The Effects of Arginine and Citrulline Supplementation in Parenteral Nutrition in Yucatan Miniature Piglets

By:

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Abstract

In situations where infants cannot tolerate oral feeding, direct intravenous infusion of nutrients, known as parenteral feeding, can be lifesaving. Prolonged parenteral feeding can induce metabolic and physiological changes ranging from reduced blood flow to the gut and gut atrophy to reduced systemic protein synthesis. Gut atrophy hinders the intestinal synthesis of arginine, an amino acid that plays a crucial role in protein and nitric oxide synthesis, as well as guanidinoacetate and, in turn, creatine synthesis. Supplemental arginine can increase systemic arginine availability, thus enhancing arginine availability for protein, creatine and nitric oxide synthesis, and potentially ameliorating gut atrophy. However, supplemental arginine is rapidly extracted from circulation by the liver; thus, we propose supplementing citrulline, an arginine precursor that bypasses the liver, as a means to increase systemic arginine availability. The study's objective is to improve arginine availability via supplemental citrulline, a novel ingredient for parenteral nutrition (PN) solutions. We hypothesized that citrulline supplementation would enhance arginine availability, thereby increasing whole-body protein, creatine and NO synthesis to a greater extent than arginine alone. The effects of supplemental arginine and citrulline on protein, creatine and NO synthesis were investigated in 7-10 day-old Yucatan piglets. Piglets received one of three nutritional interventions: control PN, arginine-supplemented PN, or citrulline-supplemented PN for six days. On study day 6, a primed and continuous infusion of isotopically labelled phenylalanine, tyrosine, guanidinoacetate, arginine and citrulline was conducted for 6 hours. Isotope enrichment of tracers and their products, fluxes and conversions were measured and used to calculate whole-body synthesis of protein (phenylalanine and tyrosine tracers), guanidinoacetate (arginine and guanidinoacetate tracers) and nitric oxide (arginine and citrulline tracers). Supplemental citrulline increased arginine synthesis and release in the kidney, thus illustrating that citrulline is an effective means of increasing systemic arginine availability. Arginine and citrulline appeared to increase arginine availability for nitric oxide (NO). However, their effects on blood flow were not as pronounced as hypothesized, suggesting alternative metabolic fates for the increased NO produced via NOS. Furthermore, there was no treatment effect on whole-body protein synthesis; tissue-specific protein synthesis in the gut, liver, and kidneys was increased with citrulline supplementation only, thus suggesting that citrulline may improve arginine availability for protein synthesis. Arginine supplementation enhanced liver creatine synthesis, and citrulline showed promise in maintaining gut and kidney function, potentially due to its role in sustaining protein

synthesis rates, compared to arginine supplementation. Due to the complex regulation of inter-organ protein, creatine and NO synthesis, more research is needed to fully understand the effects arginine and citrulline supplementation have on parenterally fed neonates during a critical period in neonatal development.

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Abbreviations

ADC – Arginine decarboxylase
ADMA – Asymmetric dimethylarginine
ADP – Adenosine diphosphate
AGAT – Arginine-glycine aminotransferase
APE – Atom percent excess
ARG – Arginase
ASL – Argininosuccinate lyase
ASS – Argininosuccinate synthase
ATP – Adenosine triphosphate
BH_4 – Tetrahydrobiopterin
Ca^{2+} - Calcium ion
CAD – Collisional deactivated dissociation
CAT – Cationic amino acid transporter
CAT-1 – isoform of cationic amino acid transporter
CDS – Creatine deficiency syndromes
cGMP – Cyclic guanosine monophosphate
CIT – Citrulline
CK – Creatine kinase
CO_2 – Carbon dioxide
DNA – Deoxyribonucleic acid
eNOS – endothelial nitric oxide synthase
EP – Entrance potential
ESI – Electrospray ionization
FAD – Flavin adenine dinucleotide
FMN – Flavin mononucleotide
GAA – Guanidinoacetate
GAMT – Guanidinoacetate *N*-methyltransferase
GC – Gas-chromatography
GLN – Glutamine
GLU – Glutamic acid
GTP – Guanosine triphosphate
HCl – Hydrochloric acid
HPLC – High-performance liquid chromatography
iNOS – Inducible nitric oxide synthase
IS – Ionization spray
K_m – Michaelis-Menten constant
MAT – Methionine adenosyltransferase
MAT1/3 – Isoforms of methionine adenosyltransferase
MRM – Multiple reaction monitoring

mRNA – messenger ribonucleic acid
NADPH – Nicotinamide adenine dinucleotide phosphate
NAG – *N*-acetylglutamate synthase
NH_3 – Ammonia
nNOS – Neuronal nitric oxide synthase
NO – Nitric Oxide
NOHA – *N*-hydroxy-1-arginine
NOS – Nitric oxide synthase
NRC – National research council
OAT – Ornithine aminotransferase
ODC – Ornithine decarboxylase
ORN – ornithine
OTC – Ornithine carbamoyltransferase
P5CS – Delta-1-pyrroline-5-carboxylate synthase
PC – Phosphatidylcholine
PCA – Perchloric acid
PFBBr – Pentafluorobenzyl bromide
PITC – Phenylisothiocyanate
PN – Parenteral nutrition
PRO – proline
PYCR – Pyrroline-5-carboxylate reductase
PYCRL – Pyrroline-5-carboxylate reductase-like
Q – Flux
ROS – Reactive oxygen species
SAM – S-adenosyl methionine
SMA – Superior mesenteric artery
SMS – Spermine synthase
SPE – Solid phase extraction
SRM – Spermidine synthase
TCA – Tricarboxylic acid cycle
TEM – Gas temperature
TFA – Trifluoroacetic acid

1. Introduction

1.1 Rationale

1.1.1 Parenteral feeding

In situations where infants cannot tolerate oral feedings, such as in the case of premature births or neonates with intestinal dysfunction, total parenteral nutrition is necessary. Total parenteral nutrition, or parenteral nutrition (PN), is a means by which nutrients are delivered directly to the blood via intravenous infusion, bypassing absorption by the gut. The use of PN, although lifesaving, impacts the gut's physiological characteristics and metabolic capacity (Bertolo et al., 2003). A substantial complication of prolonged PN is gut atrophy due to a reduction in blood flow in the mesenteric artery, which is the main artery that supplies blood to the gut. Impaired gut function leads to reduced interconversion of proline, ornithine and arginine, thereby affecting arginine availability during a critical period in neonatal development (Bertolo et al., 2003). Arginine plays a crucial role in whole-body protein synthesis (growth), nitric oxide synthesis (NO), creatine synthesis and polyamine synthesis (essential for gut health) (Curis et al., 2005; McCormack & Johnson, 1991; Wu, 2009). In the case of parenterally fed neonates, reduced arginine availability can have severe repercussions for neonatal growth and blood flow regulation due to impaired protein and nitric oxide synthesis, respectively (Dinesh et al., 2014). Thus, improving arginine availability in parenterally-fed neonates is important for proper growth and blood flow regulation during development. However, the liver rapidly utilizes arginine in the urea cycle; hence, it is not available for protein and nitric oxide synthesis in the rest of the body. Research has shown that citrulline is the most efficient arginine precursor and may prove to be an alternative method for increasing arginine availability in parenterally-fed neonates (Urschel et al., 2006).

1.1.2 Dietary Composition of Total Parenteral Nutrition

The nutrient composition of parenteral nutrition is mainly modelled on the complete oral nutrient requirements for neonates, although some are based on cord blood nutrient concentrations. Parenteral nutrition contains macro- and micronutrients required to support growth and development during times when enteral nutrition is not feasible. Essential and non-essential amino acids are provided as crystalline amino acids at an approximate rate of 3-4 g/kg/day, with an elevated essential to non-essential amino acid ratio to maintain an estimated protein retention of approximately 70%. However, some dispensable amino acids are either unstable (ie, glutamine, asparagine) or have limited solubility (ie, tyrosine) and are sometimes not included. However, although unstable and with limited solubility, cysteine is typically added to parenteral solutions to maintain adequate protein synthesis rates during an essential period in neonate growth and development (Johnson, 2013; Schutzman et al., 2008). Carbohydrates are typically provided as dextrose (ie, glucose), which is acquired from corn or wheat, while the lipids are provided as a 20% sterile lipid emulsion that historically was formulated from soybean oil (Schutzman et al., 2008), but more recently have been formulated from mixed oils including fish oil. Understanding the total energy needs is essential when providing PN as a sole nutrient source. A total caloric intake, via enteral or parenteral sources, of 85-127 kcal/kg/day should ensure an adequate neonate growth rate of approximately 15 g/kg/day. Lastly, micronutrients such as electrolytes, trace minerals and vitamins are provided as part of a PN nutrient regimen to ensure adequate support of neonatal growth (Johnson, 2014).

1.1.3 The Effects of Parenteral Nutrition (PN) on Nutrient Absorption

The use of PN in a clinical setting can greatly improve the nutrition status of a wide range of patients, from premature neonatal infants who cannot tolerate oral feeding to adults who suffer from intestinal dysfunction or malnourishment. Although lifesaving, PN feeding impacts the physiological characteristics and metabolic capacity of the gut caused by reductions in blood flow to the gut, ultimately leading to PN-induced gut atrophy (Bertolo et al., 2003). A study by Niinikoski et al. (2004) demonstrated the potent effects of PN on blood flow to and from the gut. The study evidenced that after only 8 hours of nutrients being delivered intravenously in three-week-old piglets, blood flow in the portal vein (from the gut to liver) and superior mesenteric artery (from the heart to the gut) decreased more than 30% compared to piglets fed orally (enterally) (Niinikoski et al., 2004). Furthermore, intravenous administration of nutrients, as opposed to oral administration, leads to a reduction in trophic factor stimulation in the lumen of the gut, thereby contributing to gut atrophy. PN-induced gut atrophy subsequently leads to morphological changes in the gut, such as decreased crypt cell proliferation, decreased villus height and mucosal surface area, which in turn leads to decreased protein synthesis and turnover and increased apoptosis resulting in further atrophy of the gut (Burrin et al., 2000; Goldstein et al., 1985; Shulman, 1988). A study by Bertolo et al. (1999) evidenced villous atrophy and reduced crypt depth after only eight days of feeding 3-day-old piglets via intravenous and intra-portal routes, the latter of which bypassed only the gut; however, gut morphology was preserved in the piglets fed via an intra-gastric catheter (Bertolo et al., 1999). From a metabolic standpoint, PN-induced gut atrophy hinders the absorption and metabolic capacity of the gut, thus leading to insufficient nutrient absorption upon refeeding and reduced synthesis of many nutrients, such as arginine, thereby further exacerbating gut atrophy (Yang et al., 2009). Since the gut plays a critical

role in arginine synthesis, impairments of the gut due to PN feeding could lead to reduced quantities of arginine available for the multitude of arginine metabolic pathways (Bertolo et al., 2003).

1.2 Arginine

Arginine is a critically important amino acid required for many metabolic pathways. Arginine catabolism is controlled by four predominant categories of catabolic enzymes: nitric oxide synthases (NOS) for NO synthesis; arginases, specifically arginase I and arginase II, in the urea cycle for detoxification of ammonia and synthesis of ornithine, proline and glutamate; arginine decarboxylase for agmatine synthesis; and finally, arginine-glycine aminotransferase (AGAT) for the synthesis of guanidinoacetate, and ultimately creatine (Hsu & Tain, 2019). The arginase catabolic pathways utilize the greatest proportion of circulating arginine. Arginase I is abundantly found within hepatocytes, whereas arginase II is widely expressed in all mitochondria-containing extrahepatic cells (Morris, 2007). Both arginase I (cytosolic) and arginase II (mitochondrial) are responsible for the detoxification of ammonia within the body through the formation of urea, which, in turn, is excreted as waste. Ornithine produced via the arginase pathway can be subsequently converted to proline, glutamate and polyamines; thus, in addition to its direct role in protein synthesis, arginine catabolism also plays a substantial role in protein synthesis by synthesizing other key amino acids and trophic factors in tissues.

