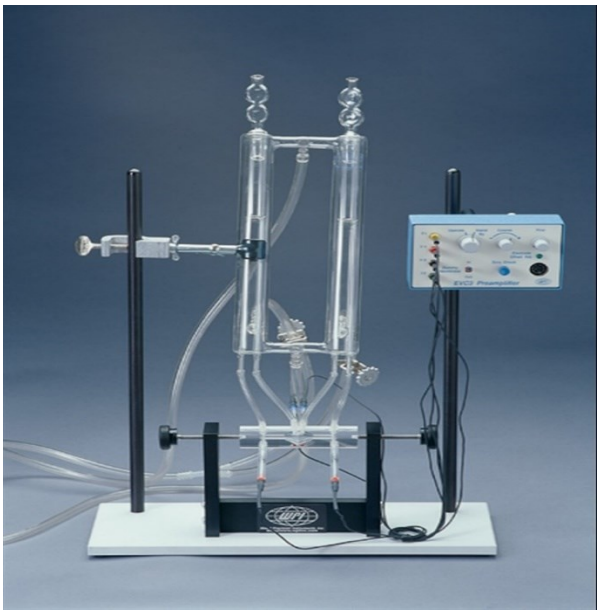


Figure 5.1B

Figure 5.1 (A) Ussing chamber compartment and (B) Ussing chamber system (Ussing System Instruction Manual)

The tissues positioned within the Ussing chambers were incubated on both the luminal and serosal sides, using 6 mL of a modified Kreb's buffer solution (Winckler et al., 1999) (Table 5.1). The pH was adjusted to 6.0 for the luminal solution and 7.4 for the serosal solution accomplished through the utilization of TRIS buffer. The luminal side buffer also contained mixture of amino acids with GAA alone or with 1.07, 1.6, 3.2, 6.4, 9.6 mM creatine (GAA only, GAA: cre 1:0.3, GAA: cre 1:0.5, GAA: cre 1:1, GAA: cre 1:2, GAA: cre 1:3). The treatment composition is detailed in Supplemental Table 7. Additionally, ^{14}C-GAA was added to the luminal buffer solutions to determine the efficiency of GAA transport across the small intestine. The Ussing chamber with GAA and no creatine was used as the control condition. The ratio of sample solution to modified Kreb's buffer solution utilized (1: 9) was determined to be appropriate for maintaining a comparable osmotic environment between the luminal solution and intracellular fluids (300–310 mOsm· L^{-1}) in order to minimize luminal cell lysis (Awati et al., 2009). In the serosal solution, 10 mM glucose was added as an energy source, while 10 mM mannitol was incorporated into the luminal solution to ensure osmotic equilibrium. The gas lift system ensured a continuous supply of oxygen and recirculation for the incubation solutions, while the temperature-controlled water jacket maintained all solutions at a constant temperature of 37°C (Figure 5.2).

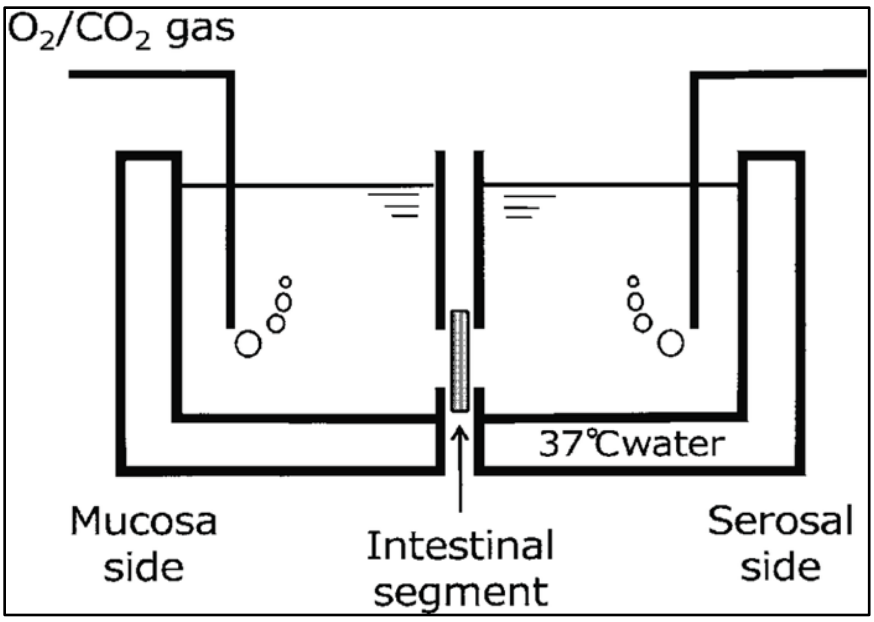


Figure 5.2 Schematic drawing of Ussing chamber (Inagaki-Tachibana et al., 2009)

During the experiment's commencement and at 15-minute intervals thereafter, 0.5 mL samples were collected from both the serosal and luminal buffers. The aliquots were replaced by an equal volume of fresh buffer solution. The complete experiment was conducted over a duration of 2 hours.

5.3.3.2 Measuring ^{14}C-GAA appearance in the serosal buffer

GAA transport was determined by ^{14}C-GAA appearance in the serosal buffer. A 50 µL sample was mixed with 5 mL of EcoLite™ Liquid Scintillation Cocktail (MP Biomedicals) and the radioactivity was measured using liquid scintillation counter. ^{14}C-GAA appearance was determined using the following calculation:

GAA appearance= $(n_0/ DPMt_0) * DPMt_n$

$DPMt_0$: DPM counted in the luminal compartment buffer before the experiment was started

$DPMt_n$: DPM counted in serosal compartment taken after t_n

n_0: total GAA (nmol) present in the luminal buffer before the experiment was started

5.3.3.3 Measuring ^{14}C-GAA disappearance from the luminal buffer

GAA disappearance was determined by measuring the ^{14}C-GAA radioactivity in the luminal buffer. A 50 µL sample was mixed with 5 mL of EcoLite™ Liquid Scintillation Cocktail (MP Biomedicals) and the radioactivity was measured using liquid scintillation counter.

^{14}C-GAA disappearance was calculated using the following calculation:

GAA disappearance= $(n_0/ DPMt_0) * DPMt_n$

$DPMt_0$: DPM counted in the luminal compartment buffer before the experiment was started

$DPMt_n$: DPM counted in luminal compartment taken after t_n

n_0: total GAA (nmol) present in the luminal buffer before the experiment was started

Subsequently, GAA transportation rates were determined by calculating the slopes from the GAA appearance and disappearance graphs over a 2-hour period.

5.3.3.4 Measuring the tissue viability

The viability of the intestinal tissue affixed to the Ussing chambers was evaluated using the EKl electrode kit (WPI, Sarasota, FL) in conjunction with the DVC-1000 voltage/current clamp (WPI, Sarasota, FL). Smaller blue voltage electrodes were inserted into the "V1" and "V2" terminals of the DVC-3 preamplifier (WPI, Sarasota, FL), and these electrodes were linked to luer ports situated close to the tissue. The larger red current electrodes were joined to the "I1" and "I2" terminals of the DVC-3, and then they were linked to luer ports situated further away from the intestinal mucosal tissue. Subsequently, the DVC-3 was linked to the DVC-1000 electrode clamp. The DVC-1000 electrode clamp detected the voltage gradient across the intestinal mucosal tissues. The amplified signals were then transmitted to a computer using the Lab-Trax data acquisition system and visualized through the Labscribe data recording software (iWorx Systems Inc).

5.3.3.5 Analysis of tissue radioactivity

Tissue samples (0.1 g) were manually homogenized and combined with 1 mL of Solvable tissue solubilizer. Following digestion of the samples at 50°C for 2 hours, they were allowed to equilibrate to room temperature. Subsequently, 10 mL of EcoLite™ liquid scintillation cocktail was added to each sample and left overnight for stabilization. Radioactivity levels were then measured by counting for 10 minutes using a liquid scintillation counter.

5.3.3.6 Amino acid analysis

Amino acid concentrations in luminal buffer at the end of the experiment were measured by HPLC following derivatization with phenylisothiocyanate with norleucine as the internal standard

(Bidlingmeyer et al., 1984). Apparent amino acid disappearance rate across the jejunum was determined using the following equation:

Apparent amino acid disappearance rate (%/min) = $[(AA_0 - AA_{t120})/AA_0] \times 100/120$

AA_0= amino acid concentration in the luminal solution at time 0

AA_{t120} = amino acid concentration in the luminal solution at time t = 120 min

5.4 Statistical analyses

5.4.1 Experiment 1

Data were analyzed using one-way ANOVA followed by Newman-Keuls or Tukey's post hoc test to detect the differences between treatments. Significant differences were recognized when the P-value was less than 0.05 (GraphPad Prism; Graph Pad Software Inc.).

5.4.2 Experiment 2

Data were analyzed using one-way ANOVA followed by Newman-Keuls Multiple Comparison to detect the differences between treatments. Significant differences were recognized when the P-value was less than 0.05. Correlation between GAA transportation rate and time was analyzed by linear regression (GraphPad Prism; Graph Pad Software Inc.). Polynomial contrasts were carried out to determine the linear and quadratic effects of increasing creatine levels (IBM SPSS Statistics; Version 27).

5.5 Results

5.5.1 Experiment 1

5.5.1.1 Creatine concentration in tissues

The creatine concentration in the jejunum was notably higher in both creatine and methionine supplemented groups. However, there was no significant difference in jejunal creatine concentration between the control group and the GAA group (Figure 5.3A).

The liver creatine concentration was significantly higher in the GAA + Cre group compared to the other groups. In contrast, the GAA group and the GAA + Met group exhibited similar creatine concentrations to the control group (Figure 5.3B).

The brain creatine concentration showed a significant increase in both the groups that received supplements of methionine and creatine. However, there was no significant difference among the GAA + Met group and the GAA + Cre group. Additionally, the brain creatine concentration in the GAA group was found to be similar to that in the control group (Figure 5.3C).

There were no significant differences in muscle and kidney creatine concentrations among the groups (Figure 5.3D, Figure 5.3E).

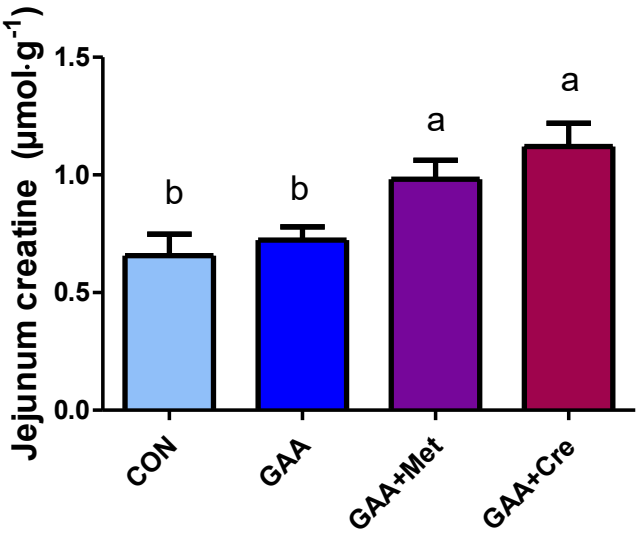


Figure 5.3A

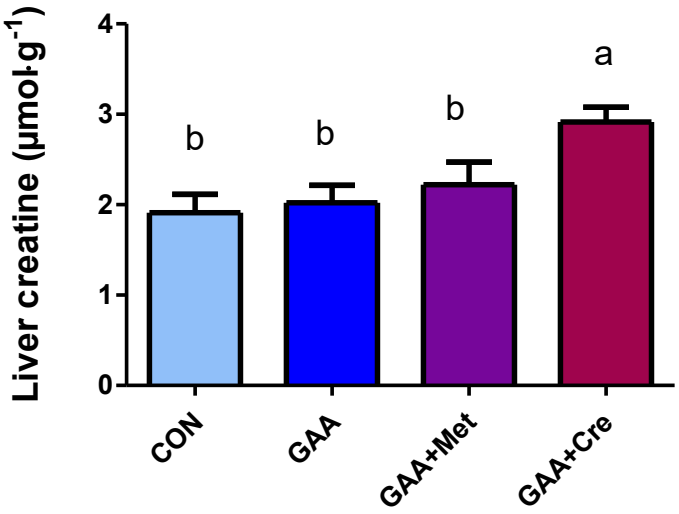


Figure 5.3B

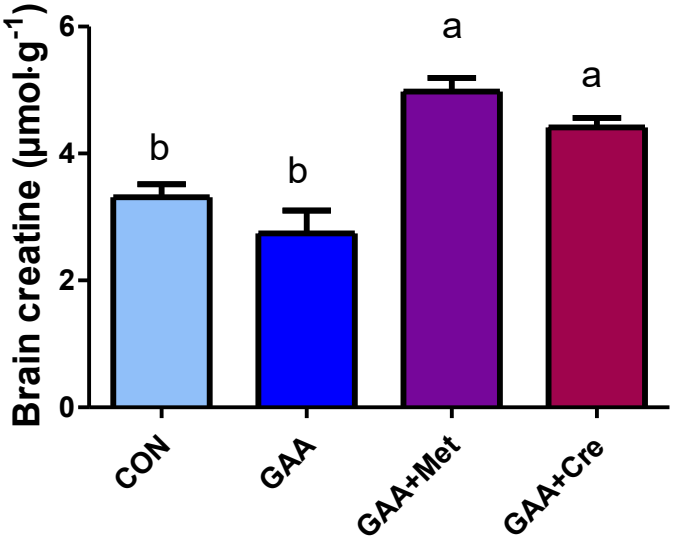


Figure 5.3C

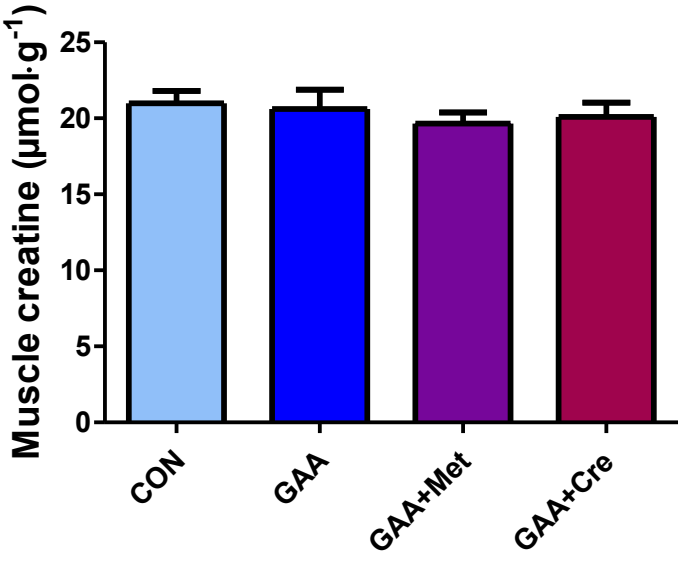


Figure 5.3D

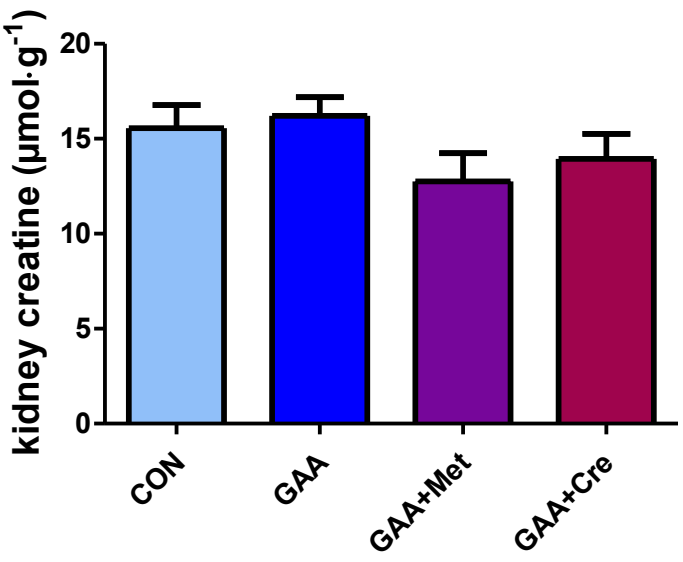


Figure 5.3E

Figure 5.3 Creatine concentration in jejunum (A), liver (B), brain (C), muscle (D), kidney (E) following 3 h duodenal infusion with diets containing no GAA, GAA only, GAA + Met, GAA + Cre in piglets (n=6). Values are means ± SEM; P values were calculated by 1-way ANOVA followed by Newman-Keuls post test. Bars with different letters are significantly different ($P < 0.05$).

5.5.1.2 Creatine concentration in plasma

Portal vein creatine concentration was higher at 3 h in all 3 treatment groups compared to the control group. Similarly carotid creatine concentration was also higher in treatment groups compared to the control group (Figure 5.4A, Figure 5.4B). There were no significant difference in the levels of creatine in the carotid artery or portal vein across the different treatment groups.

5.5.1.3 Plasma amino acid concentrations

Piglets that received diets supplemented with methionine showed significantly elevated plasma methionine levels after 3 h across all sampled blood vessels compared to other treatment groups, except for methionine levels in the carotid artery of the control group (Table 5.2). Plasma arginine concentrations remained consistent across treatments in all vessels except for the renal vein, where the control group showed a significantly higher concentration compared to the GAA and GAA + Cre groups (Table 5.2). Plasma citrulline levels showed consistency across treatment groups in the carotid artery, renal vein, and femoral vein. However, citrulline levels in the portal vein of the control group were notably lower compared to the other treatment groups. Conversely, citrulline levels in the jugular vein of the control group were significantly higher than those in the methionine-supplemented group (Table 5.2).

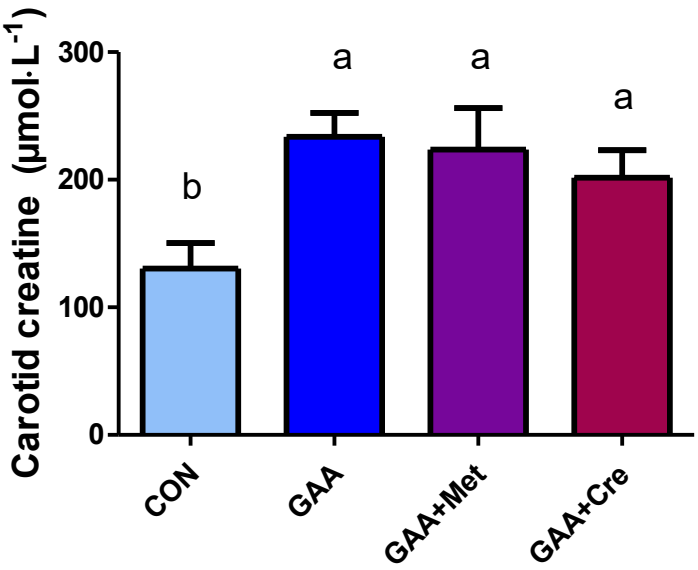


Figure 5.4A

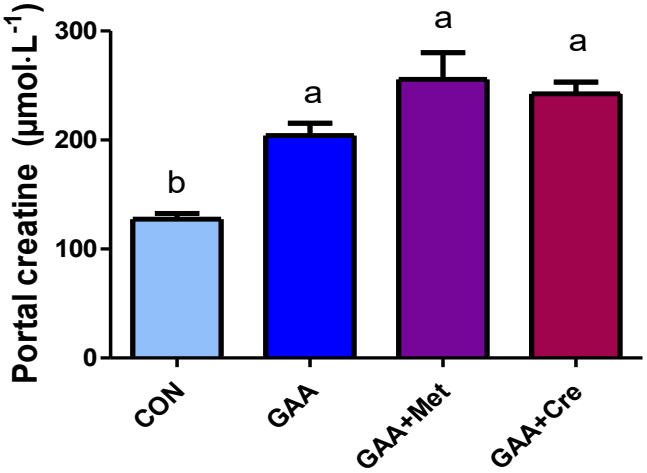


Figure 5.4B

Figure 5.4 Creatine concentration in carotid artery (A) and portal vein (B) following 3 h duodenal infusion with diets containing no GAA, GAA only, GAA + Met, GAA + Cre in piglets (n=6). Values are means ± SEM; P values were calculated by 1-way ANOVA followed by Newman-Keuls post test. Bars with different letters are significantly different ($P < 0.05$)

Table 5.2 Selected plasma amino acid concentrations associated with the production of creatine in the carotid artery, portal, renal, jugular, and femoral veins.

	Control	GAA	GAA + Met	GAA + Cre	P value*
	Methionine (μmol·L^{-1})				
Carotid Artery	106 ± 40.8^{b}	68 ± 9.0^{b}	148 ± 18a	52 ± 9.5^{b}	0.001
Portal Vein	56 ± 14.5^{b}	53 ± 7.4^{b}	150 ± 11.1a	66 ± 10.2^{b}	0.0001
Renal Vein	77 ± 13.0^{b}	78 ± 19.1^{b}	159 ± 36.9a	68 ± 25.4^{b}	0.0001
Jugular Vein	82 ± 18.0^{b}	69 ± 12.0^{b}	156 ± 33.1a	74 ± 6.1^{b}	0.0001
Femoral Vein	73 ± 12.1^{b}	67 ± 22.3^{b}	141 ± 27.2a	68 ± 16.4^{b}	0.0001
	Arginine (μmol·L^{-1})				
Carotid Artery	221 ± 116.0	120 ± 81.1	184 ± 47.8	150 ± 108.1	0.3
Portal Vein	166 ± 75.0 ^{a}	46 ± 12.0 ^{b}	65 ± 14.5 b	57 ± 11.5 ^{b}	0.001
Renal Vein	211 ± 65.3^{a}	117 ± 29.0^{b}	150 ± 46.4ab	122 ± 65.1^{b}	0.01
Jugular Vein	225 ± 140.5	95 ± 31.8	89 ± 55.0	97 + 71.5	0.3
Femoral Vein	175 ± 43.3	61 ± 22.7	83 ± 82.1	61 ± 19.9	0.4

	Citrulline (μmol·L^{-1})				
Carotid Artery	96 ± 14	226 ± 140.9	140 ± 23.5	131 ± 51	0.1
Portal Vein	131 ± 57.2^{b}	233 ± 36.5^{a}	286 ± 61.1^{a}	262 ± 63.5^{a}	0.001
Renal Vein	300 ± 35.1	282 ± 130.4	202 ± 110.8	231 ± 51.0	0.3
Jugular Vein	202 ± 93.8^{a}	139 ± 45.6^{ab}	96 ± 20.0^{b}	117 ± 20.6^{ab}	0.02
Femoral Vein	134 ± 28.8	162 ± 45.4	147 ± 28.3	181 ± 111.4	0.6

*P-value for overall treatment effect by one-way ANOVA. Different superscript letters represent differences in group means by Tukey's post hoc test ($P < 0.05$).

**AA concentrations were analyzed by Julian Edwards for his honours thesis.*

5.5.2 **Experiment 2**

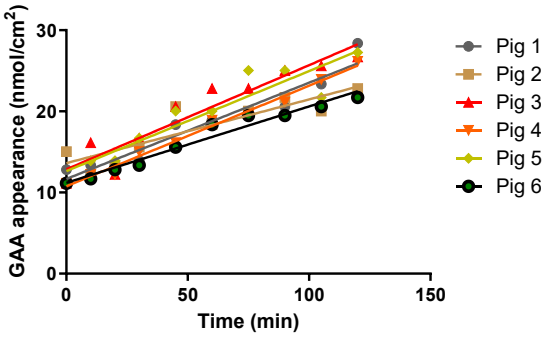
5.5.2.1 GAA transport across the jejunal section with differing concentrations of creatine

The transepithelial GAA appearance from lumen to serosa across the jejunal sections, assessed in vitro using Ussing chambers, exhibited an increasing linear trend from 0 to 120 minutes ($R^2 >$ 0.32). Examples of the linear relationship are demonstrated in Figure 5.5A-F, while an overall comparison between treatment groups is illustrated in Figure 5.6. (Correspondingly, disappearance of GAA from the luminal side with different concentrations of the creatine was also found to be linear from 0 to 120 min ($R^2 > 0.77$). Linear relationships are illustrated in Figure 5.7A- F, with a comprehensive comparison between all treatment groups presented in Figure 5.8. The linear relationship of GAA transport with time indicated the constant movement of GAA out of the intestinal lumen.

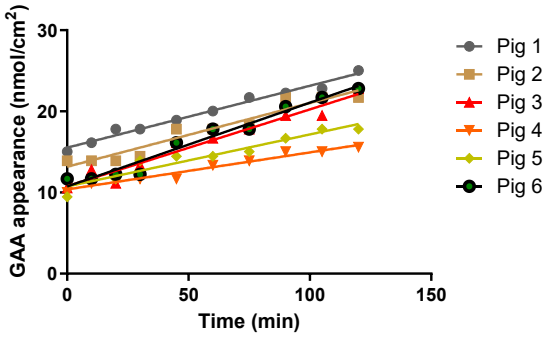
5.5.2.2 GAA appearance rate

GAA transport rates (slopes) from lumen to serosa through the jejunum were calculated and GAA: Cre 1:3 group had the highest GAA appearance rate at 0.2 ± 0.08 $nmol \cdot cm^{-2} \cdot min^{-1}$ (Figure 5.9). There were no significant differences in GAA appearance rates from lumen to serosa observed among the other groups. GAA appearance rates from lumen to serosa were linearly increased with the increasing levels of creatine (Figure 5.10).

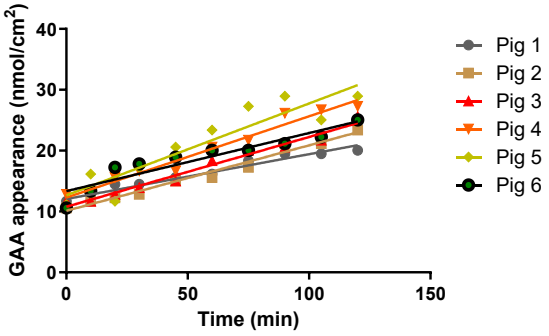
In addition, a portion of GAA undergoes conversion into creatine within the jejunum, and the ^{14}C DPM counts recorded in serosal buffer samples include both the GAA present and newly synthesized ^{14}C creatine in the jejunum.