1.2.1 Arginine Biosynthesis

Endogenous arginine synthesis is confined primarily to three main organs within the body: the liver, the small intestine, and the kidneys. Arginine is synthesized from the precursor citrulline via

two successive enzymatic reactions, argininosuccinate synthase (ASS) and argininosuccinate lyase (ASL) (Figure 1.1). In the liver, arginine is subsequently acted upon by cytosolic arginases and converted to ornithine and urea. Due to the exceedingly high activity of hepatic cytosolic arginases, there is no net synthesis of arginine in the liver. Therefore, the kidney is the main location for net arginine synthesis (Marini et al., 2017; Windmueller & Spaeth, 1981). Similar to the liver, citrulline is converted to arginine through the actions of ASS and ASL in the kidney. However, due to a lack of renal arginase activity, arginine synthesized in the kidney is subsequently transported out of the cell through cationic amino acid transporters (CAT) and is released into systemic circulation. The CAT-1 isoform is the predominant arginine transporter in the body and is found in almost all cell types. Moreover, in the small intestine, citrulline is replenished by enzymes carbamoyl phosphate synthetase I and ornithine transcarbamoylase, which utilize ornithine, NH_3 and CO_2 to recycle citrulline (Marini et al., 2017). Citrulline can also be replenished through the synthesis of NO by NOS. Resynthesized citrulline from both ornithine (urea cycle) or arginine (NO synthesis) is acted upon once again by ASS and ASL to produce arginine, thereby creating a metabolic cycle which replenishes both citrulline and arginine stores (Mori & Gotoh, 2004).

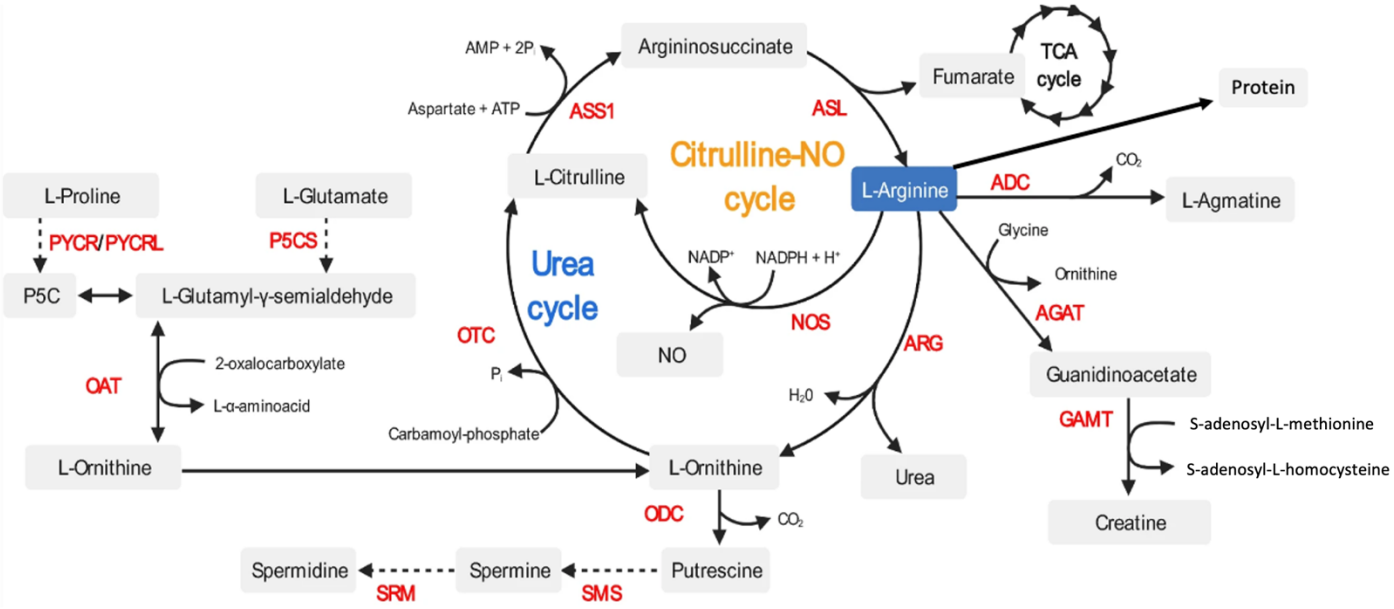

Figure 1.1: Arginine metabolic pathways. Abbreviations: ADC, arginine decarboxylase; AGAT, arginine:glycine amidinotransferase; GAMT, guanidinoacetate *N*-methyltransferase; ARG, arginase; ODC, ornithine decarboxylase; SMS, spermine synthase; SRM, spermidine synthase; OTC, ornithine carbamoyltransferase; ASS1, argininosuccinate synthase-1; ASL, argininosuccinate lyase; NOS, nitric oxide synthase; PYCR, pyrroline-5-carboxylate reductase; PYCRL, pyrroline-5-carboxylate reductase-like; P5CS, delta-1-pyrroline-5-carboxylate synthase; OAT, ornithine aminotransferase. Modified from (Martí i Líndez & Reith, 2021)

1.2.2 Metabolic roles of arginine in adults and neonates

Arginine is a semi-essential or conditionally essential amino acid that plays a role in a plethora of metabolic pathways throughout the body; thus, arginine is considered one of the most versatile amino acids. As mentioned, arginine serves as a precursor for the synthesis of nitric oxide, protein, creatine, polyamines, and agmatine and plays a crucial role in producing urea for the excretion of ammonia as a waste product (Morris, 2007). In mammals, arginine is derived from three predominant sources: consumption through the diet, protein turnover within the body, and endogenous synthesis. Net arginine synthesis is almost exclusively contained in the intestinal-renal axis, which relies on the proper function of the small intestine and kidneys. However, in special circumstances such as inflammation, infection, and intestinal or renal dysfunction, arginine's endogenous synthesis does not meet the physiological and metabolic demands for arginine, rendering it as semi-indispensable (Featherston et al., 1973).

Moreover, arginine essentiality varies throughout the stages of physiological development, with neonatal infants requiring a notably higher quantity of arginine to meet rapid growth and metabolic demands. In neonates, endogenous arginine synthesis is insufficient to meet the higher requirements to sustain ideal growth; therefore, infants are prone to developing an arginine deficiency, particularly those on parenteral feeding with gut atrophy (Wu et al., 2004). To overcome arginine deficiency or insufficiency, citrulline, a precursor to arginine, has been proposed as an effective alternative to increase systemic arginine availability (Urschel et al., 2006).

1.3 Citrulline

Citrulline is a non-essential α-amino acid that plays a significant role in arginine metabolism as a precursor and product, thus regenerating arginine for arginine's many metabolic pathways, such as excretion of toxic ammonia through urea production. Although non-proteinogenic, citrulline indirectly contributes to protein synthesis by increasing systemic arginine availability (Urschel et al., 2006). Citrulline can be consumed through food sources such as watermelon, citrus fruits and leafy greens or generated by *de novo* synthesis. The enzymes responsible for *de novo* synthesis of citrulline correspond to the two major metabolic pathways that citrulline is involved in, namely ornithine carbamoyltransferase (OTC) in the urea cycle, and NO synthase (NOS) in NO synthesis. OTC catalyzes the conversion of ornithine and carbamoyl phosphate to citrulline and phosphate (Curis et al., 2005). In adults, net synthesis of citrulline occurs in the small intestine, due to low activities of ASS and ASL, from dietary arginine, proline, glutamate and glutamine and is subsequently released to the kidneys, whereby it is converted to arginine by ASS and ASL (Bertolo & Burrin, 2008). Further, the synthesis of citrulline is regulated by pyrrolic-5-carboxylate (P5C) synthase (glutamate to P5C), proline oxidase (proline to P5C) and *N*-acetylglutamate (NAG) synthase (glutamate to citrulline, multistep process) in the small intestine (Figure 1.2) (Wu, 2009). In the small intestine, glutamine is a critically important precursor for the amino acid synthesis of glutamate, ornithine, arginine and citrulline. Glutamate is subsequently converted to P5C by P5C synthase, followed by the conversion of P5C to ornithine by ornithine aminotransferase (OAT), and finally, the carbamoylation of ornithine to citrulline by OTC (Figure 1.2) (Bertolo & Burrin, 2008)).

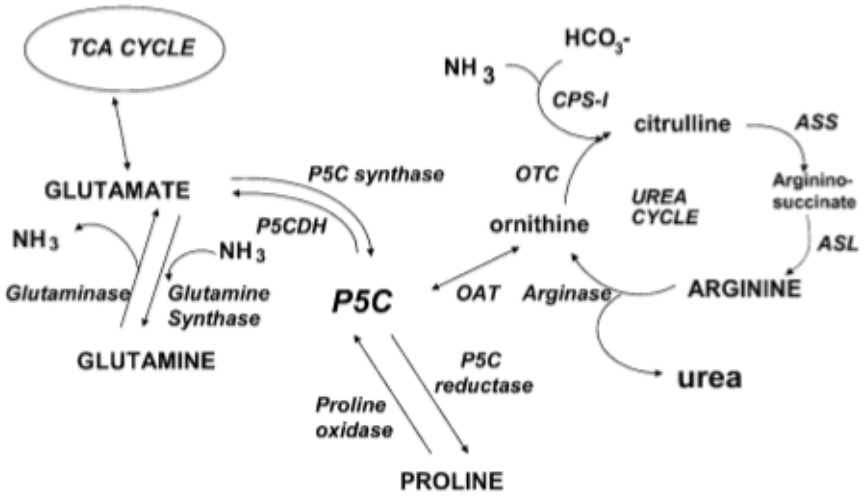

Figure 1.2: Metabolic pathways of glutamate, glutamine, proline, ornithine, citrulline and arginine Abbreviations: TCA cycle, tricarboxylic acid cycle; NH_3, ammonia; P5CDH, pyrroline-5-carboxylate dehydrogenase; P5C synthase, pyrroline-5-carboxylate synthase; P5C pyrroline-5-carboxylate; P5C reductase, pyrroline-5-carboxylate reductase; OAT, ornithine aminotransferase; OTC, ornithine carbamoyltransferase; CPS-, carbamoyl phosphate synthetase; HCO_{3-}, hydrocarbonate; ASS, argininosuccinate synthase-1; ASL, argininosuccinate lyase. Adapted from (Bertolo & Burrin, 2008).

Due to the low activities of ASS and ASL in the adult small intestine, the citrulline synthesized through the P5C synthase pathway is released into portal circulation, whereby it is extracted by the kidneys, as opposed to portal arginine, which is catabolized in the liver by arginase, for ammonia detoxification in the urea cycle. Due to the lower renal activity of arginase, the majority of net arginine synthesized in the proximal tubules of the kidneys from citrulline is released to the systemic circulation (Bertolo & Burrin, 2008). Therefore, since circulating arginine is readily taken up by the liver and catabolized by arginase, the citrulline released from the gut may play an important role in increasing renal arginine synthesis for systemic use. However, in neonates, ASS and ASL are present in small intestinal enterocytes (Figure 1.3) (Bertolo & Burrin, 2008)). ASS and ASL present in enterocytes utilize arterial citrulline to produce arginine locally in the gut, thereby bypassing the uptake and breakdown of arginine by the liver. Therefore, when the gut is atrophied from parenteral nutrition, supplemental citrulline is likely necessary and more effective than arginine for enhancing arginine availability in neonates (Schwedhelm et al., 2008).