GAA appearance (nmol/cm^2)
Time (min)
Pig 1
Pig 2
Pig 3
Pig 4
Pig 5
Pig 6

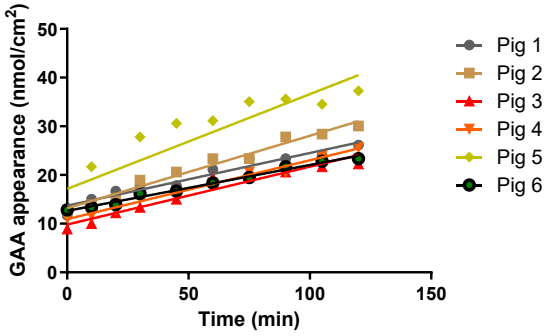
(A)


GAA appearance (nmol/cm^2)
Time (min)
Pig 1
Pig 2
Pig 3
Pig 4
Pig 5
Pig 6

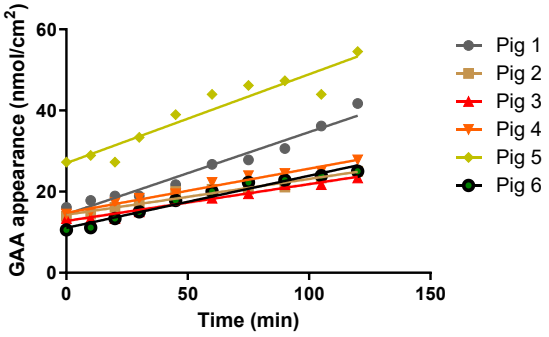
(B)


GAA appearance (nmol/cm^2)
Time (min)
Pig 1
Pig 2
Pig 3
Pig 4
Pig 5
Pig 6

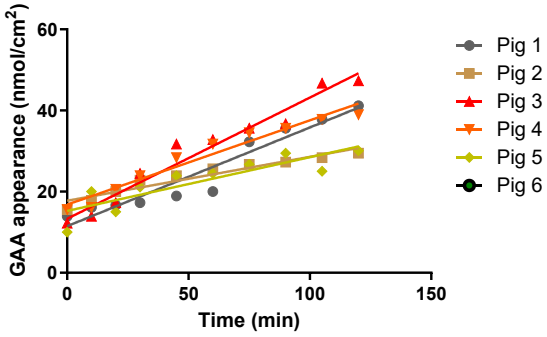
(C)


GAA appearance (nmol/cm^2)
Time (min)
Pig 1
Pig 2
Pig 3
Pig 4
Pig 5
Pig 6

(D)


GAA appearance (nmol/cm^2)
Time (min)
Pig 1
Pig 2
Pig 3
Pig 4
Pig 5
Pig 6

(E)


GAA appearance (nmol/cm^2)
Time (min)
Pig 1
Pig 2
Pig 3
Pig 4
Pig 5
Pig 6

(F)

Figure 5.5 GAA appearance in serosal chamber through neonatal pig jejunum over time. Each data point represents the values measured from one piglet at a specific point in time. Data were linearly regressed over time (Figure 5.5A; GAA only group, Figure 5.5B; GAA: Cre 1:0.3, Figure 5.5C; GAA: Cre 1:0.5, Figure 5.5D; GAA: Cre 1:1, Figure 5.5E; GAA: Cre 1:2, Figure 5.5F; GAA: Cre 1:3).

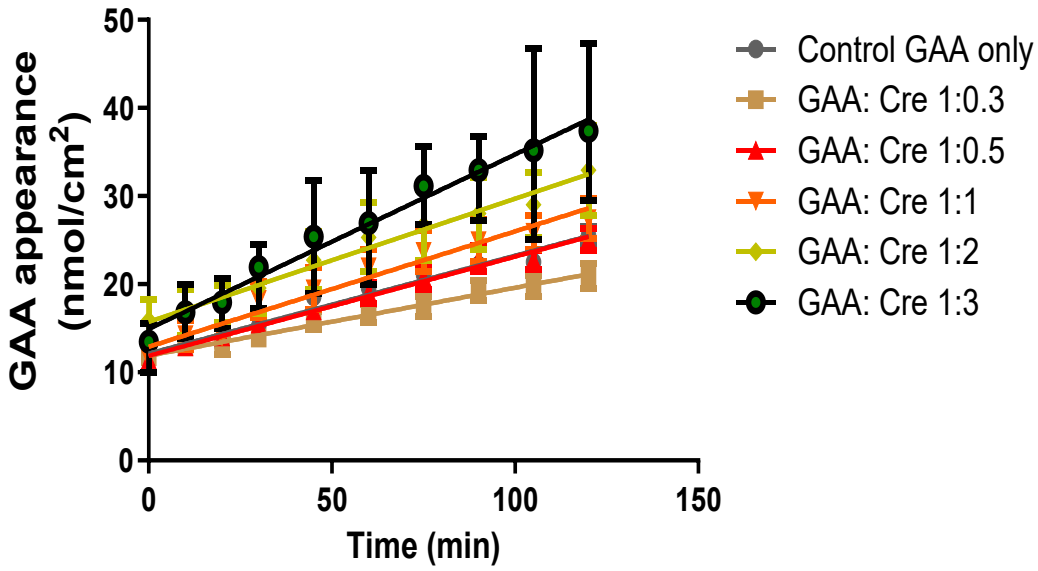
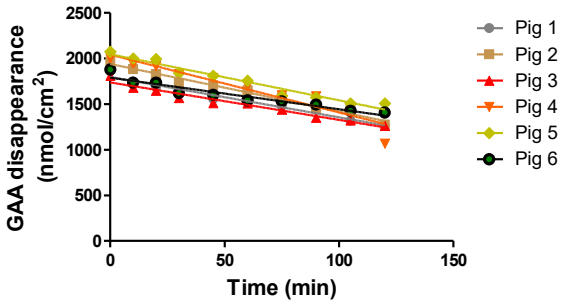
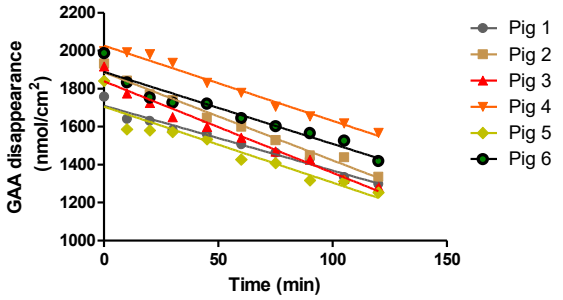


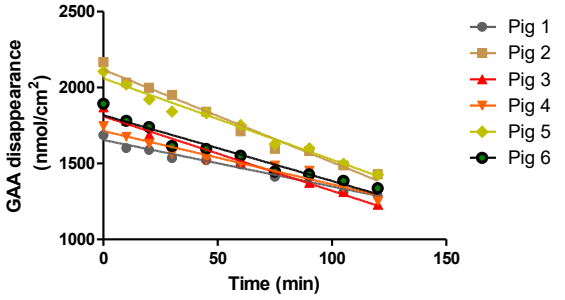
Figure 5.6 GAA appearance in serosal chamber through neonatal pig jejunum over time. Values are means ± SEM; each data point represents 5 or 6 piglets. Data were linearly regressed over time.


GAA disappearance (nmol/cm2)
Time (min)
Pig 1
Pig 2
Pig 3
Pig 4
Pig 5
Pig 6

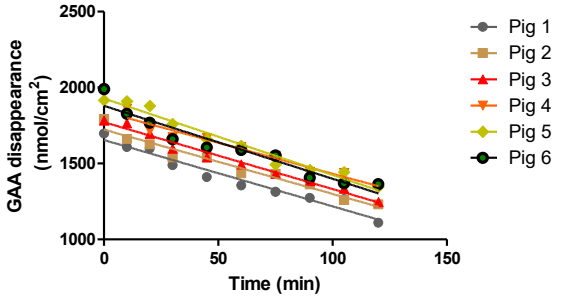
(A)


GAA disappearance (nmol/cm2)
Time (min)
Pig 1
Pig 2
Pig 3
Pig 4
Pig 5
Pig 6

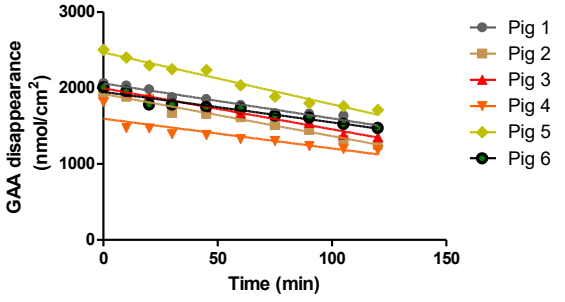
(B)


GAA disappearance (nmol/cm2)
Time (min)
Pig 1
Pig 2
Pig 3
Pig 4
Pig 5
Pig 6

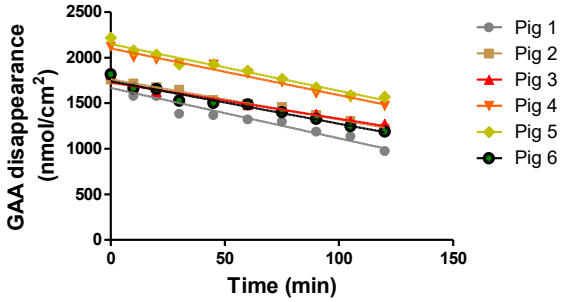
(C)


GAA disappearance (nmol/cm2)
Time (min)
Pig 1
Pig 2
Pig 3
Pig 4
Pig 5
Pig 6

(D)


GAA disappearance (nmol/cm2)
Time (min)
Pig 1
Pig 2
Pig 3
Pig 4
Pig 5
Pig 6

(E)


GAA disappearance (nmol/cm2)
Time (min)
Pig 1
Pig 2
Pig 3
Pig 4
Pig 5
Pig 6

(F)

Figure 5.7 GAA disappearance from lumen into neonatal pig jejunum over time. Each data point represents the values measured from one piglet at a specific point in time. Data were linearly regressed over time (Figure 5.7A; GAA only group, Figure 5.7B; GAA: Cre 1:0.3, Figure 5.7C; GAA: Cre 1:0.5, Figure 5.7D; GAA: Cre 1:1, Figure 5.7E; GAA: Cre 1:2, Figure 5.7F; GAA: Cre 1:3).

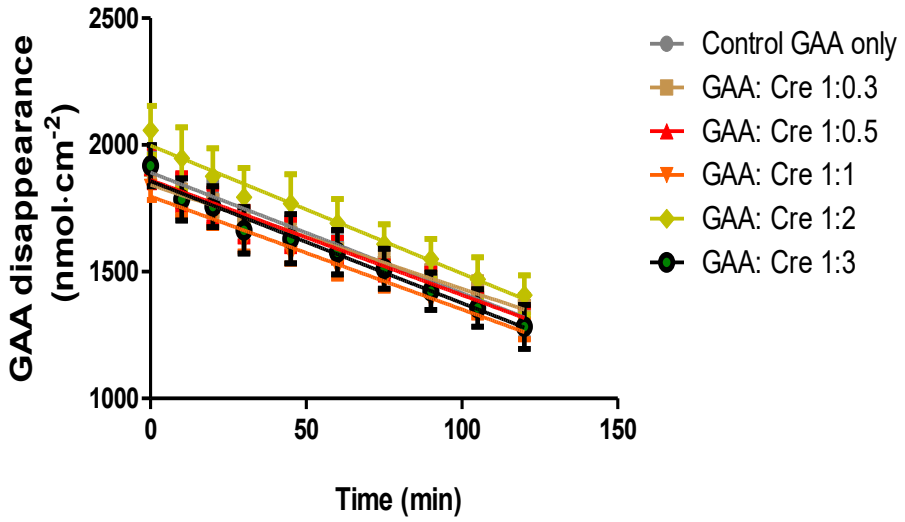


Figure 5.8 GAA disappearance from lumen into neonatal pig jejunum over time. Values are means ± SEM; each data point represents 5 or 6 piglets. Data were linearly regressed over time.

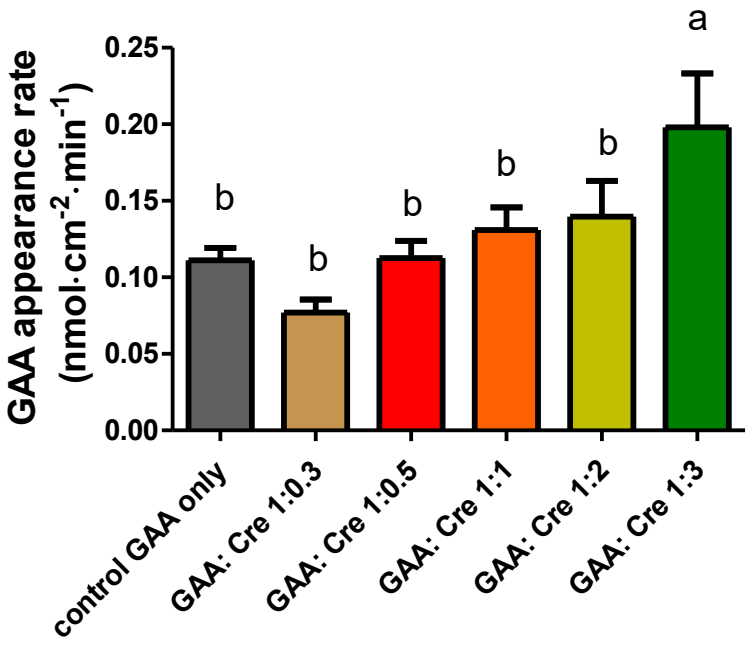


Figure 5.9 GAA appearance rate in the serosal chamber through neonatal pig jejunum with varying concentrations of creatine. Values are means ± SEM; each bar represents 5 or 6 Ussing chamber experiments, with tissues taken from 5 or 6 piglets. P values were determined by one-way ANOVA. Groups with differing superscripts are significantly different at $P < 0.05$ using Newman-Keuls Multiple Comparison Test.

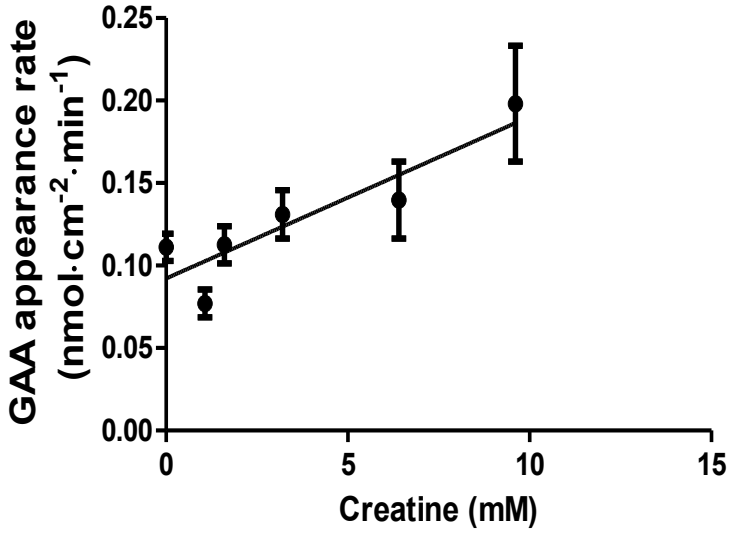


Figure 5.10 GAA appearance rate across neonatal pig jejunum with varying concentrations of luminal creatine. Data were analyzed using linear regression and P values were calculated by ANOVA with polynomial contrasts for linear trend. (P value for linear effect = 0.01 Quadratic=0.06)

5.5.2.3 GAA disappearance rate

GAA disappearance rate (slope) from the lumen was also measured in sections of piglet jejunum that were mounted in Ussing chambers, to determine if increasing levels of creatine would have a greater effect in the GAA disappearance. However, GAA disappearance rates were not significantly different among the groups (Figure 5.11).

5.5.2.4 Area under the curve (AUC) of GAA appearance rate

AUC corresponds to the amount of dietary GAA that appeared in the serosal side over the 120 min period. The AUC up to 120 minutes was calculated to assess the appearance of GAA through the piglet jejunum with different concentrations of creatine. GAA: Cre 1:3 group had the highest AUC (3243± 411.6 $nmol \cdot min^{-1} \cdot cm^{-2}$) among the groups (Figure 5.12, Table 5.3).

5.5.2.5 Jejunal tissue radioactivity

At the end of the experiment, the remaining radioactivity in the jejunum sample was measured. The jejunum tissue contained both ^{14}C-GAA and newly synthesized ^{14}C-creatine from the ^{14}C-GAA introduced into the lumen. The results showed that there were no significant differences among the different experimental groups in terms of this residual radioactivity in the jejunum (Table 5.4).

5.5.2.6 Apparent amino acid disappearance rate

There was no significant difference of the amino acid transportation across the jejunum among the groups (Table 5.5).

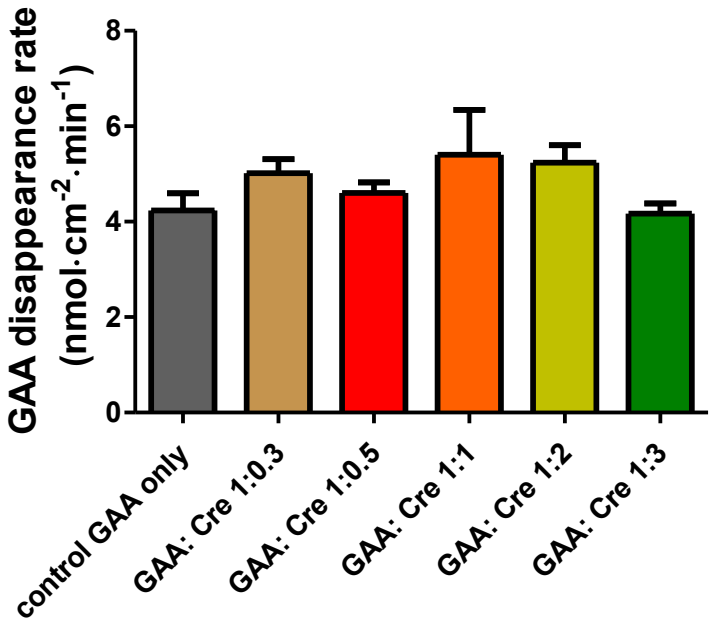


Figure 5.11 GAA disappearance rate from the luminal chamber into the neonatal pig jejunum with varying concentrations of creatine. Values are means ± SEM; each bar represents 5 or 6 Ussing chamber experiments, with tissues taken from 5 or 6 piglets. P values were determined by one-way ANOVA. Groups with differing superscripts are significantly different at $P < 0.05$ using Newman-Keuls Multiple Comparison Test.

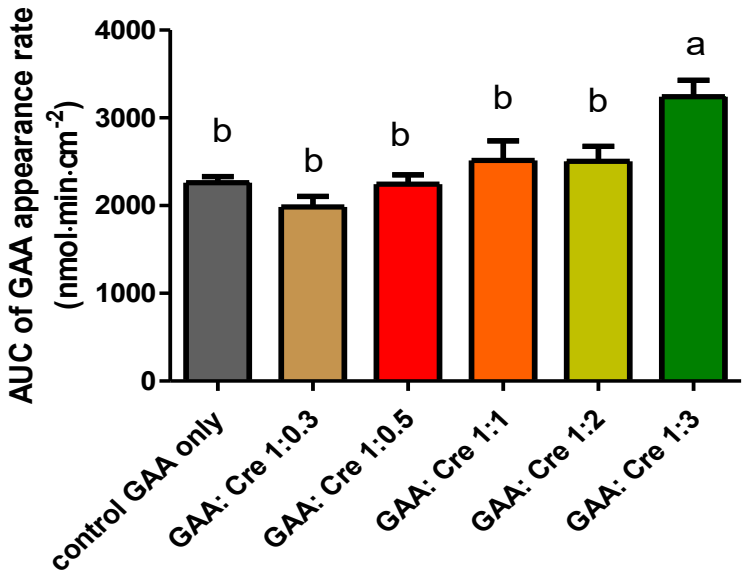


Figure 5.12 Area under the curve to 120 min for the appearance of GAA in the serosal chamber through the piglet jejunum with different concentrations of creatine. Values are means ± SEM; each bar represents 5 or 6 Ussing chamber experiments, with tissues taken from 5 or 6 piglets. P values were determined by one-way ANOVA. Groups with differing superscripts are significantly different at $P < 0.05$ using Newman-Keuls Multiple Comparison Test.

Table 5.3 AUC for the appearance of GAA in the serosal chamber through the piglet jejunum, with different concentrations of creatine

	Control GAA only	GAA: Cre 1:0.3	GAA: Cre 1:0.5	GAA: Cre 1:1	GAA: Cre 1:2	GAA: Cre 1:3	*P
Area under the curve to 120 min (nmol·min ·cm^{-2})	2263± 171.8^{b}	1985± 292.2^{b}	2244± 267.4^{b}	2516± 548.4^{b}	2506± 381.7^{b}	3243± 411.6^{a}	0.0001

Data are presented as mean± SEM

*P values were determined by one-way ANOVA. Groups with differing superscripts are significantly different at $P < 0.05$ using Newman-Keuls Multiple Comparison Test.

Table 5.4 Jejunal tissue radioactivity at the end of the 2 h Ussing chamber experiment.

	Control GAA only	GAA: Cre 1:0.3	GAA: Cre 1:0.5	GAA: Cre 1:1	GAA: Cre 1:2	GAA: Cre 1:3	*P
Tissue radioactivity DPM·mg^{-1}	55.35± 22.20	47.81± 24.30	48.33± 21.50	46.45± 30.64	37.08± 14.71	56.11± 20.66	0.73

Data are presented as mean± SEM

*P-value for overall treatment effect by one-way ANOVA.

Table 5.5 Apparent amino acid disappearance rate through the piglet jejunum with different concentrations of creatine

	Control	GAA: Cre 1:0.3	GAA: Cre 1:0.5	GAA: Cre 1:1	GAA: Cre 1:2	GAA: Cre 1:3	*P
	(%·min^{-1})						
Aspartate	0.65±0.02	0.68±0.04	0.64±0.02	0.66±0.04	0.66±0.06	0.69±0.05	0.5
Serine	0.45±0.06	0.54±0.11	0.46±0.04	0.47±0.07	0.48±0.11	0.52±0.13	0.8
Glycine	0.33±0.10	0.46±0.15	0.34±0.06	0.34±0.08	0.41±0.26	0.49±0.24	0.6
Taurine	0.13±0.08	0.20±0.08	0.11±0.05	0.10±0.01	0.11±0.07	0.10±0.04	0.1
Histidine	0.31±0.12	0.49±0.16	0.31±0.09	0.39±0.08	0.48±0.18	0.32±0.06	0.08
Threonine	0.10±0.03	0.08±0.03	0.07±0.04	0.09±0.07	0.06±0.03	0.11±0.05	0.3
Alanine	0.34±0.11	0.48±0.19	0.32±0.10	0.46±0.13	0.47±0.24	0.47±0.21	0.5
Arginine	0.16±0.06	0.22±0.17	0.09±0.02	0.11±0.06	0.14±0.06	0.13±0.11	0.3
proline	0.57±0.07	0.61±0.15	0.61±0.10	0.55±0.12	0.53±0.21	0.58±0.09	0.9
Tyrosine	0.68±0.14	0.79±0.02	0.68±0.16	0.66±0.19	0.74±0.05	0.67±0.23	0.7
valine	0.40±0.16	0.47±0.16	0.36±0.19	0.39±0.23	0.41±0.10	0.45±0.18	0.9
Methionine	0.62±0.04	0.67±0.11	0.63±0.04	0.56±0.08	0.61±0.09	0.68±0.10	0.3
Isoleucine	0.39±0.07	0.41±0.21	0.33±0.05	0.33±0.09	0.36±0.14	0.60±0.24	0.08
leucine	0.15±0.05	0.14±0.09	0.07±0.05	0.10±0.08	0.13±0.07	0.17±0.08	0.4
Phenylalanine	0.31±0.12	0.48±0.15	0.31±0.10	0.35±0.06	0.34±0.12	0.37±0.16	0.3
Tryptophan	0.55±0.11	0.65±0.06	0.57±0.06	0.52±0.19	0.48±0.08	0.53±0.26	0.5
Lysine	0.69±0.03	0.72±0.05	0.68±0.03	0.66±0.01	0.69±0.05	0.68±0.03	0.3

Data are presented as mean±SD, *P-value for overall treatment effect by one-way ANOVA.

5.6 Discussion

5.6.1 Experiment 1

GAA and creatine play vital roles in energy metabolism, with creatine supplementation being widely recognized for its potential to enhance physical performance in sport and support animal performance in the commercial animal industry. GAA is emerging as a more stable substitute for creatine supplementation in both humans and animals (Asiriwardhana et al., 2022).

In the first experiment, our main goal was to identify the most efficient combination of delivering GAA supplementation to improve GAA absorption and transformation into creatine in piglets. Consequently, our objective was to assess the effects of supplementing creatine or methionine alongside GAA to increase creatine levels in piglets. To achieve this objective, we used four dietary interventions, including a control diet to compare supplementation of GAA alone or in combination with creatine or methionine. The GAA diet assessed baseline GAA absorption and conversion to creatine, with the rate of GAA supplementation sufficient to meet the total creatine accretion. The GAA + Met diet examined methionine's role in creatine synthesis by adding excess methionine to ensure maximal GAA methylation. Finally, the GAA + Cre diet determined maximum creatine delivery, via both direct supplementation and its synthesis, with half of the creatine accretion coming from each supplemented GAA and creatine.

In the jejunum, liver, and brain of the GAA group, where no additional methionine is provided, creatine levels are similar to those of the control group, suggesting GAA alone was not very effective, highlighting the significance of available methyl groups for the conversion of GAA to creatine. However, the GAA group had higher creatine levels in the carotid and portal plasma than in the control group, suggesting that absorbed GAA is converted to creatine in the gut using the body's available methyl groups and then circulated to other organs. The absence of a significant

increase in jejunal creatine levels in the GAA group suggests the possibility of rapid export of converted creatine. Jejunum, a significant site for amino acid absorption and metabolism in the small intestine, plays a crucial role in creatine biosynthesis (Brosnan et al., 2009).

AGAT facilitates a reversible transamidination process, transferring the guanidino group from arginine to glycine, which leads to the synthesis of GAA and ornithine (Walker et al., 1979). In both the GAA + Cre and GAA + Met groups, a greater amount of GAA was converted to creatine, simultaneously lowering plasma GAA levels and decreasing the conversion of GAA to arginine, as indicated by lower arginine concentration in the renal vein. This observation indicates that the supplemented GAA is effectively converted to creatine in the GAA treatment groups, compared to the control group. While the plasma arginine levels did not indicate an apparent arginine-sparing effect by GAA, there are indications of a possible sparing effect based on the citrulline plasma levels. Citrulline concentrations in the portal vein were higher in all three GAA groups. This may imply that the gut retains less arginine when it is not necessary for creatine synthesis in intestinal tissue (Dinesh et al., 2020).

Our study demonstrated that GAA combined with methionine or creatine improves body creatine pools. When considering the supplementation of GAA with methionine, it is important to recognize that such a combination can remarkably impact GAA absorption and enhance tissue creatine synthesis. Several studies have indicated the potential benefits of this approach. For example, Dinesh et al., 2021 have shown that providing GAA with adequate methionine led to improved creatine synthesis. This finding highlights the importance of methionine in facilitating the effective utilization of GAA for creatine production.