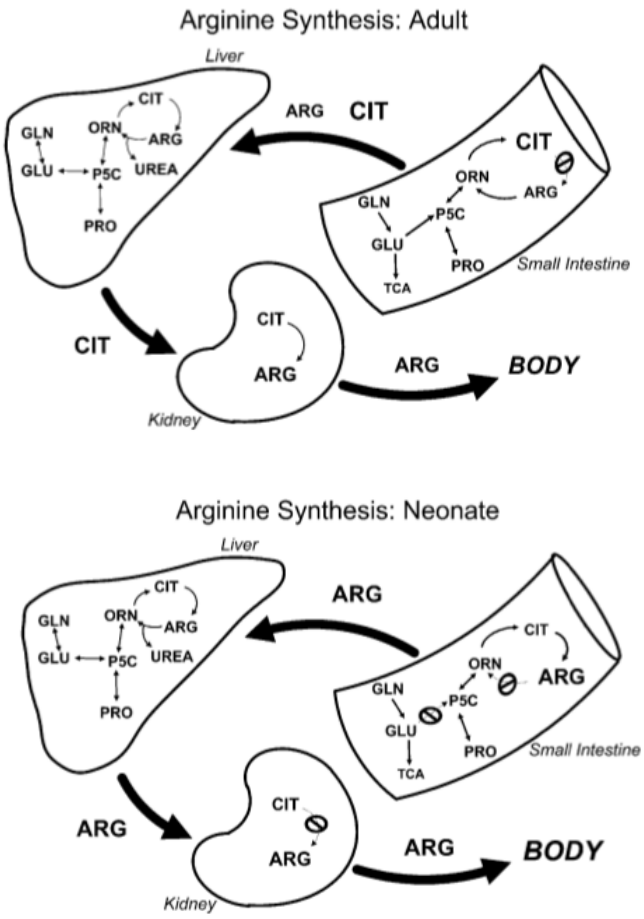

Figure 1.3: Interorgan synthesis of arginine in adults and neonates. Abbreviations: GLN, glutamine; GLU, glutamic acid; P5C, pyrroline-5-carboyxlate; PRO, proline; ORN, ornithine; CIT, citrulline; ARG, arginine; TCA, tricarboxylic acid cycle Adapted from (Bertolo & Burrin, 2008).

1.4 Nitric Oxide

Nitric oxide (NO) is an important signalling molecule in vascular regulation that functions by stimulating vasodilation. At physiological temperatures, NO is present as a gas that can readily diffuse into cells. NO interacts with oxygen and reactive oxygen species (ROS) to elicit a plethora of biological functions ranging from immunmodulation via ROS to vasoregulation of smooth and cardiac muscle via cGMP; thus, NO aids in the regulation of immune responses and vasodilation systemically (Siervo et al., 2011). Moreover, NO freely binds to enzymes such as oxidases, guanylyl cyclase, and myoglobin via metals (i.e. copper, molybdenum and iron) incorporated within the enzymes, thus influencing enzymatic activity (Ichimori et al., 1999; Samuel & Gitlin, 2006). The mechanism by which NO stimulates vasodilation is through the activation of soluble guanylate cyclase in vascular smooth muscle. The activated guanylate cyclase subsequently converts guanosine triphosphate (GTP) to cyclic guanosine monophosphate (cGMP), which in turn results in vasodilation, thereby increasing blood flow (Klinger et al., 2009). NO synthesized in and near smooth muscle cells acts as an autocrine and paracrine regulator of vascular tone and thus is responsible for the control of localized blood flow to tissues and permits tissues to modulate blood flow under various conditions to ensure adequate nutrient delivery and tissue metabolism (Chen et al., 2008).

1.4.1 Nitric Oxide Biosynthesis via Nitric Oxide Synthase

De novo synthesis of NO is an important pathway for maintaining smooth muscle vascular tone, cell-to-cell signalling, angiogenesis, and immunological and neuronal processes (Schwedhelm et al., 2008). The biosynthesis of NO occurs in multiple tissues and consists of the conversion of arginine to citrulline, releasing NO as a by-product. The enzyme responsible for

converting arginine to citrulline and producing NO is nitric oxide synthase (NOS). The stoichiometric conversion of arginine to citrulline via NOS utilizes cofactors oxygen, flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), and tetrahydrobiopterin (BH4), in addition to the electron donor nicotinamide adenine dinucleotide phosphate (NADPH). Three tissue-specific isoforms of NOS are present in humans: inducible NOS (iNOS), neuronal NOS (nNOS) and endothelial NOS (eNOS) (Schwedhelm et al., 2008). iNOS, nNOS, and eNOS differ in catalytic properties, inhibitor sensitivity, coding genes, and locations within the body. Neuronal nitric oxide synthase (nNOS) is present in neuronal tissues throughout the body; inducible nitric oxide synthase (iNOS) is present in a wide range of cells, however, it is found in abundance in immune cells. eNOS is present in vascular endothelial cells. nNOS and eNOS are controlled by the availability of calcium (Ca2+) to bind to the calmodulin domain, whereas iNOS is controlled at the level of gene transcription (Alderton et al., 2001)Click or tap here to enter text.. Activated NOS, in turn, catalyzes a hydroxylation reaction between arginine and oxygen, producing *N*-hydroxy-1-arginine (NOHA), an enzyme-bound intermediate. NOHA is further oxidized to produce NO and citrulline. Regulation of vascular tone is the most pronounced signalling effect of NO and is attributed to NO produced mainly by endothelial cells in vascular smooth muscles (Siervo et al., 2011).

Moreover, due to competitive between substrate availability between arginases and NOS for arginine, only a small proportion (approximately 1.5-2%) of total arginine is utilized for NO synthesis (Castillo et al., 1996). Elevated arginase activity is marked by decreased NOS activity; however, elevated extracellular arginine concentration increases NO production through eNOS and iNOS by promoting BH4 synthesis in endothelial cells and mRNA translation in immune cells, respectively. The relationship between arginase and NOS indicates a potential dose-dependent

response between circulating arginine and augmented NOS activity, ultimately leading to elevated NO production (Wu & Morris, 1998).

1.4.2 Arginine Paradox and Citrulline Supplementation

The arginine paradox refers to the phenomenon that despite intracellular physiological concentrations of arginine being 100 – 800 μmol/L, far exceeding the Michaelis-Menten constant (Km) of NOS (~3 μmol/L), NO production via NOS is ultimately controlled by extracellular concentration of arginine (Shen et al., 2005). The arginine paradox has yet to be fully understood. However, several theories have been proposed to explain the phenomenon. One proposed theory is inhibitory regulation by endogenous asymmetric dimethylarginine (ADMA), an arginine analogue. ADMA is derived from protein breakdown and methylation of released arginine residues by *N*-methyltransferase, an S-adenosylmethionine-dependent protein and is associated with inflammation-induced endothelial dysfunction (Antoniades et al., 2011). Dimethylarginine dimethylaminohydrolase is responsible for regulating the breakdown of AMDA, thereby working as an modulating ADMA levels and promoter of NO synthesis via NOS. Regulation of ADMA levels via *N*-methyltransferase and dimethylarginine dimethylaminohydrolase depends on oxidative and inflammatory status (Sibal et al., 2010) . Moreover, increased arginine to ADMA ratios result in increased NO synthesis and promote improved endothelium function (Sibal et al., 2010).

However, although theories such as ADMA inhibition of NOS have been proposed, the exact mechanistic control of NOS has yet to be fully established and may be multifaceted involving citrulline regulation. Theoretically, high extracellular arginine should maximize NO production via NOS, while low extracellular arginine will hinder NO production. Therefore, supplementing

citrulline as an alternative exogenous source to arginine may increase arginine availability for NOS, thereby enhancing NO production. Moreover, within endothelial cells, citrulline recycling via ASS and ASL is closely coupled to NO production through NOS via co-localization of ASS, ASL and NOS, thus allowing citrulline to be rapidly converted to arginine for NOS utilization (Flam et al., 2007). Furthermore, compared to arginine, citrulline is used in fewer metabolic pathways thus may provide a better means for supplying arginine for NOS compared to arginine supplementation.

1.5 Creatine

1.5.1 Creatine Biosynthesis – Amino Acids and Metabolites Involved

Creatine synthesis via guanidinoacetate (GAA) is quantitatively the second most demanding arginine catabolic pathway. A large proportion of circulating arginine is converted to GAA via AGAT in the kidneys and ultimately to creatine in an interorgan process involving the pancreas, liver and skeletal muscle (Mori et al., 1998). The multistep process for *de novo* synthesis of creatine is initiated in the kidneys by the enzymatic transfer of a terminal amino group from arginine to glycine, catalyzed by arginine:glycine amidinotransferase (AGAT), subsequently producing ornithine and guanidinoacetate (GAA) (Clarke et al., 2020) (Figure 1.4). The newly synthesized GAA is then exported from the kidneys to the liver, whereby GAA is methylated by guanidinoacetate methyltransferase (GAMT) through the transfer of a methyl group from S-adenosyl methionine (SAM) to GAA, producing creatine and S-adenosyl-L-homocysteine (Brosnan & Brosnan, 2016; Humm, 1997) (Figure 1.4). Synthesis of creatine is also reliant on methionine adenosyltransferase (MAT), which transfers a methyl group from methionine to SAM, the methyl donor for the methylation of GAA. MAT is found in greatest abundance in the liver,

and liver-specific isoform MAT1/3 generates substantial quantities of *S*-adenosylmethionine for methyl transferase pathways (Brosnan et al., 2011).

1.5.2 Functional role of creatine during neonatal development

Creatine and phosphocreatine are critical fuel sources for cellular energetics in many tissues, especially the muscles and the brain (Wyss & Kaddurah-Daouk, 2000). The energy buffer established by the conversion of creatine and adenosine triphosphate (ATP) to phosphocreatine and adenosine diphosphate (ADP) is catalyzed by creatine kinase (CK), an enzyme that removes a singular phosphate from ATP to phosphorylate creatine to phosphocreatine (Figure 1.5). The energy buffering system enables rapid mobilization of high-energy phosphate to various sites, including the cytosol, energy utilization in mitochondria, and energy production (Wallimann et al., 1992). The importance of the creatine buffering system for normal neurological development in neonates has been evidenced in postnatal studies examining cerebral creatine stores in the first three months of life in neonates born at term (Blüml et al., 2013, 2014). In situations whereby neonates cannot produce or utilize adequate creatine, such as in the cases of inherited creatine deficiency syndromes (CDS), AGAT- or GAMT-deficiency disorders or due to organ prematurity (i.e. preterm birth), neonates rapidly become creatine deficient once they can no longer rely on maternal creatine supply *in utero*. Neonatal creatine deficiency or inadequacy has been shown to result in impaired psychomotor function, seizures and rapidly deteriorating neurological functions (Battini et al., 2006; Rostami et al., 2020). Dysfunctional guanidinoacetate methyltransferase results in increased GAA to neurotoxic levels, while dysfunctional AGAT results in low GAA and low creatine concentrations, both of which result in similar developmental outcomes such as delayed neurological development, thus suggesting creatine, as well as GAA, play important roles

in neurological development, especially in preterm births (Battini et al., 2002). Moreover, the mechanism and relationship between creatine and neurological development in neonates has yet to be fully understood; however, ideal GAA and creatine synthesis in humans requires normal renal, pancreatic and hepatic function. In the case of premature births (less than 37 weeks gestation), renal, pancreatic and hepatic function may not be sufficient to meet the increased creatine demand during such a vulnerable developmental period in neonatal growth (Brosnan et al., 2011; Brosnan & Brosnan, 2016).

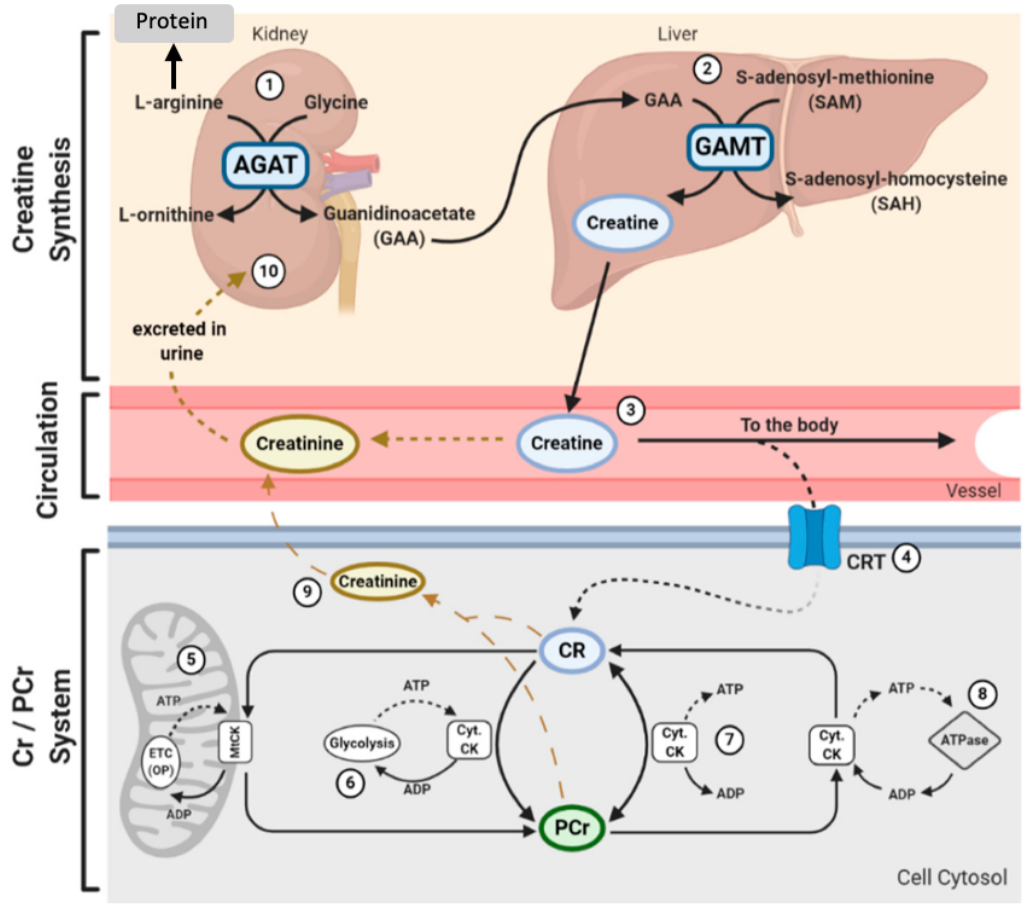

Figure 1.4: Interorgan creatine synthesis and creatine energy buffering system. AGAT; arginine:glycine amidinotransferase; GAA, guanidinoacetate; GAMT, guanidinoacetate methyltransferase; CRT. Creatine transporter; Cr, creatine; PCr, phosphocreatine; ADP, adenosine diphosphate; ATP, adenosine triphosphate; ATPase, adenosine triphosphate-hydrolase; MiCK, mitochondrial creatine kinase; ETC, electron transport chain. Modified from (Clarke et al., 2020)

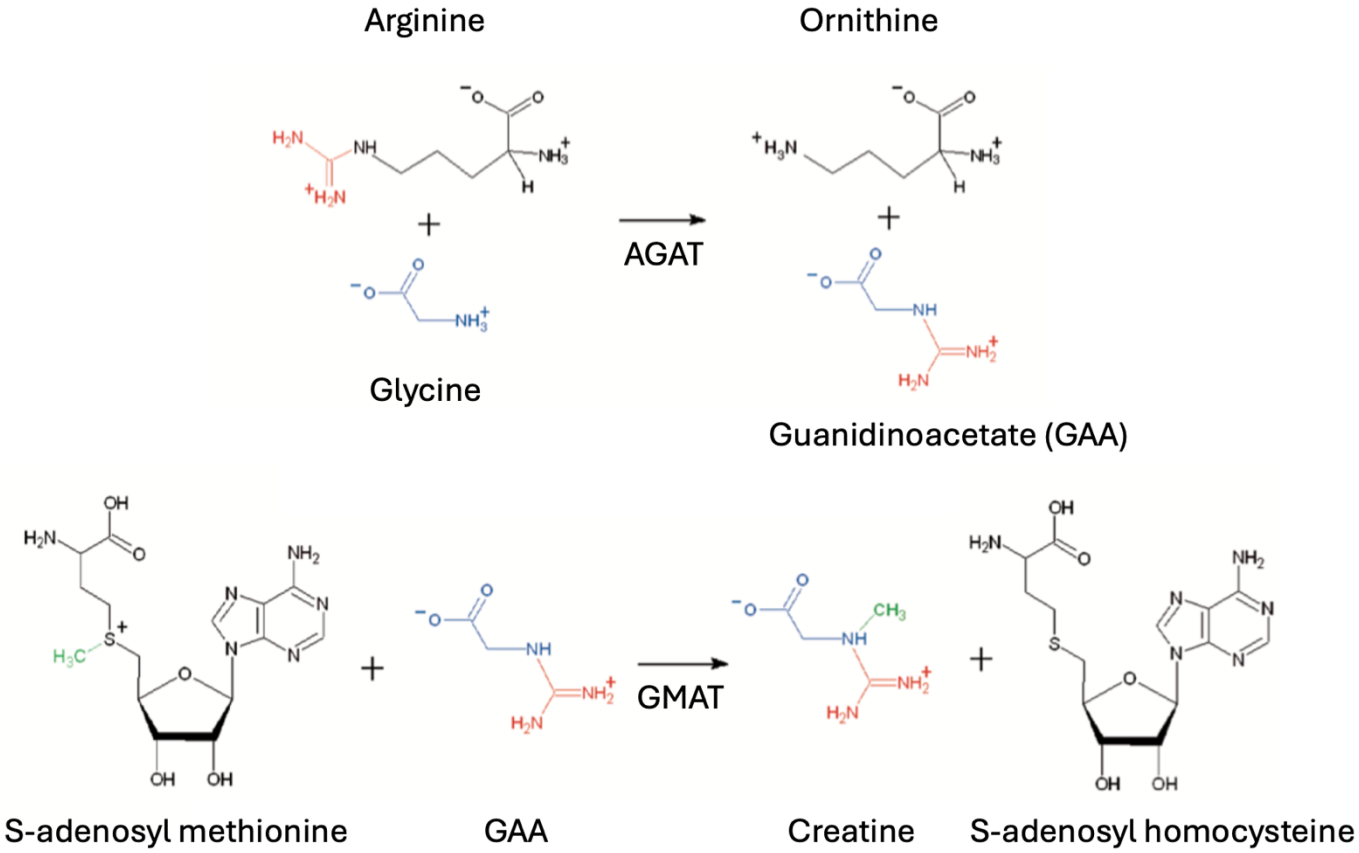

Figure 1.5: The stoichiometric conversion of arginine and glycine to creatine**.** Abbreviations: AGAT, arginine:glycine amidinotransferase; GAA, guanidinoacetate; GMAT, guanidinoacetate methyltransferase. Modified from (Brosnan & Brosnan, 2007).

1.5.3 The role of AGAT in GAA synthesis

AGAT's tissue localization and activity are complex due to the varying degrees of tissue enzymatic activity. While AGAT is found in the pancreas, testes, lungs, spleen and brain, the kidneys have been shown to be the major contributor to GAA synthesis in the body, whereby it is exported to the liver for methylation and terminates in creatine synthesis (Brosnan & Brosnan, 2007). Although the kidneys are the major source of GAA, pancreatic acinar cells express AGAT activity proportional to that of the kidneys. However, pancreatic GAMT and MAT enzyme activities are only approximately 1% of those found in the liver and thus only account for a small proportion of the transmethylation enzyme as well as the methyl donor required for creatine synthesis, respectively (Brosnan et al., 2009; Edison et al., 2007; Sorenson et al., 1995). Furthermore, methylation of GAA by GAMT has been shown to consume much of the available methyl groups; if GAA is supplemented, GAA methylation can limit methionine for protein synthesis as well as other transmethylation pathways, such as those for phosphatidylcholine (PC) synthesis and methylation of DNA, when methionine is in limited supply (McBreairty et al., 2013). Thus, arginine availability may indirectly influence transmethylation metabolism in the body by changing flux through creatine synthesis pathways. Further, the availability of methyl groups for the methylation of GAA to produce creatine plays a role in creatine synthesis; specifically, if more GAA is present, then more methyl groups are consumed, as this is not a feedback-regulated pathway (Robinson et al., 2016). Moreover, an arginine-sparing effect has been evidenced in agricultural animals. In poultry, the addition of GAA to the diet spared arginine, thus leading to increased arginine availability for metabolic pathways other than GAA synthesis (DeGroot et al., 2019; Majdeddin et al., 2020; Portocarero & Braun, 2021).

1.6 Protein Synthesis in Neonatal Piglets

Creatine synthesis via AGAT and GAMT is metabolically demanding and requires adequate availability of amino acids such as arginine and methionine as a methyl donor. In addition to creatine synthesis, arginine and methionine are essential for protein turnover in piglets (Brosnan et al., 2009). When external creatine sources do not provide adequate creatine to meet demands, *de novo* creatine synthesis must be higher to maintain creatine status. However, increasing *de novo* creatine synthesis leads to increased arginine and methionine use, thus may limit arginine and methionine available for protein synthesis (McBreairty & Bertolo, 2016). Dinesh et al. (2021) demonstrated that when precursor amino acids (arginine and methionine) were limited, deficits in plasma GAA and creatine arose, however, protein synthesis was preserved (Dinesh et al., 2021). Further, since PN-induced gut atrophy hinders the metabolic capacity of the gut, arginine becomes even more of a limiting factor since the endogenous synthesis of arginine by the gut is impaired. Therefore, providing arginine either through adequate arginine sources or through precursor availability (i.e. citrulline) must be assessed and considered when formulating PN formulations.

1.7 Purpose of Investigation

1.7.1 Purpose of Investigation

Little is known about the exact neonatal nutrient requirements during the first critical period of life, especially if the neonate requires exclusive parenteral nutrition. Hence, determining the role of citrulline in neonates could be key to enhancing arginine availability. The rationale behind the study is that by supplementing citrulline, arginine availability for NO, creatine and protein synthesis in parenterally fed neonates will be augmented, thereby improving clinically relevant outcomes such as growth, mesenteric blood flow, and intestinal function.

1.7.2 Piglets as a Model for Infants

Due to the practical and ethical limitations of studying amino acid metabolism in human neonates, a neonatal Yucatan miniature piglet model was used to study neonatal metabolism. Many similarities in developmental physiology, nutrient metabolism and dietary requirements have been reported between infants and piglets (Shulman, 1993). Moreover, piglets are relatively immature at birth, undergo rapid growth and have proportionately similar nutrient requirements to humans, thus making piglets an ideal model for studying the effects of parenteral feeding in premature neonates (Chapman et al., 2009).

1.8 Hypotheses

I hypothesized that supplemental citrulline in parenteral nutrition would mitigate reductions in mesenteric blood flow and improve whole-body and tissue-specific protein synthesis, nitric oxide synthesis and creatine synthesis to a greater extent than supplemental arginine alone.

1.9 Objectives

The objectives were:

- to improve systemic arginine availability for nitric oxide synthesis, systemic and tissue-specific protein synthesis, and creatine synthesis in parenterally fed neonatal piglets by providing additional arginine and citrulline to parenteral feeding regimens.

- to determine whether citrulline, an arginine precursor, augments arginine availability for the aforementioned outcomes to a greater extent than arginine supplementation alone.

2.0 Materials and Methods

2.1 Animal Model and Surgical Procedures

To test the study objectives, miniature Yucatan piglets were used as a model for parenterally fed neonates. Animal care and handling procedures were approved and conducted in accordance with the guidelines of Memorial University of Newfoundland Animal Care Committee and the Canadian Council on Animal Care.

Twenty-seven, 10-14 day old, suckling Yucatan miniature piglets (N=27) were obtained from the Memorial University of Newfoundland breeding colony (St. John's, NL, Canada). A sample size of 7 per group was calculated based on a significance level of 0.05 (alpha) and power of 0.80 (beta) to detect a 15% difference in blood flow through the superior mesenteric artery that supplies the small intestine (Niinikoski et al., 2004). The sample size of 8 was determined using previous variance estimate from data for arginine metabolic products (Dinesh et al., 2020; Niinikoski et al., 2004); however, all treatment groups in the study had a sample size of 9 to ensure sufficient power to determine statistical differences in other parameters as well.