In the group supplemented with GAA and methionine, a higher level of creatine synthesis occurred in the gut than in the control group and GAA-only group, as indicated by jejunum creatine

concentration. This highlights the significance of the methyl group's presence in facilitating the conversion of GAA to creatine. However, the increased jejunum creatine levels might also be due to the recirculated creatine originating from other organs. While we anticipated elevated creatine levels in the liver in the GAA + Met group, we did not detect a difference compared to the control and GAA groups. We hypothesized that when GAA is converted to creatine in the liver using excess methyl groups from an abundance of methionine, newly synthesized creatine is promptly exported to other organs rather than accumulating in the liver. The elevated creatine levels observed in the carotid artery support this notion, as the carotid artery transports and distributes creatine to other organs. Furthermore, in the GAA + Met group, high creatine levels were detected in the portal vein, brain, and carotid artery, highlighting the necessity of adequate methionine levels for creatine synthesis and the absorption of GAA.

GAA with a sufficient amount of methionine has the potential to act as a valuable supplement for enhancing various creatine-related functions in both humans and animals. As predicted, the supplementary methionine provided through the GAA + Met treatment significantly boosted methionine availability. Our measurements revealed elevated plasma methionine concentrations in arterial and venous blood samples compared to nearly all other treatment groups except for arterial blood in the control pigs.

Our data showed that the GAA + Cre group had a significantly higher level of liver creatine than other groups, as the liver is the major site of creatine accumulation and synthesis in piglets (Brosnan 2009). This finding further supports the opinion that supplementing with both GAA and creatine enhances hepatic creatine pools, likely due to the synergistic effects of these compounds in promoting GAA and creatine absorption, as well as hepatic creatine production. Our data also revealed that feeding creatine along with GAA enhanced creatine levels in the jejunum, suggesting

that creatine can spare GAA for intestinal creatine synthesis and improve GAA absorption when given together as a supplementary strategy.

The supplementation of GAA along with methionine or creatine to piglets increased brain creatine levels compared to the group that received GAA alone. Additionally, we found that the brain creatine concentration in the GAA group was similar to that in the control group. Unlike other organs, the expression of AGAT, GAMT, and creatine transporters within the central nervous system (CNS) implies that the CNS has the capability to produce creatine from precursor amino acids and GAA (Braissant and Henry, 2008). Nevertheless, it is significant that the levels of AGAT, GAMT, and creatine transporters differ across various brain regions. Moreover, other organs could synthesize creatine and transport it to the brain, or supplementary GAA might be converted to creatine within the brain utilizing accessible methyl groups. Excess creatine may spare GAA for creatine synthesis in the group receiving both GAA and creatine, aiming to elevate brain creatine levels.

In addition to providing a competitive advantage for enhancing tissue creatine levels, the combination of GAA and creatine might potentially alleviate adverse effects linked to isolated GAA administration. For example, the mixture of creatine and GAA appeared to mitigate hyperhomocysteinemia, an undesirable side effect associated with GAA supplementation (Setoue et al., 2007; Ostojic et al., 2019), which is a recognized individual risk factor for cardiometabolic diseases. However, currently, there are no pig studies examining the impacts of this combination on homocysteine levels, although this is being measured by another student for this study. Having established the impact of combining GAA and creatine in augmenting body creatine pools through short-term duodenal infusion in our current study, conducting additional research with longer

feeding trials would be advantageous for investigating the potential of this combination in alleviating side effects.

Despite our expectations, we observed no significant differences in the creatine concentrations in muscle or kidney tissues. We had anticipated that the groups supplemented with methionine and creatine would exhibit higher muscle or kidney creatine levels. However, our findings did not support this hypothesis, suggesting that 3 h of duodenal infusion may not be sufficient to see a change in creatine level or factors other than supplementation alone may contribute to creatine levels in these tissues. Indeed, we observed a doubling of muscle and kidney creatine when GAA is supplemented in weaned pigs for 3 weeks (McBreairty et al., 2015).

The comparable portal and carotid creatine concentrations observed across GAA treatment groups suggest that the presence of creatine or methionine did not significantly influence circulating creatine levels during the specified time frame. However, it is noteworthy that all GAA-supplemented groups exhibited elevated plasma creatine levels compared to the control group, highlighting the active conversion of supplemented GAA to creatine within the body, irrespective of the presence of creatine or methionine. However, plasma methionine concentrations were not different from control in the GAA and GAA + Cre treatment groups, suggesting the methionine needed for this conversion must have come from increased remethylation of homocysteine, or from breakdown of protein.

Although GAA supplementation has many benefits, some studies highlight scarcity of information on GAA transport. GAA present in the diet is absorbed by the gastrointestinal tract and transported through the portal blood into the liver, where it undergoes synthesis into creatine. Various transporters responsible for GAA uptake in vivo have been identified, including SLC6A8, SLC6A6, and SLC6A13 (Tachikawa et al., 2009; Braissant et al, 2012). Among these transporters,

SLC6A8 exhibits higher expression levels in the small intestine compared to the large intestine. Furthermore, few studies indicated that SLC6A8 shows an inhibitory effect at high substrate concentrations (Garcia-Miranda et al., 2009; Loike et al., 1986. Dai et al., 1998). Moreover, gastrointestinal contents containing higher concentrations of GAA might hinder the functional expression of relevant transporters (SLC6A8 and SLC6A6) in small intestine epithelial cells (Zhang et al., 2022). Consequently, these findings underscore our rationale for combining creatine or methionine with GAA instead of solely depending on a high dietary intake of GAA to elevate creatine levels in the body. This approach could potentially avoid the limitations associated with exclusively relying on dietary GAA intake, thereby offering a promising avenue for optimizing creatine levels and improving overall metabolic function.

5.6.2 **Experiment 2**

In the first experiment, we anticipated that the group receiving both GAA and creatine would have higher creatine levels in the body due to synergistic transport. Consequently, we developed the second experiment to examine the absorption kinetics of GAA across the jejunum, particularly when supplemented with different concentrations of creatine.

In the second experiment, we investigated the transportation rates of GAA across the jejunum of Yucatan miniature piglets under in vitro conditions using the Ussing chamber model. This model provides a controlled environment that allows researchers to investigate the mechanisms and dynamics of absorption across biological barriers such as epithelial membranes. Using this model, researchers can gain valuable insights into the transport mechanisms, kinetics, and factors influencing the absorption of substances of interest, contributing to our understanding of physiological processes and potential therapeutic interventions (Winkler et al., 1999; Tennakon, 2013).

The Ussing chamber experiment revealed that the rate of GAA appearance from the luminal to the serosal side, across the jejunum, was highest in the treatment group with a GAA to creatine ratio of 1:3, highlighting that a higher level of creatine enhances GAA absorption across the jejunum. Although the mechanism is unclear, we can speculate that sharing the same transporter with creatine may influence GAA transportation. While creatine transporters can transport GAA, studies suggest GAA exhibits a lower affinity for these transporters than creatine (Tachikawa et al., 2009). Hence, supplementing creatine alongside GAA can potentially saturate the creatine transporters, thereby increasing GAA transport through alternative transporter systems. This interaction may lead to a synergistic absorption effect, ultimately augmenting whole body concentrations of both GAA and creatine.

Moreover, according to the AUC data, the amount of dietary GAA that appeared on the serosal side over the 120 minutes was highest when GAA to creatine ratio was 1:3 compared to other groups. However, in the present study, the measurement of ^{14}C radioactivity in the serosal side includes both transported ^{14}C- GAA from the luminal side, as well as ^{14}C-creatine that was newly synthesized by the enterocytes during the experiment. Despite using various creatine analysis techniques to isolate creatine and employing fraction collection methods to detect newly synthesized ^{14}C-creatine in the serosal side, we could not distinguish ^{14}C-GAA and ^{14}C-creatine in the serosal side. We do not have a method to isolate ^{14}C-GAA, so we tried to isolate ^{14}C-creatine by HPLC and fraction collection; however, we could not detect any creatine peaks on the serosal side, nor could we detect any DPM counts for ^{14}C-creatine fraction in the serosal solution. We concluded from these results that a conversion of GAA to creatine was very low in the jejunum throughout the experiment and that the counts on the serosal side were almost entirely due to appearance of ^{14}C-GAA. To better understand creatine transportation across the jejunum when

supplemented with GAA, a follow-up study was developed utilizing ^{14}C-creatine tracers for easier detection. Another graduate student is conducting this study.

No significant differences were observed in the remaining radioactivity in jejunal tissue among treatment groups. These data suggest that the retention of ^{14}C-GAA or ^{14}C-creatine was not different with changing luminal creatine concentrations. Although we could not detect ^{14}C-creatine in jejunal tissue, we presume little was converted. Given the 2-hour duration of the experiment, it is likely that GAMT (and AGAT) enzyme activities were similar across all treatment groups, as there was insufficient time for enzymes to respond to dietary concentrations. Moreover, because of the lack of treatment effect in luminal disappearance, we can conclude that most of the effect of creatine on GAA transport was due to interactions at the basolateral membrane level.

We also investigated the influence of a mixture containing GAA and creatine on the absorption rate of other amino acids. Our findings indicate that increasing the level of creatine in the lumen did not significantly impact the apparent disappearance rate of amino acids from the luminal chamber. Given that GAA and creatine utilize distinct sets of transporters compared to other amino acids, their supplementation does not substantially influence the transportation of other amino acids across the jejunum. This observation underscores the specificity and independence of GAA and creatine transport processes, suggesting that their supplementation does not disrupt the overall absorption dynamics of amino acids in this intestinal segment.

To our knowledge, our study represents the first demonstration of GAA's absorption kinetics in the piglets' intestine. The in vitro Ussing chamber model has limitations, and one of its key drawbacks is the lack of a complex physiological environment in the intestinal mucosa (Clarke, 2009). The excision of tissue from the animal diminishes the impact of any endocrine or nervous stimuli on the intestinal mucosa (Clarke, 2009). This decrease in the presence of such stimuli may

contribute to reduced intestinal tonicity, potentially causing the tissue to become more permeable compared to its in vivo state. We could incorporate the findings of the Ussing experiment for comparison with our first experiment. Interestingly, we observed no significant difference in plasma creatine concentrations in GAA: Cre 1:1 group in first experiment and the GAA transportation rates in GAA: Cre 1:1 ratio group in second experiment, compared to other groups. However, given that we observed an increase in GAA absorption into the jejunum with the GAA: Cre 1:3 ratio, we could consider incorporating this ratio into our future animal experiments to investigate the overall creatine metabolism at the whole-body level.

5.7 Conclusion

We found that the GAA + Cre mixture outperformed the GAA + Met mixture in enhancing liver creatine levels. However, both supplemented groups showed increased brain creatine levels following a 3-hour duodenal infusion in piglets. Considering the summary of the results, both GAA supplemental strategies with creatine and methionine enhance body creatine levels and can effectively improve body creatine pools.

A GAA-creatine mixture with a ratio of 1-to-3 was determined to be superior compared to other ratios with lower GAA-to-creatine proportions, exhibiting enhanced absorption across the jejunum in Yucatan miniature piglets. These findings may contribute to developing effective supplements to increase creatine levels in the body. However, expanding the scope of research through additional trials with higher GAA: Cre ratios in animals will provide more comprehensive insights into the efficacy and potential risks associated with using this mixture as a dietary supplement.

5.8 References

Alraddadi EA, Lillico R, Vennerstrom JL, Lakowski TM, Miller DW. Absolute oral bioavailability of creatine monohydrate in rats: debunking a myth. Pharmaceutics 2018;10(1):31.

Asiriwardhana M, Bertolo RF. Guanidinoacetic acid supplementation: A narrative review of its metabolism and effects in swine and poultry. Front Anim Sci 2022;3: 117.

Awati A, Rutherfurd SM, Plugge W, Reynolds GW, Marrant H, Kies AK, Moughan PJ. Ussing chamber results for amino acid absorption of protein hydrolysates in porcine jejunum must be corrected for endogenous protein. Recent Adv Food Nutr Agric. 2009;89(11):1857-61.

Baker DH. Advances in protein-amino acid nutrition of poultry. Amino Acids 2009; 37:29–41.

Bidlingmeyer BA, Cohen SA, Tarvin TL. Rapid analysis of amino acids using pre-column derivatization. J Chromatogr. 1984;336:93–104.

Boudry G, The Ussing chamber technique to evaluate alternatives to in-feed antibiotics for young pigs. Anim Res. 2005;54:219–230.

Braissant O. Creatine and guanidinoacetate transport at blood-brain and blood-cerebrospinal fluid barriers. J Inherited Metab Dis 2012;35(4):655–664.

Braissant O. and Henry H. AGAT, GAMT and SLC6A8 distribu-tion in the central nervous system, in relation to creatine deficiencysyndromes: a review. J Inherit Metab Dis. 2008;31:230–239

Brosnan JT, Wijekoon EP, Warford-Woolgar L, Trottier NL, Brosnan ME, Brunton JA, Bertolo RFP. Creatine synthesis is a major metabolic process in neonatal piglets and has important implications for amino acid metabolism and methyl balance. J Nutr 2009;139:1292-7.

Brunton JA, Baldwin MP, Hanna RA, Bertolo RF. Proline supplementation to parenteral nutrition results in greater rates of protein synthesis in the muscle, skin, and small intestine in neonatal Yucatan miniature piglets. J Nutr 2012;142(6):1004-8.

Clarke LL. A guide to Ussing chamber studies of mouse intestine. Am J Physiol Gastrointest Liver Physiol. 2009;296:G1151-1166.

Dai W, Vinnakota S, Qian X, Kunze DL, Sarkar HK. Molecular characterization of the human CRT-1 creatine transporter expressed in Xenopus oocytes. Arch Biochem Biophys. 1999;361(1): 75–84.

Dinesh OC, Kankayaliyan T, Rademacher M, Tomlinson C, Bertolo RF, Brunton JA. Neonatal piglets can synthesize adequate creatine, but only with sufficient dietary arginine and methionine, or with guanidinoacetate and excess methionine. J Nutr. 2021;151:531-539.

Dinesh OC, Brunton JA, Bertolo RF. The kidneys are quantitatively more important than pancreas and gut as a source of guanidinoacetic acid for hepatic creatine synthesis in sow-reared Yucatan miniature piglets. J Nutr. 2020;150: 443-9.

Dodge ME, Bertolo RF, Brunton JA. Enteral feeding induces early intestinal adaptation in a parenterally fed neonatal piglet model of short bowel syndrome. J Parenter Enteral Nutr. 2012;36:205-12.

Ducroc R, Voisin T, El Firar A and Laburthe M, Orexins control intestinal glucose transport by distinct neuronal, endocrine, and direct epithelial pathways. Diabetes 56:2494–2500 (2007).

Garcia-Miranda P, Garcia-Delgado M, Peral MJ, Calonge ML, Ilundain AA. Ontogeny regulates creatine metabolism in rat small and large intestine. J Physiol Pharmacol. 2009;60(3):127–133.

Grøndahl ML and Skadhauge E. Effect of mucosal amino acids on SCC and Na and Cl fluxes in the porcine small intestine - a review of the active Surveillance data. Comp Biochem Physiol. 1997;118A:233–237.

Inagaki-Tachibana E, Tsukahara T, Kaji K, Eguchi R, Kanazawa H, Hayashi H, Suzuki Y. Involvement of DNA fragmentation of enterocytes in mucosal injury to a mouse jejunum incubated in ussing chambers. Nagoya J Med Sci. 2009;71(1-2):11-18.

Kreider RB, Kalman DS, Antonio J, Ziegenfuss TN, Wildman R, Collins R, et al. International Society of Sports Nutrition position stand: safety and efficacy of creatine supplementation in exercise, sport, and medicine. J Int Soc Sports Nutr. 2017;14:18

Lamarre SG, Edison EE, Wijekoon EP, Brosnan ME, Brosnan JT. Suckling rat pups accumulate creatine primarily via de novo synthesis rather than from dam milk. J Nutr. 2012;140:1570–3.

Loike JD, Somes M, Silverstein SC. Creatine uptake, metabolism, and efflux in human monocytes and macrophages. Am J Physiol. 1986;251(1):C128–C135.

Loike JD, Zalutsky DL, Kaback E, Miranda AF, Silverstein SC. Extracellular creatine regulates creatine transport in rat and human muscle cells. Proc Natl Acad Sci. 1988;85(3):807–811.

Tachikawa M, Kasai Y, Yokoyama R, Fujinawa J, Ganapathy V, Terasaki T, Hosoya KI. The blood–brain barrier transport and cerebral distribution of guanidinoacetate in rats: involvement of creatine and taurine transporters. J Neurochem. 2009;111(2):499–509.

McBreairty LE, Robinson JL, Furlong KR, Brunton JA, Bertolo RF. Guanidinoacetate is more effective than creatine at enhancing tissue creatine stores while consequently limiting methionine availability in Yucatan miniature pigs. PLoS One. 2015;10(6):e0131563.

Moazed B and Hiebert LM, An in vitro study with an Ussing chamber showing that unfractionated heparin crosses rat gastric mucosa. J Pharm Exp Therap. 2007;322:299–305.

Ostojic SM, Ostojic J, Drid P, Vranes M. Guanidinoacetic acid versus creatine for improved brain and muscle creatine levels: a superiority pilot trial in healthy men. Appl Physiol Nutr Metab. 2016;41:1005–7

Ostojic SM. Advanced physiological roles of guanidinoacetic acid. Eur J Nutr 2015;54:1211–15.

Ostojic SM. Co-administration of creatine and guanidinoacetic acid for augmented tissue bioenergetics: a novel approach? Biomed Pharmacother. 2017;91:238–40.

Semeredi S, Stajer V, Ostojic J, Vranes M, Ostojic SM. Guanidinoacetic acid with creatine compared with creatine alone for tissue creatine content, hyperhomocysteinemia, and exercise performance: A randomized, double-blind superiority trial. Nutrition. 2019;57:162-6.

Setoue M, Ohuchi S, Morita T, Sugiyama K. Hyperhomocysteinemia induced by guanidinoacetic acid is effectively suppressed by choline and betaine in rats. Biosci Biotechnol Biochem. 2008;72:1696–703

Shoveller AK, Brunton JA, House JD, Pencharz PB, Ball RO. Dietary cysteine reduces the methionine requirement by an equal proportion in both parenterally and enterally fed piglets. J Nutr. 2003;133(12):4215-24.

Soderholm JD, Olaison G, Peterson KH, Franzen LE, Lindmark T, Wiren M, Tagesson C & Sjodahl R .Augmented increase in tight junction permeability by luminal stimuli in the non-inflamed ileum of Crohn's disease. Gut 2002;50:307-313.

Stockler-Ipsiroglu S, van Karnebeek CD. Cerebral creatine deficiencies: a group of treatable intellectual developmental disorders. Semin Neurol. 2014;34:350–6

Tachikawa M, Ikeda S, Fujinawa J, Hirose S, Akanuma SI, Hosoya KI. γ-Aminobutyric acid transporter 2 mediates the hepatic uptake of guanidinoacetate, the creatine biosynthetic precursor, in rats. PloS one. 2012;7(2):e32557.

Tachikawa M, Kasai Y, Yokoyama R, Fujinawa J, Ganapathy V, Terasaki T, Hosoya KI. The blood–brain barrier transport and cerebral distribution of guanidinoacetate in rats: involvement of creatine and taurine transporters. J Neurochem. 2009;111(2):499–509.

Tennakoon, BC. Dipeptide transport in Yucatan miniature piglet intestine. Master's thesis 2013, Memorial University of Newfoundland, St. John's, Canada.

Ussing System Instruction Manual. World Precision Instruments Inc., 2014.

Walker JB. Creatine: biosynthesis, regulation, and function. Adv Enzymol Relat Areas Mol Biol. 1979;50(177):2.

Winckler C, Breves G, Boll M, Daniel H. Characteristics of dipeptide transport in pig jejunum in vitro. J Camp Physiol B. 1999;169:495-500.

Wu-Pong S, Livesay V, Dvorchik B and Barr WH, Oligonucleotide transport in rat and human intestine Ussing chamber models. Biopharm Drug Dispos. 20:411–416 (1999).

Wyss M, Kaddurah-Daouk R. Creatine and creatinine metabolism. Physiol Rev. 2000;80:1107–1213.

Zhang S, Zang C, Pan J, Ma C, Wang C, Li X, Cai W, Yang K. Effects of dietary guanidinoacetic acid on growth performance, guanidinoacetic acid absorption and creatine metabolism of lambs. PLoS One. 2022;17(3):e0264864.

Chapter 6: General discussion

One of the major goals of my PhD program was to determine the methionine requirement for non-protein pathways and the specific pathways that are compromised when methionine is deficient in the diet of neonatal piglets. Furthermore, this research explored the significance of methionine in converting GAA into creatine in neonates, as well as the interplay between GAA and creatine to augment the body's creatine reserves. The research outlined in this thesis centers on methionine's metabolism and developmental needs, with a concurrent examination of creatine synthesis and the metabolism of its precursor, GAA.

The thesis includes three research experiments, all directed toward addressing metabolic queries. In the first study, we tackled a metabolic question: Does the current estimation of methionine requirement fall short when we consider the demands of methionine for synthesizing other non-protein-related products? The second metabolic inquiry revolved around understanding the influence of methionine availability on the absorption of GAA and its conversion into creatine in piglets administered diets with differing amounts of methionine. In the third study, the metabolic question under investigation centered on the role of creatine in the absorption of GAA across the gut in a neonatal piglet model. Additionally, we designed this study to determine which combination of amino acids or products is more effective in increasing the body's creatine reservoirs.

These research studies used piglets as a model for human infants and as representatives of swine production. The subsequent discussion aims to emphasize and connect the main discoveries of this thesis while also tackling inquiries that require additional exploration in future studies.

6.1 Methionine requirement for non-protein pathways in neonates

6.1.1 Significance and the overview of results

Determining the methionine requirement is a complex task because it involves considering both the high demand for transmethylation products and the availability of remethylation nutrients. These factors can collectively impact the availability of methionine for protein synthesis in growing neonates, highlighting the intricate balance required to meet their nutritional needs effectively. Moreover, methionine has been proposed as the first limiting amino acid in piglets, primarily because the gastrointestinal tract substantially uses methionine, especially as the gut extracts amino acids on first pass from the diet (Stoll et al., 1998).

Studies have demonstrated the significance of methionine in fulfilling the demand for transmethylation products such as creatine, PC, and methylated DNA. However, no research has identified the methionine requirement for methylated products beyond protein synthesis. The following examples highlight the significance of discovering methionine requirements for critical metabolites. Suckling piglets receive approximately 25% of the creatine they need from sow milk, but they must endogenously synthesize the remaining portion of their creatine requirement (Brosnan et al., 2009). Similarly, infants fed infant formulas with minimal creatine content, such as soy-based formulas, are required to synthesize most of their creatine needs internally (Edison et al., 2013). Consequently, these circumstances place a demand on precursor amino acids, particularly methionine. These scenarios can compromise the production of other metabolites like PC and methylated DNA because more methionine is directed towards creatine synthesis in infants fed formula lacking creatine.

Therefore, chapter three was structured to investigate which specific methylated product or tissue-specific proteins might experience compromise when there is a deficiency of methionine in neonatal piglets. We conducted a six-day intragastric feeding trial to test these objectives and determined that more methionine was required to fulfill methionine needs for creatine and PC synthesis compared to tissue-specific proteins and methylated DNA. Moreover, hepatic protein synthesis was conserved even under extremely low methionine concentrations in the diet.

By examining breakpoints in various tissues, we assessed how dietary methionine requirement is distributed among specific organs for protein synthesis. Inadequate methionine intake in the diet can have detrimental effects on the overall growth, function of specific essential tissues, and critical functions related to transmethylated products. As we reduced methionine levels in the diet, we identified which tissues decreased their protein synthesis, conserving methionine for use in other tissues. Distinctly, different tissues have varying dietary methionine requirements, and our findings provide insights into why growth restrictions occur prior to issues with intestinal function and transmethylated products. Moreover, we can approximate the quantity of methionine needed to sustain protein synthesis in each tissue and transmethylated product by identifying specific dietary methionine requirements based on breakpoints.

6.1.2 **Implications and modifications**

The amino acid requirement data obtained from piglets can be applied to human infants after clinical trials, accounting for the differences in growth rates. Piglets and human infants exhibit distinct developmental stages and metabolic demands, necessitating careful experimental design and interpretation adjustments.

Given the latest research discoveries and the constraints identified within the study, we can explore a few potential future directions. Since this study focuses on neonates, it would be valuable to quantify the methionine requirements for both protein synthesis and methylated products in growing pigs. This is particularly relevant because the proportion of methionine requirement allocated to non-protein pathways may increases significantly in older animals and comparing these demands across various age groups could provide valuable insights. Furthermore, exploring how these dietary methionine requirements for individual tissues might alter when different methyl donors are introduced would be interesting. For example, low methionine levels can impair liver function and contribute to hepatic steatosis and other liver disorders. Moreover, methionine and its derivatives are important for neurotransmitter synthesis and brain function, so inadequate levels can affect neurological development and function. Therefore, gathering data on methionine requirements in specific tissues would be invaluable for establishing therapeutic levels necessary to support the optimal function of these tissues. Conducting a comparison of methionine kinetics in piglets fed different types of formula could yield additional insights into finding the most suitable diet for neonates. As specific commercial infant formulas, like soy-based ones, contain very little creatine, while cow-milk-based formulas have varying creatine content, it might be worth considering the addition of supplemental arginine and methionine to such formulas to address the potentially elevated amino acid needs for increased creatine synthesis. In future studies, it is advisable to assess methionine requirements while considering the presence of various methylated products, such as creatine, in the diet.

Moreover, future research endeavors should focus on assessing the enzymatic capacity to metabolize homocysteine, particularly concerning enzymes such as BHMT involved in remethylation and CGL in the transsulfuration pathway. While homocysteine levels gradually

increase with rising methionine levels, we have not observed a corresponding increase in cysteine or glutathione levels. Therefore, it is crucial to investigate whether homocysteine accumulates in the liver, whether the gut utilizes glutathione rapidly, or if the liver utilizes it for antioxidant functions. Future studies should incorporate measurements of antioxidant activities and indicators of methionine toxicity to gain a more comprehensive understanding of these processes. In addition, it will be necessary to assess the enzyme activities such as GAMT, DNMT, and PEMT to determine whether these enzyme activities reach their maximum levels with increasing methionine concentrations in the diet.

Analyzing the impact of altering dietary methionine levels on PC and fatty acid profiles would be beneficial in understanding the potential influence of methionine on fatty acid metabolism. While the Kennedy pathway accounts for the synthesis of approximately 70% of hepatic PC, it is important to note that the fatty acid composition of PC produced through the PEMT pathway differs from that of the Kennedy pathway. PEMT plays a unique role in the mobilization of essential fatty acids, specifically 20:4(n-6) and 22:6(n-3), from the liver into the bloodstream (Watkins et al., 2003). Indeed, these fatty acids, such as DHA (docosahexaenoic acid), hold significant importance for early-life brain and eye development. Future studies should investigate the fatty acid composition of PC to determine whether any changes occur depending on methionine availability.