On the morning of surgery (study day 0) piglets were fasted for 8hrs and transported from the breeding colony by Animal Care Services to the surgical suite in the Biotechnology building on Memorial University of Newfoundland St. John's campus (St. John's, NL, Canada). Since three surgeries typically took place per surgery day, piglet deliveries were staggered by approximately 2 hours (9am, 11am and 1pm delivery) thus allowing the second and third piglet to stay with the sow thereby minimizing stress and the risk of adverse health effects due to prolonged fasting, such as hypoglycemia. Upon arrival to the Biotechnology building, piglets were weighed and an intramuscular injection of ketamine hydrochloride (20 mg/kg body weight; Rogarsetic Rogar STB, Montreal Canada) and acepromazine (0.5 mg/kg body weight; Atravet:Ayerst Laboratories,

Montreal, Canada) was given to induce anesthesia. A subcutaneous injection of atropine (0.05 mg/kg body weight; Rafter Dex Calgary, Canada) was also given. Additionally, buprenorphine analgesic (0.03 mg/kg body weight Temgesic, Reckitt Benckiser Healthcare, UK) was given prior to and after surgery (10-12 hours apart) for peri- and post-surgical pain management. Once the piglets were anesthetized via intramuscular injection, the piglets were intubated with an endotracheal tube to facilitate and maintain general anesthesia throughout the surgical procedure. 0.8-1.5% isoflurane (Abbot Laboratories Ltd.) was used as the anesthetizing agent and was delivered with 1.5 L/min medical grade oxygen. Heart rate and oxygen saturation was monitored using a pulse oximeter attached to the piglet's ear. Respiratory rate and respiratory gases (oxygen and carbon dioxide) were monitored using an inline pressure and flow sensor attached to the proximal end of the endotracheal tube. Lastly a rectal thermometer was inserted to monitor the piglet's body temperature throughout the procedure.

Once anesthetized, the piglets and incision areas were cleaned with a three step process: step one consisted of scrubbing with Prepodyne solution; step two consisted of washing with 70% isopropanol (Fisher Scientific, Ottawa, ON, Canada); and step three consisted of a final wash of Prepodyne solution prior to moving the piglet to the surgical table. All surgical equipment, gowns and drapes were sterilized by autoclave sterilization, while the catheters and blood flow probes were sterilized using ethylene oxide gas sterilization.

The sterile surgical procedure was a three-step process that started with a 2-2.5 cm dorsal incision between the left scapula and spine, parallel to the spine whereby the jugular and femoral catheters were anchored. The jugular catheter was tunneled subcutaneously and cranially towards the left temporomandibular joint whereby it was tunneled caudally towards the left external jugular

vein. The femoral catheter was tunneled subcutaneously caudally, in parallel with the spine towards the left hind leg whereby it was tunneled around the knee joint to the left femoral vein.

The second step of the surgical procedure was the implantation of the jugular and femoral catheters. Firstly, the jugular vein was isolated by blunt dissection through a 2 cm paramedial incision and a silastic catheter (ID 0.76 mm, OD 1.65 mm) was advanced into the external jugular vein to the junction with the cranial superior vena cava, thereby providing central venous access immediately cranial to the heart. Secondly, the femoral vein was isolated via a 2.5 cm diagonal incision between the left last nipple and the knee. The second silastic catheter (ID 0.63 mm, OD 1.19 mm) was implanted and advanced cranially to the caudal inferior vena cava, providing central venous access caudal to the heart. The jugular and femoral catheters were anchored by subcutaneous suture under the dorsal incision and all incisions were closed via interrupted sutures. Prior to insertion of the superior mesenteric artery (SMA) blood flow probe, the piglet was attached to a continuous saline infusion (6 mL/kg body weight) to replenish and maintain fluid levels throughout the rest of the surgery. Intravenous injection of 0.5 mL of antibiotic Borgal (Trimethoprim 40 mg/mL and sufadoxine 200 mg/mL; Intervet Canada Ltd, Canada) diluted in 9.5 mL of saline was given to prevent the development of infection during and post-surgery. Additional doses of Borgal were given daily for the first two days of the study after surgery.

The third and final step of the procedure was the implantation of the SMA blood flow probe. A 3-3.5 cm diagonal ventral to dorsal incision along the costal margin was made with blunt dissection in line with the muscular fibers to dissect through the three muscle layers covering the peritoneum to minimize damage to the abdominal muscles thus improving surgical recovery. Once localized to the peritoneum, blunt dissection was performed in the dorsal direction towards the renal capsule whereby separation of the peritoneum from the renal fascia was performed to provide

a location to isolate the SMA below the left kidney, adjacent to the inferior vena cava and abdominal aorta. The SMA was isolated by localizing the hepatic vascular bundle coming out of the kidney and tracing the abdominal aorta caudally until the abdominal aorta branches off to supply the small intestine via the SMA. Once the SMA was blunt dissected and isolated, a 4 mm perivascular ultrasonic blood flow probe (Transonic System Inc, Ithaca, USA) was secured around the vessel, ultrasound gel was injected between the vessel and the blood flow probe to facilitate ultrasonic transmission and blood flow was assessed and recorded before incision closure. The cable of the perivascular ultrasonic probe was anchored via purse string suture to the three abdominal muscle layers and excess cable was coiled and anchored via sutures subcutaneously in a tunneled pocket. The remaining cable was then tunnelled cranial-dorsally towards the dorsal incision where the catheters were anchored, all skin incisions were then closed with interrupted sutures. Hibitane (1% Chlorhexidine acetate), an antibacterial cream, was applied to all incisions post-surgery to prevent infections and was reapplied daily over the course of the study. Post-surgery, piglets were transferred to metabolic animal housing cages and recovery was monitored.

2.2 Experimental Protocol and Diets

Piglets were housed for 6 days in individual metabolic cages fitted with a swivel and tether system (Lormir Biomedical, Notre-Dame-de-I'Île-Perrot, Quebec, Canada) that connected to the dorsal side of jackets worn by the piglets and allowed for free movement while also protecting the catheter and probe exit and permitting continuous intravenous access for diet infusion to the jugular vein catheter. Room temperature was maintained at 27^{O}C to help piglets maintain adequate thermoregulation, and supplemental heat was also provided directly into the metabolic cages via

heat lamps. A 12-hr light-dark cycle was programmed in the rooms lighting unit to simulate a natural light-dark cycle.

Immediately post-surgery, piglets were randomized into one of three complete elemental parenteral diets; 1) a control PN containing 0.87 g/kg/day arginine and no citrulline, 2) a high arginine PN containing 1.75 g/kg/day arginine and no citrulline and 3) a citrulline PN containing 0.87 g/kg/day arginine and 0.88 g/kg/day citrulline (Table 2.1). All diets were mixed from free crystalline L-amino acids (Ajinomoto, Japan; Evonik Industries AG, Hanau-Wolfgang, Germany; Sigma-Aldrich, Oakville, Canada) that were weighed and mixed in 4 L beakers with 1.5 L of pyrogen free water at a temperature of 50-70^{O}C under nitrogen gas, aseptic preparation described by Dodge et al. (2012). All vitamins and minerals were provided at 120% of the requirement for neonatal piglets as defined by the National Research Council (2012). Prior to intravenous infusion of the parenteral formulas, multivitamins (Multi12/K1 Pediatric, Baxter Corporation, Mississauga, On, Canada), iron dextran (Bimeda-MTC Animal Health, Cambridge, ON, Canada), trace elements (200% of NRC; Sigma-Aldrich Canada, Oakville, ON, Canada), and intralipid were added to each diet bag, as per Wykes et al. (1993). Each bag was weighed periodically to ensure accurate dietary infusion rates. Post-surgery, study day 0, all piglets underwent an adaptation period whereby diets were infused by dual channel infusion pumps (Baxter Healthcare Corporation, Deerfield, USA) at 50% of the target study rate of 13.5 mL/kg/h. The following study day (study day 1), infusion rates were increased to 75% for 6 h and then infused at 100% (13.5 mL/kg/h) for remainder of the study period (days 2-6). Piglets were weighed each morning and infusion rates adjusted accordingly.

2.3 Parenteral Diet Treatments

2.3.1 Control Diet

The purpose of the control diet was to deliver all amino acids and nutrients at a level adequate for maintaining neonatal piglet nutritional needs, but was not supplemented with additional arginine or citrulline. The amino acid pattern is based on human breast milk protein (ie Vaminolact) but is modified for piglet growth rates (Wykes et al., 1993); this control PN diet contained 0.88 g/kg/d arginine and no citrulline.

2.3.2 High Arginine (High Arg) Diet

The high arginine diet is formulated to match the control diet, but the arginine is increased by 2 fold from 0.87 g/kg/d to 1.75 g/kg/d. The purpose of the high arginine (and citrulline) diets was to determine if supplementation of additional arginine (or citrulline) would impact outcomes in a clinical setting, thus the diets were not isonitrogenous and the control diet was used as the "base" or "standard of care" diet with the supplemented arginine (or citrulline) added to the control. The high arginine treatment did not contain additional citrulline.

2.3.3 Citrulline (Cit) Diet

Citrulline plays a major role in resynthesizing arginine in the urea cycle and is also used to increase arginine availability for nitric oxide synthesis via NOS in a 1:1 molar fashion. The citrulline treatment group contained the same quantity of arginine as the base control diet (0.87 g/kg/day) but contained additional citrulline at an equimolar ratio to the arginine quantity (0.88 g/kg/day). The citrulline treatment group was used to investigate if citrulline, an arginine

precursor, would be as efficient as the additional arginine for increasing arginine availability for arginine's metabolic pathways.

2.3.4 Trace Minerals, Vitamins and Lipids

Trace minerals and vitamin mixtures were both prepared in the laboratory prior to the start of the study (Bray, 2017). Minerals and vitamins were provided at more than 120% of the NRC requirements for piglets to ensure all nutritional needs (National Research Council, 2012). Lipid emulsions were supplied by Baxter Intralipid 20% (20% Intralipid, Baxter Corporation, Mississauga, ON, Canada) while total energy was provided at 1.1 MJ energy/kg/day. Furthermore, lipid and carbohydrates each provided 50% of the non-protein energy in the diets (Wykes et al., 1993).

Table 2.1: Amino acid profiles of treatment diets

Amino Acids	Control	High Arginine	Citrulline
	g/L	g/L	g/L
Alanine	5.89	5.89	5.89
Arginine	**3.65**	**7.30**	**3.65**
Citrulline	**0.00**	**0.00**	**3.67**
Aspartate	3.32	3.32	3.32
Cysteine	0.76	0.76	0.76
Glutamate	5.72	5.72	5.72
Glycine	1.47	1.47	1.47
Histidine	1.69	1.69	1.69
Isoleucine	2.51	2.51	2.51
Leucine	5.67	5.67	5.67
Lysine-HCl	5.58	5.58	5.58
Methionine	1.04	1.04	1.04
Phenylalanine	3.00	3.00	3.00
Proline	4.52	4.52	4.52
Serine	3.11	3.11	3.11
Taurine	0.27	0.27	0.27
Tryptophan	1.14	1.14	1.14
Tyrosine	0.44	0.44	0.44
Valine	2.89	2.89	2.89
Threonine	2.23	2.23	2.23

Table 2.2: Total composition of treatment diets

Composition of Elemental Diets	**g/L**
L-Amino Acids	56.0
Dextrose	90.3
K_2HPO_4 Trihydrate	1.57
KH_2PO_4 Monobasic	1.085
Potassium Acetate	1.47
Sodium Chloride	2.17
Magnesium Sulfate	0.78
Zinc Sulfate	0.089
Calcium Gluconate	6.41
Composition of Added Mixtures	***Dose*/kg BW/d**
Fat Soluble Vitamins, *Dose*	
Vitamin A, *ug*	273
Vitamin D, *ug*	2.20
Vitamin E, *mg*	1.50
Vial 1	
Ascorbic acid, *mg*	17.4
Thiamine (as hydrochloride), *mg*	0.26
Riboflavin (as phosphate), *mg*	0.30
Pyridoxine hydrochloride, *mg*	0.22
Niacinamide, *mg*	3.70
d-Panthenol, *mg*	1.10
Vitamin K_1, *mg*	0.04
Vial 2	
Biotin, *ug*	4.35
Folic Acid, *ug*	30.46
Vitamin B_{12}, *ug*	0.22
Trace Elements	**mg/kg BW/d**
Zinc (as $ZnSO_4$)	10.07
Copper (as $CuSO_4$)	0.87
Manganese (as $MnSO_4$)	0.66
Chromium (as $CrCl_3$)	0.01
Selenium (as SeO_2)	0.05
Iodine (as NaI)	0.02
Iron Dextran	
Iron (as $FeHO_2$)	3.0

2.4 Daily Animal Care and Day to Day Procedures

From study day 1-6, daily animal care was performed between 8-9 am Newfoundland Standard Time and consisted of daily body weight recordings, 1 mL blood sample from the femoral venous catheter and superior mesenteric arterial (SMA) blood flow measurements via Transonic T403 flow meter (Transonic Systems). As mentioned, additional doses of diluted Borgal antibiotic (0.1 mL/kg) were administered daily and a 0.03 mg/kg dose of buprenorphine analgesic (Temgesic, Reckitt Benckiser Healthcare, UK) was administered twice daily for study day 1 (post-surgery), and then as needed if piglets demonstrated pain or discomfort.

SMA blood flow measurements were taken 3 times daily at 9 am, 3 pm and 9 pm to ensure an accurate daily average can be attained throughout the course of the study. To further ensure that the blood flow measurements were accurate, the piglets were awoken if sleeping, and blood flow was measured 10 times over 10 minutes over a range of positions (i.e. standing, sitting and laying). It was not uncommon for the SMA blood flow probe to have to settle into position and give an inaccurate or nil reading for the first day post-surgery, however, once the flow probe settled and the incision started to heal, the flow readings would become more accurate.

2.5 Stable Isotope Infusions and Necropsy

On Study Day 5, a five-hour isotope infusion of stable isotopically labelled amino acids (L-Phenylalanine (Ring-D5), L-Tyrosine (Ring-3,5-D2), L-Tyrosine (Ring-D4), L-Arginine:HCl (15N4, 13C6), L-Citrulline (Ureido-13C, 3,3,4-D3) and Guanidinoacetate (2,2-D2)) was performed, and blood samples were drawn every 30 min to measure phenylalanine oxidation (to calculate whole-body protein synthesis), creatine synthesis and nitric oxide synthesis. Three baseline blood samples were taken from the femoral catheter at time point -60, -40, and -20 min

followed by a priming dose of 0.51 mg/kg labelled L-Tyrosine (Ring-D4) to prime the product pool thus allowing steady state kinetics to be reached more quickly. The prime dose of L-Tyrosine as well as a 5-hour constant infusion were infused into a Y-connector attached to the PN line infusing into the jugular catheter. Immediately following the priming dose administration, a syringe pump was used to continually infuse the mixed stable isotope infusion of L-Phenylalanine (Ring-D5) (3.40 mg/kg/h), L-Arginine:HCl (15N4, 13C6) (3.40 mg/kg/h), L-Citrulline (Ureido-13C, 3,3,4-D3) (0.60 mg/kg/h) and Guanidinoacetate (2,2-D2) (0.20 mg/kg/h) (Table 2.3).

Due to the insolubility of the L-Tyrosine isotope in the volume of saline required to dissolve the isotopes for the constant infusion, the 6-hour constant dose of isotopes was dissolved in the parenteral diet. The treatment diets infused during the constant infusion were adjusted so the total grams of amino acids were constant. The quantity of labelled amino acids required for the isotope kinetics were calculated in g/L. Infusion diets were then calculated by subtracting the labelled amino acids (g/L) from the unlabelled amino acid profile (g/L) to allow for the addition of the labelled amino acid isotopes during the isotope infusion, thus maintaining the same amino acid profile as treatment days.

Every 30 minutes, 0.8-1.0 mL of blood was sampled from the femoral catheter and directly transferred into ethylenediamine tetraacetic acid (EDTA) tubes (BD Vacutainers, Mississauga, ON, Canada). Plasma was separated immediately after centrifugation at 5,000 RPM for 5 minutes and stored at -80^{o}C until further analysis.

Table 2.3: Prime and constant dose for stable isotopes

Stable Isotope	Prime (mg/kg)	Constant (mg/kg/h)	Purpose
L-Arginine:HCl (15N4, 99%; 13C6 99%)	2.00	3.40	Arg (M+10) to Cit (M+9) for NO synthesis, Arg (M+10 to GAA (M+3) for GAA Synthesis
L-Citrulline (Ureido-13C, 99% 3,3,4-D3, 98%)	0.15	0.60	To determine Cit flux
Guanidinoacetate (2,2-D2, 99%)	0.10	0.20	To determine GAA flux
L-Phenylalanine (Ring-D5, 98%)	1.09	3.40	Phe (M+5) to Tyr (M+4) for whole-body protein synthesis
L-Tyrosine (Ring-3,5-D2, 98%)	-	1.65	To determine Tyr flux
L-Tyrosine (Ring-D4, 98%)	0.51	-	Prime for the Tyr (M+4) product pool

On study day 6, the piglets were anesthetized with 5% isoflurane mixed with oxygen (1.5 L/min) and received a flooding bolus dose of unlabelled L-phenylalanine (1.5 mmol per kg by body weight; Sigma-Aldrich) and labelled L-phenylalanine (D8, 98%, dose 0.15 mmol per kg body weight, Cambridge Isotopes Laboratories, Inc.) over the course of 5 minutes. A 30-minute lag time (between infusion and first blood sample) was started once bolus dose of Phenylalanine was administered, during which time, blunt dissection of the neck was performed to isolate the carotid artery to allow for direct sampling of arterial blood, and a 4 mm ultrasonic probe was used to determine carotid blood flow. A laparotomy was then performed to gain access to the visceral organs, right kidney and blood vessels (portal and renal vein) required for the trans-organ metabolite balance calculations. Blunt dissection was performed to isolate the renal artery/vein, and the portal vein, and 4 mm and 6 mm ultrasonic probes were used to measure blood flow through both vessels, respectively. At the end of the 30-minute lag time, a 2-3 mL blood sample was taken from the carotid artery, jugular, portal and renal veins. Following blood sample collection, small intestine length was assessed by measuring the whole small intestine from the pyloric sphincter to the ileocecal valve using a 30 cm ruler. The first 15 cm of small intestine proximal to the pyloric sphincter was considered the duodenum and small intestine weight was assessed by weighing 50 cm of proximal jejunum immediately after the 15 cm of duodenum. The 50 cm of proximal jejunum was sliced and opened vertically, and gut diameter was measured. The opened proximal jejunum was flushed with saline, and the mucosa scraped using a glass microscope slide separating the mucosa from the muscularis externa. The mucosa along with liver, pancreas, kidney, brain, longissimus dorsi and bicep were dissected, weighed, flash frozen with liquid nitrogen and stored at -80^{o}C until analyzed. The piglets were euthanized via exsanguination as part of the necropsy procedure.

2.6 Analytic Procedures

2.6.1 Blood Flow

As mentioned in "*2.1 Animal Model and Surgical Procedures*", the piglets were fitted with a perivascular ultrasonic blood flow probe (Transonic System Inc, Ithaca, USA) which allowed for long term monitoring of blood flow through the SMA, which supplies the small intestine. Blood flow measurements were taken every minute for 10 minutes, three times daily, thus ensuring an accurate representation of any changes in blood supply to the small intestine throughout the course of the study. Terminal blood flow readings were also taken during the necropsy procedure using either a 6 mm, 4 mm or 2 mm perivascular ultrasonic blood flow probe (Transonic System Inc, Ithaca, USA), depending on vessel size (carotid/SMA – 4 mm, portal – 6 mm, renal – 2 mm) in the carotid and superior mesenteric arteries (SMA) as well as in the portal and renal veins.

2.6.2 Morphological and Histological Analysis of the Duodenum and Jejunum

The diameter, length and weight of the gut were measured. Duodenal and jejunal sections of the gut were placed in formalin for histology. The samples were set in paraffin wax and sent to the histology medical laboratories at the Health Science Center for sectioning, hematoxylin and eosin staining and slide mounting. Villus height and crypt depth were assessed using a compound microscope and Toupeview Imaging software.

2.6.3 Organ Hydration Status and Lipid Quantification

2.6.3.1 Hydration status was assessed by weighing approximately 1g of frozen tissue sample and recording the pre-dried weight. The samples were then dried at 100^{o}C for 24 h, reweighed and the post-dried weight was recorded. The pre-dried and post-dried weight difference is representative of water loss and thus tissue hydration status.

2.6.3.2 Tissue Lipid Composition was assessed using the lipid extraction method reported by (Folch et al., 1957). Briefly, 100 mg of tissue sample was weighed and homogenized with 50 mmol/L NaCl. Subsequently, 2 mL of 2:1 chloroform:methanol (%v/v) solution was added to each sample and samples were vortexed for 1 minute and left overnight at 4^{o}C. The following day, samples were centrifuged at 3000 RPM for 10 minutes and the lipid layer was extracted and transferred to a glass culture tube. The glass culture tube was weighed prior and after the lipid layer was transferred. The extracted lipid layer was subsequently evaporated under nitrogen gas for 15-20 minutes until the lipid layer was completely dried. Post-dried weight was recorded, and the pre-dried and post-dried weight difference is representative of the lipid quantity in the tissues.

2.6.4 Plasma and Tissue Metabolite Analyses

2.6.4.1 Plasma Amino Acid Concentrations were analyzed by reverse-phase high-performance liquid chromatography (HPLC) following phenylisothiocyanate (PITC) method by (Bidlingmeyer et al., 1984). The HPLC system used for plasma amino acid concentrations contained a Waters 2487 Dual I absorbance detector, a column heater, Waters 717 plus Auto Sampler, Waters 1525 Binary HPLC pump and a reverse-phase C18 Pico-Tag column (60Å, 4 µm, 3.9 x 300 mm) (Waters Corporation, Milford, MA, USA). Two mobile phases were used in the analyses (A and B); mobile phase A was 70 mM sodium acetate in 97.5 % HPLC grade water/ 2.5% acetonitrile adjusted to a pH of 6.55 using acetic acid while mobile phase B was 40% HPLC grade water/ 45% acetonitrile/ 15% methanol. Derivatized amino acids were detected at UV 254 nm absorbance and peaks were integrated using Empower 3 software and concentrations calculated against an internal standard, norleucine (Waters Corporation, Milford, MA, USA)

2.6.4.2 Plasma and Tissue Creatine Concentration was analyzed by reverse-phase high-performance liquid chromatography (HPLC) following a modified method reported by (Lamarre et al., 2010). Tissues were homogenized with 50 mM Triss buffer (pH 7.4) and kept for 30 minutes to allow for spontaneous conversion of phosphocreatine to creatine, especially important for skeletal muscle tissues. Whereas 50 mM Tris buffer (pH 7.4, Fisher Scientific, Ottawa, ON, Canada) was added directly to the plasma samples in a 1.5 mL microcentrifuge tube. Tissue homogenates and plasma samples were then deproteinized by trifluoroacetic acid (TFA; Thermo Fisher Scientific, Mississauga, ON, Canada) and centrifuged at 13,500 *g* for 10 minutes. The HPLC system used for the analyses was the same system as described above (Section 2.6.4.1), however, an isocratic mobile phase of 0.1% TFA in 3% methanol / 97% HPLC grade water and N/RP-HPLC Hypercarb (7 μm, 4.6 x 100 mm) column (Termo Fisher Scientific, Mississauga, ON, Canada) were used to elute the creatine peaks. The creatine peaks were detected at UV 210 nm absorbance and peaks were also integrated using Empower 3 software and concentrations calculated against a standard curve (Waters Corporation, Milford, MA, USA).