If the available research funding permits conducting a larger-scale study, it would be advantageous to examine various levels of methionine across groups of piglets. This approach could help verify the breakpoints by evaluating a series of groups. Additionally, if we aim to observe chronic changes in growth, plasma homocysteine levels, and protein synthesis in response to increasing methionine levels, extending the feeding duration beyond 24-48 hours will be necessary. Nonetheless, it is

important to emphasize that we should exercise caution and avoid extreme increases or decreases in methionine concentrations during these experiments.

There are some limitations of using piglets as a human neonatal model. Piglets mature faster than human infants, meaning their developmental stages do not align perfectly with those of humans. This difference can make it challenging to correlate certain outcomes in piglets directly to equivalent stages in human infants. Piglets are often housed in controlled research environments and provided with diets tailored for experimental consistency, which may not fully reflect the varied environmental exposures and dietary conditions human infants experience. Additionally, using piglets can be labour-intensive and costly compared to other laboratory animals. Their larger size and specific housing, feeding, and handling requirements demand more resources and specialized care, which can increase the overall expense and complexity of studies.

6.2 GAA supplementation and methionine in animal production

6.2.1 Significance and the overview of results

Amino acids represent a significant expense, constituting fifty percent of the overall feed costs for domestic animal producers. Hence, providing precursors or derivatives of these amino acids in the feed can lower production costs. GAA, a direct precursor of creatine may potentially spare more expensive precursor amino acids, thereby reducing feed-related expenses. Therefore, we are conducting a comprehensive study on the metabolism and absorption of GAA, which will provide valuable data that could lead to more cost-effective and efficient feeding strategies. In this study, we aimed to assess how methionine impacts the absorption of GAA and the synthesis of creatine. In piglets, supplementing with GAA successfully augments creatine reserves, but this effect is observed only when methionine is in excess. This increase in creatine comes at the cost of PC

synthesis but does not affect hepatic protein synthesis (Kankayaliyan 2014). When incorporating GAA into the diet, it is essential to take into account the demand for methionine. This consideration is crucial to ensure no potential limitations on methyl groups. In brief, findings from our research indicated that when piglets received supplemental GAA and surplus methionine, creatine production in different tissues increased. Conversely, when methionine intake was insufficient, the supplemented GAA was not only synthesized poorly, but is was also not absorbed effectively, resulting in a low GAA appearance in the portal vein. Additionally, due to the limited availability of methyl groups for converting GAA into creatine in the liver, GAA accumulated in the livers of piglets fed the lowest methionine level. However, we are uncertain about the toxic effects that might result from the accumulation of GAA in various tissues.

Although the gut possesses the capability to produce creatine, the specific fate and significance of this synthesized creatine remain relatively unexplored. Several investigations have indicated potential advantageous impacts of creatine within the gastrointestinal tract. The current research finding suggests that combining GAA with sufficient methionine can boost creatine reservoirs, specifically in the jejunum, potentially offering a valuable alternative to traditional creatine supplements for supporting gut functionality. In addition to its recognized role in gastrointestinal health, research has consistently showcased the advantageous and safe nature of GAA as a dietary supplement for humans and animals. In commercial animal farming, GAA has emerged as a valuable tool for promoting growth, enhancing physical performance, optimizing reproductive parameters, and improving the quality of meat products. Its ability to facilitate creatine synthesis contributes significantly to these outcomes, enabling animals to reach their growth potential more efficiently and effectively.

In summary, this study elucidated that neonatal piglets require excess methionine to achieve complete absorption of GAA. However, it is crucial to note that while these findings highlight the importance of methionine levels for GAA absorption in this context, they do not definitively establish the exact methionine requirement for GAA absorption throughout the gastrointestinal tract.

Piglets receiving methionine at roughly double the estimated whole-body requirement exhibited superior creatine stores. However, it is important to exercise caution before advocating for a doubling of methionine requirements, considering its potential impact on elevating homocysteine levels, a recognized risk factor for cardiovascular disease. Balancing the benefits of enhanced creatine stores with the potential health risks associated with elevated homocysteine concentrations is essential in formulating dietary recommendations for optimal piglet health and development.

6.2.2 **Implications and future directions**

In forthcoming studies, exploring the long-term effects of methionine and GAA supplementation on protein synthesis and growth would be valuable. Such an assessment could be effectively carried out during a few weeks of the feeding trial to understand how these nutritional interventions influence physiological processes related to transmethylated products, protein metabolism, and overall growth in neonatal piglets. Moreover, considering that prolonged administration of GAA and methionine can result in hyperhomocysteinemia, it is essential to conduct pilot studies to determine the optimal levels of GAA and methionine to prevent long-term alterations in homocysteine levels in the body. Since we could not detect changes in muscle creatine levels, possibly due to the shorter duration of duodenal infusion, a long-term feeding trial would offer more comprehensive data on how GAA and methionine impact muscle mass and muscle creatine levels.

Considering the lack of a precise mechanism elucidating how methionine enhances GAA absorption, conducting transporter studies would be invaluable in further investigating the findings from the current study. For example, future studies should focus on studying the activities of transporters involved in GAA and methionine transportation in the gut. Moreover, to recognize the fate of GAA supplementation in the body, it will be necessary to examine GAA excretion through various methods as well. Analyzing urine samples for direct GAA excretion could provide vital data to help bridge the gap between the observed outcomes and the underlying physiological processes. These suggested research paths show the ability to advance our understanding of the complex mechanisms underlying GAA absorption and metabolism.

Furthermore, we could conduct breakpoint analysis to determine the optimum methionine level to maximize transmethylated products and protein synthesis when methionine is administered with GAA as a supplement. By extrapolating from the current experimental design, a similar approach can be applied to studying this supplementation regimen's effects on growing commercial pigs. Through such research, we could gain valuable information on how it influences their growth rates, overall health, and, ultimately, their market value. This exploration could provide significant evidence for optimizing the management and productivity of commercial pig farming operations, leading to more efficient and profitable outcomes.

6.3 GAA and creatine co-administration on creatine metabolism

6.3.1 Significance and the overview of results

Previous research findings from our lab have shown that GAA supplementation for 18 days is more efficient in increasing creatine levels in the liver and muscle than equimolar supplementation with creatine (McBreairty et al., 2015). However, this study, as well as other studies in humans, have

shown that administering GAA in the long term may lead to side effects such as hyperhomocysteinemia (McBreairty et al., 2015; Ostojic et al., 2014). Therefore, finding the best supplemental strategy to improve creatine stores in the body while reducing the possible side effects is the primary goal of such research.

Exploring the co-administration of GAA and creatine on creatine metabolism presents an interesting path for research on creatine supplements. Moreover, investigating how these two compounds interact and influence the metabolic pathways involved in creatine synthesis, storage, and utilization could provide a significant understanding of optimizing their combined effectiveness as nutritional supplements. This line of inquiry will help uncover novel strategies to enhance creatine metabolism and potentially improve athletic performance, muscle function, and overall health outcomes.

In chapter five, we investigated the effect of different combinations of GAA and creatine to enhance creatine levels in the body. Moreover, another objective was to determine the efficiency of GAA transportation across the gut when administered with different levels of creatine. Because of recent research demonstrating that creatine has diverse benefits for various diseases and its well-known role as an ergogenic nutrient, our study aimed to assess and compare the effectiveness of creatine and GAA in increasing tissue stores of creatine. We conducted this investigation using neonatal pigs, and our findings show that GAA plus creatine mixture enhanced hepatic creatine levels, while GAA plus creatine and GAA plus methionine groups led to increased brain creatine levels. Moreover, we could not detect significant differences in muscle creatine levels from the different supplemental strategies. However, information regarding the muscle's ability to synthesize creatine is currently limited. Research findings have shown that piglets have low levels of GAMT activity in muscles, and the activity of AGAT is undetectable (Brosnan et al., 2009).

These findings suggest that the synthesis and availability of GAA may limit the capacity for creatine synthesis in muscle tissue. It is worth considering the possibility that SAM could be limiting GAA methylation in the context of creatine synthesis, as the MAT activity is also low in the muscles of pigs (Brosnan et al., 2009).

Our ex vivo experiments using the Ussing chamber revealed that creatine enhances GAA appearance from lumen to serosa with a 1:3 GAA to creatine ratio. However, we did not see any changes in GAA disappearance from the lumen in relation to increasing creatine levels. Moreover, we found no significant difference in other amino acid disappearance rates related to increased creatine levels. Considering all these data, we could conclude that administering GAA with creatine is an efficient supplementary strategy to improve GAA absorption and creatine levels in the body.

6.3.2 **Implications and future directions**

GAA and creatine combination can be considered a novel approach to improve creatine pools and creatine-related functions in the body. This strategy holds promise for athletes, fitness enthusiasts, and individuals seeking to optimize their physical performance and muscle function through supplements. Furthermore, it opens up opportunities for further research into the synergistic effects of GAA and creatine on various aspects of human physiology, potentially leading to advancements in sports nutrition and performance enhancement protocols.

Future experiments should investigate this process when GAA supplementation is administered orally for a longer duration to understand further the effects of supplements and muscle capacity for creatine synthesis and usage. At the same time, enzyme activities such as GAMT, AGAT, and MAT should be analyzed to differentiate the direct effect of the supplement and the effect due to

the saturated capacity of the enzymes involved in creatine metabolism. Moreover, future studies can further investigate parameters such as creatine kinase activity and ATP and ADP levels to understand how the synergistic effect impacts energy metabolism.

Furthermore, different gut regions have variable levels of enzymes and GAA transporter activities. For example, SLC6A8 expression in the small intestine is much higher than in the large intestine and has an inhibiting effect at high substrate concentrations (Zhang et al., 2022). Since no studies have explored the GAA transporter levels in pigs, we should investigate the relative expression of SLC6A6 and SLC6A8 mRNA in gut and brain to determine the transportation of GAA across different tissues and organs. As we have analyzed the absorption of GAA across jejunum, the same approach can be used to study how transportation differentiates in different parts of the gut. Likewise, future investigations could concentrate on conducting Ussing chamber experiments across various gastrointestinal tract segments, including the duodenum, jejunum, ileum, and colon. These experiments would elucidate which specific region exhibits better absorption rates when GAA is supplied with creatine in the diet. By pinpointing the segments of the digestive system with the highest absorption efficiency, such studies would provide more information regarding the optimal delivery methods for enhancing the bioavailability of GAA and creatine supplements.

Furthermore, GAA is transported through various other transporters such as those for GABA, creatine, and taurine. Because these transporters transport other amino acids and metabolites, investigating the effects of GAA and creatine dietary interventions on the transportation of other amino acids will help mitigate the side effects associated with these supplemental combinations. Therefore, transporter studies should be further elaborated to study other transporters' activities in different gut segments.

Overall, GAA and creatine supplements offer numerous potential health and performance benefits for humans and animals, making them valuable tools in various contexts, from sports nutrition and fitness to livestock production and agriculture. Continued research into their mechanisms of action and potential applications can further reveal their roles in promoting health and enhancing performance across diverse populations.

6.4 References

Brosnan JT, Wijekoon EP, Warford-Woolgar L, Trottier NL, Brosnan ME, Brunton JA, Bertolo RF. Creatine synthesis is a major metabolic process in neonatal piglets and has important implications for amino acids metabolism and methyl balance. J Nutr. 2009;139(7): 1292-7.

Edison EE, Brosnan ME, Aziz K, Brosnan JT. Creatine and guanidinoacetate content of human milk and infant formulas: implications for creatine deficiency syndromes and amino acid metabolism. Br J Nutr. 2013;110:1075– 1078.

Kankayaliyan T, Dinesh OC, Rademacher M, Brunton JA, Bertolo RF. Creatine de novo synthesis and methionine metabolism in neonatal piglets. Canadian Nutrition Society Annual Conference; 2014: Appl Physiol Nutr Metab, Vol. 39.

McBreairty LE, Robinson JL, Furlong KR, Brunton JA, Bertolo RF. Guanidinoacetate is more effective than creatine at enhancing tissue creatine stores while consequently limiting methionine availability in Yucatan miniature pigs. PLoS One. 2015;10(6): e0131563.

Ostojic SM, Stojanovic M, Drid P, Hoffman JR. Dose–response effects of oral guanidinoacetic acid on serum creatine, homocysteine and B vitamins levels. Eur J Nutr. 2014;53:1637–1643

Stoll B, Henry J, Reeds PJ, Yu H, Jahoor F, Burrin DG. Catabolism dominates the first-pass intestinal metabolism of dietary essential amino acids in milk protein-fed piglets. J Nutr. 1998;128(3):606-614.

Zhang S, Zang C, Pan J, Ma C, Wang C, Li X, Cai W, Yang K. Effects of dietary guanidinoacetic acid on growth performance, guanidinoacetic acid absorption and creatine metabolism of lambs. PLoS One. 2022;17(3):e0264864.

Appendix

Supplemental Table 1: Composition of diet

Composition of adaptation diet[1]	*g/L*
L-Amino acids[2]	56
D-Glucose	90.3
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
Composition of vitamin and mineral mixtures added just before use	*dose (kg $BW^{-1} \cdot d^{-1}$)*
Intralipid, *mL*[3]	52
Multi-12/K_1Pediatric[4]	
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)	1.4
Vitamin K_1, *mg*	0.04
Biotin, *µg*	4.35
Folic Acid, *µg*	30.46
Vitamin B_{12}, *µg*	0.22
Trace Element Mix[5]	*dose (mg·kg $BW^{-1} \cdot d^{-1}$)*
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[6]	
Iron (as ferric hydroxide)	3.0

[1]The 'adaptation' diet was comprised of an amino acid, glucose, mineral mixture, which was infused at a rate of 272 m.kg $BW^{-1} \cdot d^{-1}$.