2.6.4.3 Arteriovenous Metabolite Balance Calculation

Net arteriovenous balances were determined using the plasma analytes (i.e. amino acids, creatine) concentration in plasma of the blood flowing into (arterial) and out (venous) of the organ of interest (small intestine, kidney, brain) as well as the blood flow to the organ. The net balance of analyte reported for the kidney were doubled to account for the contributions of two kidneys. A positive net arteriovenous balance is representative of a net organ release of the metabolite to the plasma, whereas a negative net arteriovenous balance represents organ uptake from the plasma pool.

AV Balance $(\mu mol/\ kg_{body\ weight}//min)$ = $[(V_{Analyte} - A_{Analyte}) / 1000] \bullet$ BF $(mL/\ kg_{body\ weight}//min)$

$V_{Analyte}$ represents the venous analyte concentration and $A_{Analyte}$ represents the arterial analyte concentration. BF is the blood flow from the organ of interest measured during the terminal necropsy procedure via the perivascular ultrasonic blood flow probes.

2.6.5 In vitro AGAT activity

AGAT enzymatic activity was assessed in vitro using a method reported by Van Pilsum et al. (1970).

2.6.6 Tissue Specific Protein Synthesis

The isotopic enrichment of L-phenylalanine (D8) in tissue-free and tissue-bound fractions was determined by gas chromatography-mass spectrometry (GC-MS) using a modified method based off a method reported by Lamarre et al. (2015).

2.6.6.1 Preparation

To prepare tissue samples, 100 mg of frozen tissue samples were homogenized in 2 mL deionized water (1:20 dilution), 0.25 mL 2 M ice-cold perchloric acid (PCA) was then added to the homogenate and vortexed thoroughly. Following homogenization, the homogenate was centrifuged at 13,500 *g* for 20 minutes at 4°C. The supernatant was transferred to 7 mL scintillation vials and contained the free pool of amino acids. The pellet was also saved and contained the protein-bound pool of amino acids. Protein in the pellets was hydrolyzed in 6 N HCl at 100 degrees

Celsius for 24 h. Acid extracts contained the amino acids that were incorporated into proteins. Phenylalanine was extracted from tissue free, and tissue protein pools using solid phase extraction (SPE) as per Lamarre et al. (2015b). Bond Elute C18 SPE cartridges (100 mg, 1 mL, Agilent Technologies, Santa Clara, CA, USA) were used to filter the eluent which was subsequently dried in a vacuum concentrator and stored at -20°C. 500 µL of HPLC grade water was used to resuspend the dried eluent on the day of derivatization and quantification. 50 µL of the resuspended sample was transferred to 2 mL glass gas-chromatography (GC) vials for derivatization with 100 mM pentafluorobenzyl bromide (PFBBr) (Lamarre et al., 2015).

2.6.6.2 Quantification

Isotopic enrichment of phenylalanine (D8) in the tissue free and tissue protein fractions was determined with a model 6890 GC linked to a 5976N quadrupole MS (Agilent Technologies, Santa Clara, CA, USA) operating in electron ionization mode. To identify the potential ions required to determine the percent molar enrichments (mol%) of the fractions, a mixed sample of labelled phenylalanine (D8) and unlabelled phenylalanine was run in scan mode and ions 91 (90.70 to 91.790), 99 (98.70 to 99.70), 300 (299.70 to 300.70), 306 (305.70 to 306.70), 307 (306.70 to 307.70) and 308 (307.70 to 308.70) were identified and monitored. Corrected area of identified peaks was used for the calculation of percent molar enrichments (mol%).

2.6.6.3 Tissue-specific protein synthesis calculations

Percent molar enrichment (mol%) was determined in free (IE_{free}) and protein bound (IE_{bound}) phenylalanine using isotopic enrichments of L-phenylalanine (D8) of the PCA-insoluble (protein bound) and PCA-soluble (tissue free) phenylalanine pools. 1440 is the total minutes per

day and t is the time the sample was being labelled in minutes, which was determined by the time the tissue was frozen after the isotope flooding dose.

$$FSR = (IE_{bound} / IE_{free}) \bullet (1440 / t) \bullet 100$$

2.6.7 LC-MS/MS Analysis for Whole-body Protein, Creatine and Nitric Oxide Synthesis

2.6.7.1 Preparation

To prepare plasma samples for mass spectrometry, 25 µL of plasma and 200 µL of 100% MeOH were added to a 1.5 mL Eppendorf tube and vortexed for 10 seconds. Eppendorf tubes were then centrifuged for 10 minutes at 13,000 g. Supernatant was collected in borosilicate glass tubes (13 x 100 mm, VWR disposable culture tubes), covered with parafilm and frozen at -80ºC for 20 minutes and subsequently freeze dried for 1 hour. After drying, samples were derivatized with 100 µL of 3 N HCl (acid) in n-butanol, glass tubes were then capped and placed in an oven at 65ºC for 20 minutes. After heading for 20 minutes, samples were freeze dried for 1 hour and stored at -20ºC until they were shipped to the Analytical Facility for Bioactive Molecules (AFBM) of the Centre for the Study of Complex Childhood Diseases at the Hospital for Sick Children in Toronto, Ontario, Canada for further analyses.

2.6.7.2 LC-MS/MS and Quantification

Quantification as well as the operational protocol was performed and provided by personnel at the AFBM. Each sample was extracted and subsequently diluted with 200 uL of mobile phase B buffer, 10% acetonitrile in 90% water with 5 mM ammonium formate. Samples were processed and injected on the LC-MS/MS on the same day.

HPLC was performed using Agilent Technologies 1200 Series binary pump, auto-sampler and oven column compartment system (Agilent Technologies: Santa Clara, California, USA). The analytical column used was a HILIC MS C18 Column (4.6 x 50 mm, 2.6 µm particle size) from Kintex (Phenomenex, Torrance, California, USA). The method was optimized for peak shape for the different analytes measured; phenylalanine, tyrosine, arginine, citrulline, guanidinoacetate and creatine. Curtain gas, collisional deactivated dissociation (CAD), gas temperature (TEM), ionization spray (IS), GS1, GS2 and entrance potential (EP) were set to 35, medium, 500°C, 5500, 50, 40 and 10, respectively. A gradient program was used for the HPLC separation at a flow rate of 0.35 mL/minute. The initial solvent composition was 90% mobile phase B (10% water / 90% acetonitrile with 5 mM ammonium formate) and 10% mobile phase A (90% water / 10% acetonitrile with 5 mM ammonium formate) and held for 3.5 minutes. From 3.5-6.10 minutes, the solvent composition was 10% mobile phase B and 90% mobile phase A. From 6.1-8 minutes, the composition went back to initial conditions and equilibrated for 1 minute before the next injection total run time was 8 minutes. The HPLC system was coupled on-line to a SCIEX 5500-QTrap triple-quadrupole mass spectrometer (Sciex, Concord, Canada) equipped with a Turbo Ion Spray source. Electrospray ionization (ESI) was performed at 600ºC in the positive mode with multiple reaction monitoring (MRM) to select both parent and characteristic daughter ions specific to each analyte simultaneously from a single injection. Nitrogen was used as the nebulizing, turbo spray and curtain gas. Each target was then uniquely identified by the parent-to-daughter ion mass transition and the specific retention time. The multiple reaction monitoring mass transitions (m/z) were: arginine 231.0-70.0, arginine (M+10) 241.1-75.1, citrulline 232.15-70.0, citrulline (M+4) 236.2-73.1, citrulline (M+9) 241.11-75.1, guanidinoacetate 118.1-76.0, guanidinoacetate (M+1) 119.1-76.0, guanidinoacetate (M+2) 120.1-78.0, guanidinoacetate (M+3) 121.1-78.0, creatine

(M+1) 133.1-90.0, creatine (M+2) 134.1-92.0, creatine (M+3) 135.1-93.0, phenylalanine 166.1-120.0, phenylalanine (M+5) 171.1-125.0, tyrosine 182.1-136.0, tyrosine (M+2) 184.1-138.0, tyrosine (M+4) 186.1-140.0. Data were collected and analyzed by Analyst v 1.6.2 (AB Sciex, Concord Canada). Qualitative analysis was based on area ratios.

2.6.7.3 Isotope Kinetic Calculations

a) Isotope enrichments were determined using atom percent excess (APE) by the following equation reported by Tomlison et al. (2011)

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

R_s is the tracer:tracee enrichment in the sample and R_b represents the tracer:tracee natural abundance.

b) Whole-body Amino Acid Flux was used to calculate molar conversion in concordance with the method reported by Tomlinson et al. (2011):

$$\text{Flux (Q)} \ (\mu mol/\ kg_{body\ weight}//h) = I \times [(E_i/E_p) - 1]$$

Flux (Q) is the rate of isotope appearance within a pool. I is the tracer infusion rate in μmol/kg/h. E_i is the isotope enrichment in the infusate while E_p is the isotope enrichment in the plasma.

c) Precursor to Product Conversion Rate was calculated based on the method reported by (Tomlinson et al., 2011):

$$Q_{precursor>product}\ (\mu mol/kg_{body\ weight}/h) = (E_{product}/\ E_{precursor})\ x\ Q_{product\backslash}$$

$Q_{product}$ represents the whole-body product flux rate calculated from relevant isotope infusion; $Q_{tyrosine}$ was used for phenylalanine to tyrosine, $Q_{citrulline}$ for arginine to citrulline, Q_{GAA} for arginine to GAA. E is the enrichment of the product ($E_{product}$) and precursor ($E_{precursor}$) (Dinesh et al. 2021)

d) Fractional Conversion Rate as % of Product Flux

$$(E_{product}\ /\ E_{precursor})\ *\ 100$$

E is the enrichment of the product ($E_{product}$) and precursor ($E_{precursor}$) (Dinesh et al. 2021)

e) Percent Change for Arginine to NO

I. Whole-body conversion of Arginine to Citrulline

$$Q_{Arg \rightarrow Cit} = (E_{Cit}/\ E_{Arg})\ *\ Q_{Cit}$$

II. Percent Change from Control

$$\%\ Change = [((Q_{Arg \rightarrow Cit\ Treatment}) - (Q_{Arg \rightarrow Cit\ Control}))/\ (Q_{Arg \rightarrow Cit\ Control})]\ *\ 100$$

f) GAA de novo synthesis based off the 1:1 whole-body conversion of arginine to GAA

$$Q_{Arg \rightarrow GAA} = (E_{GAA} / E_{Arg}) * Q_{GAA}$$

g) Whole-body arginine incorporation and release from protein calculated from phenylalanine to protein synthesis and piglet amino acid composition data based on the method reported by (Wu et al., 1999)

I. **Whole-body Conversion Rate of Arginine to Protein**

Arginine → Protein = (Protein Accretion) * 1.65

II. **Arginine Release from Protein Breakdown**

Protein → Arginine = (Protein Catabolism) * 1.65

h) Arginine de novo Synthesis

Flux (Q_{arg}) – (Arg intake + Arg from Protein Breakdown)

i) Whole-body Protein Dynamics was assessed based on the method reported by (de Betue et al., 2017)

I. **Protein Synthesis** ($\mu mol/kg_{body\ weight}/h$) = $Q_{Phenylalanine}$ – (Phenylalanine to Tyrosine Conversion Rate)

II. **Protein Catabolism** ($\mu mol/kg_{body\ weight}/h$) = $Q_{Phenylalanine}$ - $I_{Phenylalanine}$

III. Protein Accretion (μmol/$kg_{body\ weight}$/h) = Protein Synthesis – Protein Catabolism

Protein synthesis was expressed as g of protein/kg of body weight per day assuming the phenylalanine content of protein to be 280 umol/g (Thompson et al., 1989).