[2]Amino acid composition described in Materials and Methods.

[3]Intralipid® 20% (Baxter, Mississauga, Canada).

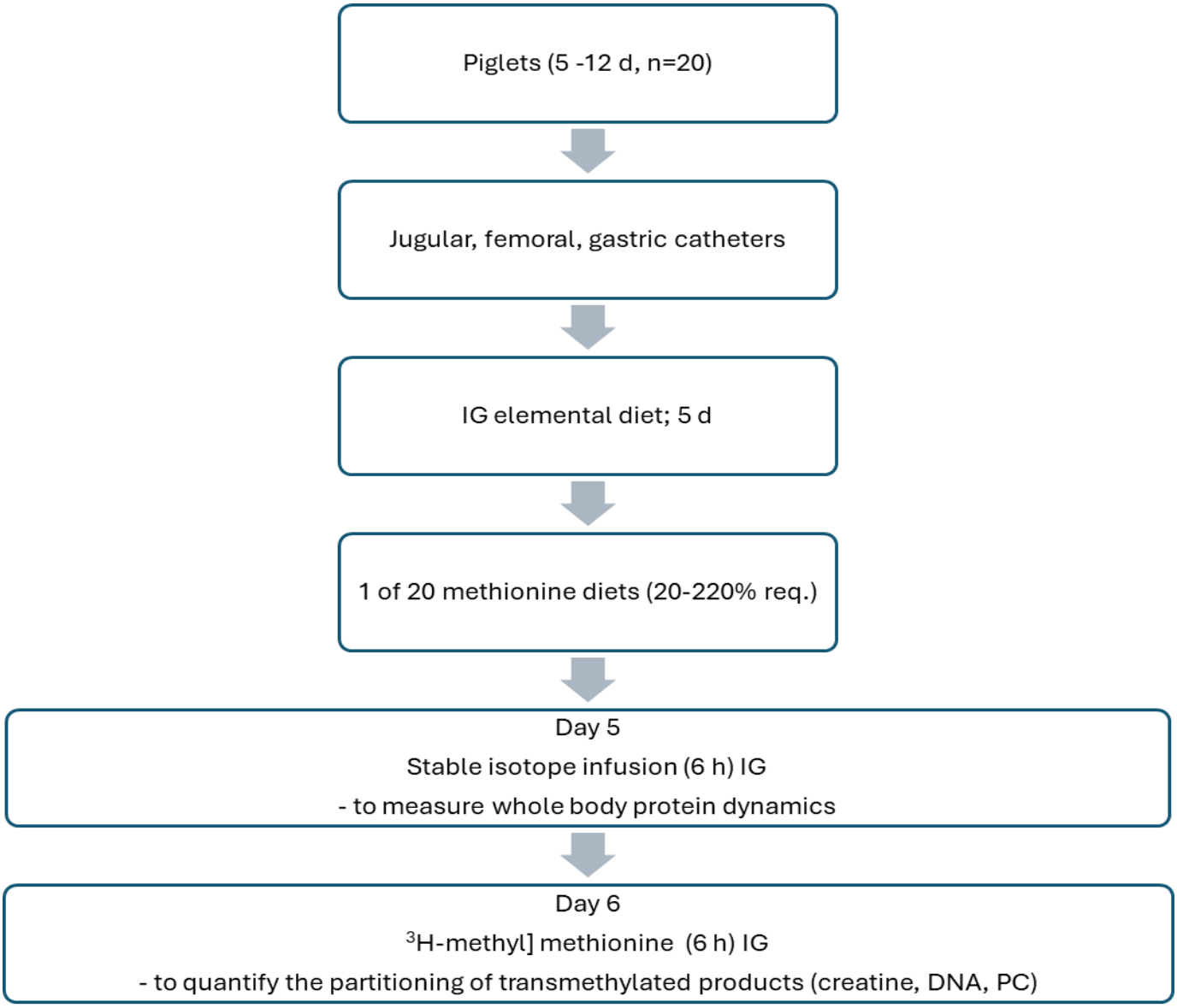
[4]Multi-12/K_1 Pediatric® multi-vitamin solution for TPN feeding (Baxter, Mississauga, Canada).

[5]Trace element mix (components from Sigma-Aldrich, Oakville, Canada) prepared in laboratory.

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

Supplemental Figure 1: Study timeline illustrating key days and isotope infusion protocols

IG, intragastric; req, requirement.



Supplemental Table 2: Summary table for dual linear regression and simple linear regression models

	Dual linear regression		Simple linear regression	
	P	r^2	P	r^2
Whole body protein	0.0014	0.5387	0.0057	0.3529
Liver Ks	0.0004	0.5992	0.0945	0.1475
Kidney Ks	0.0003	0.6214	0.0029	0.398
Biceps femoris muscle Ks	0.0005	0.5941	0.0019	0.4234
Liver creatine	<.0001	0.7164	0.0004	0.5164
Liver methylated DNA	0.0335	0.3294	0.0464	0.2026

Supplemental Table 3: Amino acid and GAA concentration in the dietary infusates.

	Dietary Infusate			
Amino Acids	**20% Met**	**80% Met**	**140% Met**	**200% Met**
	g·L^{-1}			
Alanine	6.39	6.02	5.64	5.27
Arginine	3.65	3.65	3.65	3.65
Aspartic Acid	3.32	3.32	3.32	3.32
Cysteine	0.76	0.76	0.76	0.76
Glutamic Acid	5.72	5.72	5.72	5.72
Glycine	1.47	1.47	1.47	1.47
Histidine	1.69	1.69	1.69	1.69
Isoleucine	2.51	2.51	2.51	2.51
Leucine	5.67	5.67	5.67	5.67
Lysine-HCl	5.58	5.58	5.58	5.58
Methionine[1]	0.21	0.83	1.45	2.08
Phenylalanine	3.00	3.00	3.00	3.00
Proline	4.52	4.52	4.52	4.52
Serine	3.11	3.11	3.11	3.11
Taurine	0.27	0.27	0.27	0.27
Tryptophan	1.14	1.14	1.14	1.14
Tyrosine	0.44	0.44	0.44	0.44
Valine	2.89	2.89	2.89	2.89
Threonine	2.23	2.23	2.23	2.23
GAA[2]	0.375	0.375	0.375	0.375

[1]Methionine in the dietary infusate was provided at 20%. 80%, 140% or 200% of the requirement (2.08-20.77 mg·kg^{-1} BW·h^{-1}) as determined by Shoveller et al., 2003 (24) for young pigs. Alanine concentration was manipulated to provide isonitrogenicity, with respect to amino acids. GAA, guanidinoacetic acid.

[2]GAA was provided at a rate of 3.75 mg·kg^{-1} BW·h^{-1}: this amount of GAA, if completely converted to creatine, would fulfill the piglet's total creatine accretion rate at this age (13).

Supplemental Table 4: GAA concentration in tissues after 4 h duodenal infusion with diets containing 20% Met, 80% Met, 140% Met, or 200% Met in piglets.

	Treatments					P-value[1]		
GAA	**20% Met**	**80% Met**	**140% Met**	**200% Met**	**SEM**	**Trt**	**L**	**Q**
Jejunum GAA ($\mu mol \cdot g^{-1}$)	2.20^{c}	2.68^{c}	3.64^{b}	5.52^{a}	0.10	<0.001	<0.001	0.002
Liver GAA ($nmol \cdot g^{-1}$)	357.75^{a}	241.01^{b}	217.42^{b}	171.62^{b}	14.49	0.004	<0.001	0.240
Kidney GAA ($nmol \cdot g^{-1}$)	792.92	804.86	815.78	634.14	78.09	0.824	0.515	0.544
Carotid GAA ($\mu mol \cdot L^{-1}$)	13.71	14.90	14.82	15.83	0.42	0.398	0.116	0.919
Portal GAA ($\mu mol \cdot L^{-1}$)	16.39^{b}	17.35^{b}	17.93^{b}	26.92^{a}	0.61	<0.001	<0.001	0.005
Change in GAA portal balance (%)	0^{b}	50.91^{b}	29.03^{b}	237.6^{a}	30.05	<0.001	<0.001	0.022
Portal GAA appearance (%)	31.08^{b}	27.37^{b}	26.31^{b}	104.93^{a}	5.07	<0.001	<0.001	<0.001

[1] L, linear effect; Q, quadratic effect; Trt, treatment effect.

[a–c] Differences between treatments ($P < 0.05$).

SEM, standard error of the mean

Met, methionine; 20% Met = 2.08 $mg \cdot kg^{-1}$ $BW \cdot h^{-1}$, 80% Met= 8.3 $mg \cdot kg^{-1}$ $BW \cdot h^{-1}$, 120% Met=14.53 $mg \cdot kg^{-1}$ $BW \cdot h^{-1}$, 200% Met=20.77 $mg \cdot kg^{-1}$ $BW \cdot h^{-1}$

Supplemental Table 5: creatine concentration in tissues after 4 h duodenal infusion with diets containing 20% Met, 80% Met, 140% Met, or 200% Met in piglets.

	Treatments					P-value[1]		
Creatine	**20% Met**	**80% Met**	**140% Met**	**200% Met**	**SEM**	**Trt**	**L**	**Q**
Jejunum creatine ($\mu mol \cdot g^{-1}$)	0.53^{b}	0.30^{c}	0.76^{a}	0.84^{a}	0.04	<0.001	<0.001	0.001
Liver creatine ($\mu mol \cdot g^{-1}$)	2.62^{b}	2.57^{b}	2.81^{b}	4.43^{a}	0.21	<0.001	<0.001	0.001
Kidney creatine ($\mu mol \cdot g^{-1}$)	14.80	15.03	13.78	10.63	1.76	0.069	0.032	0.107
Muscle creatine ($\mu mol \cdot g^{-1}$)	33.55	35.79	40.68	37.99	3.44	0.522	0.258	0.48
Carotid creatine ($\mu mol \cdot L^{-1}$)	586.01^{b}	784.79^{a}	843.32^{a}	913.03^{a}	31.10	0.017	0.002	0.316
Portal creatine ($\mu mol \cdot L^{-1}$)	318.85^{c}	719.56^{b}	665.65^{b}	1020.14^{a}	41.56	<0.001	<0.001	0.785
Change in creatine portal balance (%)	0^{c}	69.47^{b}	46.61^{b}	140.90^{a}	4.74	<0.001	<0.001	0.209

[1] L, linear effect; Q, quadratic effect; Trt, treatment effect.

[a–c] Differences between treatments ($P < 0.05$).

SEM, standard error of the mean

Met, methionine; 20% Met = 2.08 $mg \cdot kg^{-1}$ $BW \cdot h^{-1}$, 80% Met= 8.3 $mg \cdot kg^{-1}$ $BW \cdot h^{-1}$, 120% Met=14.53 $mg \cdot kg^{-1}$ $BW \cdot h^{-1}$, 200% Met=20.77 $mg \cdot kg^{-1}$ $BW \cdot h^{-1}$

Supplemental Table 6: Amino acid, GAA and creatine concentration in the dietary infusates.

	Dietary Infusate			
Amino Acids	**Control**	**GAA**	**GAA+Met**	**GAA+Cre**
	$g \cdot L^{-1}$			
Alanine	5.89	5.89	5.26	5.89
Arginine	3.65	3.65	3.65	3.65
Aspartic Acid	3.32	3.32	3.32	3.32
Cysteine	0.76	0.76	0.76	0.76
Glutamic Acid	5.72	5.72	5.72	5.72
Glycine	1.47	1.47	1.47	1.47
Histidine	1.69	1.69	1.69	1.69
Isoleucine	2.51	2.51	2.51	2.51
Leucine	5.67	5.67	5.67	5.67
Lysine-HCl	5.58	5.58	5.58	5.58
Methionine[1]	1.04	1.04	2.08	1.04
Phenylalanine	3.00	3.00	3.00	3.00
Proline	4.52	4.52	4.52	4.52
Serine	3.11	3.11	3.11	3.11
Taurine	0.27	0.27	0.27	0.27
Tryptophan	1.14	1.14	1.14	1.14
Tyrosine	0.44	0.44	0.44	0.44
Valine	2.89	2.89	2.89	2.89
Threonine	2.23	2.23	2.23	2.23
GAA[2]	-	0.375	0.375	0.188
Creatine	-	-	-	0.25

[1]Methionine in the dietary infusate was provided at 100% or 200% of the requirement (2.08-20.77 $mg \cdot kg^{-1}$ $BW \cdot h^{-1}$) as determined by Shoveller et al., 2003 for young pigs. Alanine concentration was manipulated to provide isonitrogenicity, with respect to amino acids. GAA, guanidinoacetic acid.

[2]GAA was provided at a rate of 3.75 $mg \cdot kg^{-1}$ $BW \cdot h^{-1}$: this amount of GAA, if completely converted to creatine, would fulfill the piglet's total creatine accretion rate at this age (Brosnan et al., 2009).

Supplemental Table 7: The initial concentrations of GAA and creatine in the luminal side (at the beginning of the Ussing chamber experiment)

	Control	GAA: Cre 1:0.3	GAA: Cre 1:0.5	GAA: Cre 1:1	GAA: Cre 1:2	GAA: Cre 1:3
GAA (mM)	3.20	3.20	3.20	3.20	3.20	3.20
Creatine (mM)	0.00	1.07	1.60	3.20	6.40	9.61