2.7 Statistical Analyses

Data were analyzed using one-way ANOVA with Tukey's multiple comparison post-hoc test. Data assessed among treatments and over time were analyzed using a two-way (or mixed model) ANOVA with Tukey's multiple comparison post-hoc test. Statistical software used for data analysis was GraphPad Prism 9 (Graph Pad Software, San Diego, CA, USA). The alpha level was set at 0.05. All data were reported as mean ± standard deviation.

A Pearson correlation was performed between plasma creatine concentration and blood flow. A significant interaction was deemed as a $P< 0.05$. The Pearson correlation r was utilized to determine a positive or negative correlation between the two variables in the case of a significant P-value.

3.0 Results

3.1 Body Weight and Growth Parameters

Supplemental arginine and citrulline to parenteral feeding regimens did not have a significant effect on piglet body weight throughout the course of the study. Similarly, mean body weight was not different among treatment groups pre-surgery (Figure 3.1) or at necropsy (Figure 3.2). However, mean piglet weight did significantly increase over the duration of the study (Figure 3.3), yet the daily growth rate did not differ among treatments (Figure 3.4).

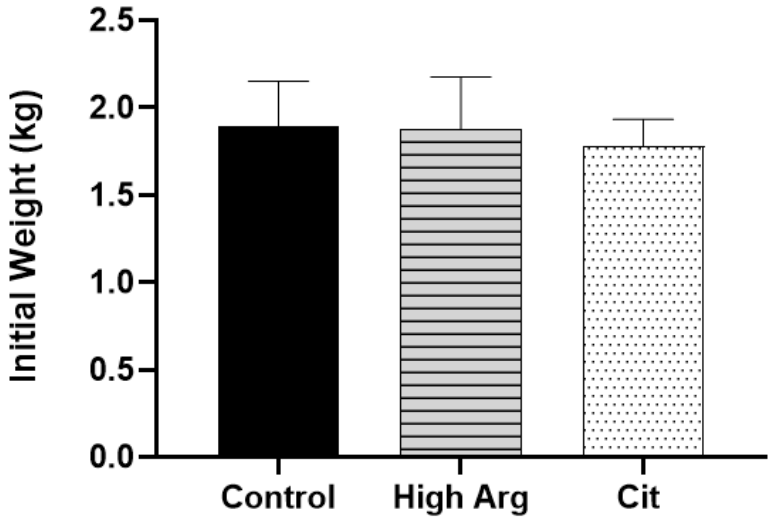

Figure 3.1: Piglet body weight pre-surgery on Study Day 0 prior to parenteral feeding. Data represent means ± standard deviation in kg. All treatments were n = 9. Treatment effect was assessed by one-way ANOVA with a Tukey's multiple comparisons test.

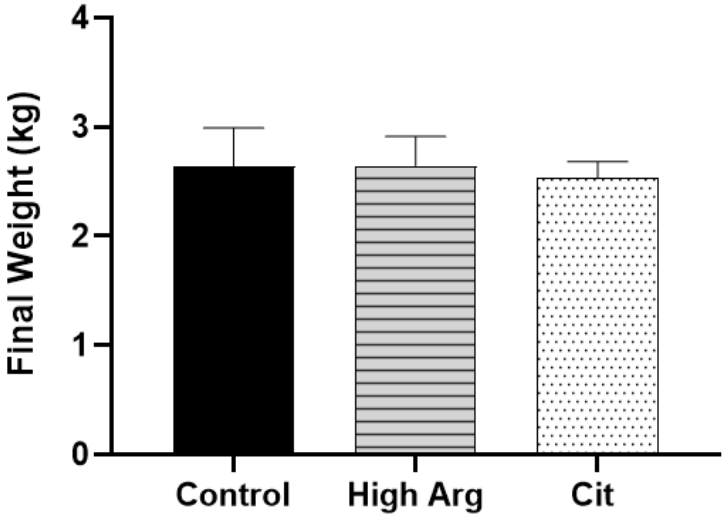

Figure 3.2: Piglet body weight at necropsy on study day 6 after parenteral feeding**.** Data represent means ± standard deviation in kg. All treatments were n = 9. Treatment effect was assessed by one-way ANOVA with a Tukey's multiple comparisons test.

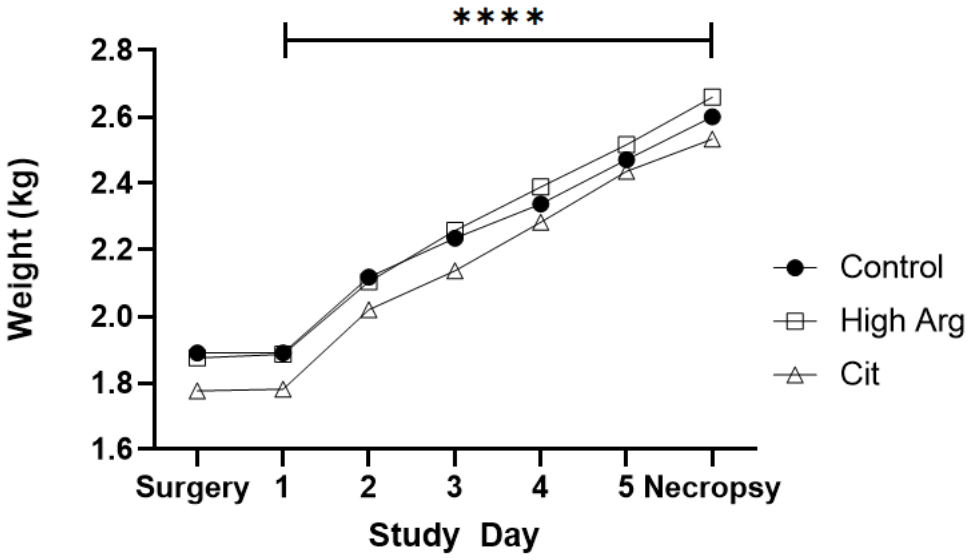

Figure 3.3: Daily piglet weight by study day. Data represent means in kg. All treatments were n = 9. Treatment effect was assessed by two-way ANOVA (among treatments and over time) with a Tukey's multiple comparisons test. There were no significant differences among treatments ($P > 0.05$) but there was a significant effect of time and of interaction between treatment and time ($p < 0.0001$).

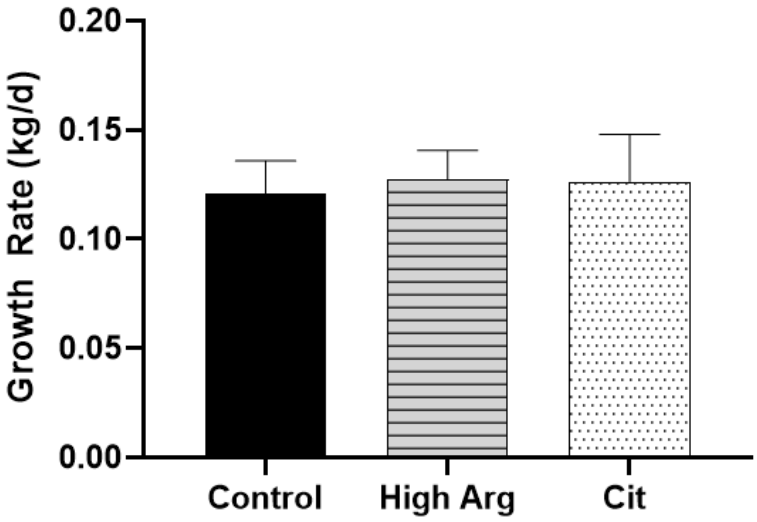

Figure 3.4: Daily growth rate of neonatal piglets from study day 0 to 6. Data represent means ± standard deviation in kg. All treatments were n = 9. Treatment effect was assessed by one-way ANOVA with a Tukey's multiple comparisons test.

3.2 Organ Morphology

3.2.1 Gross Organ Weight, Water and Lipid Composition

Supplementing additional arginine or citrulline to parenteral feeding regimens did not elicit a treatment effect on gross organ weight (Table 3.1). However, when gross organ weight was corrected for terminal piglet body weight, there was a significant treatment effect in the kidney and spleen. Piglets receiving the supplemental citrulline had significantly larger kidneys compared to control and high arginine treatments ($P = 0.038$, $P < 0.0001$, respectively). Furthermore, piglets in the high arginine treatment groups had significantly smaller spleen weights when compared to control and supplemental citrulline treatments ($P = 0.018$, $P = 0.025$, respectively) (Table 3.1). However, there was no treatment effect for renal hydration status (Figure 3.5) or lipid composition (Figure 3.6).

Table 3.1: Terminal gross organ weights after six days of parenteral feeding

	Control	High Arginine	Citrulline	P-Value
Gross Organ Weight (g)				
Small Intestine	54.8 ± 9.23	54.9 ± 8.58	51.0 ± 8.28	P > 0.05
Liver	92.1 ± 11.21	91.0 ± 11.1	93.9 ± 11.5	P > 0.05
Kidney	14.1 ± 2.13	9.11 ± 0.83	10.4 ± 0.83	P > 0.05
Brain	41.2± 18.8	34.1 ± 2.71	36.8 ± 4.59	P > 0.05
Spleen	9.90± 1.46	7.49 ± 1.82	10.6 ± 4.77	P > 0.05
Thymus	1.13 ± 0.14	0.89 ± 0.22	0.89 ± 0.32	P > 0.05
Gross Organ Weight Corrected for Terminal Body Weight (g/kg)				
Small Intestine	20.9 ± 2.70	20.8 ± 2.46	20.1 ± 3.16	P > 0.05
Liver	34.9 ± 1.63	34.4 ± 2.51	37.1 ± 4.88	P > 0.05
Kidney	**3.76 ± 0.33[b]**	**3.45 ± 0.19[b]**	**4.09 ± 0.26[a]**	**P < 0.0001**
Brain	15.8 ± 7.48	12.9 ± 1.12	14.5 ± 1.83	P > 0.05
Spleen	**3.79 ± 0.61[a]**	**2.83 ± 0.60[b]**	**4.19 ± 1.86[a]**	**P < 0.01**
Thymus	0.45 ± 0.09	0.34 ± 0.10	0.36 ± 0.14	P > 0.05

Data represent means ± standard deviation in g and g/kg body weight. All treatments were n = 9 for small intestine, liver, kidney, brain, spleen and n = 6 for thymus. Reported P-value represents overall treatment effect by one-way ANOVA with a Tukey's post-hoc test (superscript). P-value was deemed significant if $P < 0.05$. Means within rows not sharing a superscript letter represent a significant treatment effect between treatments.

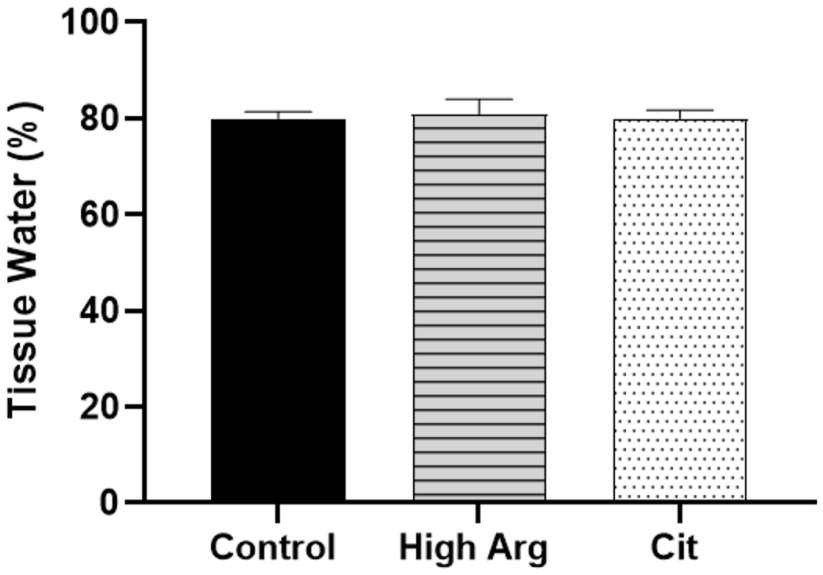

Figure 3.5: Renal hydration status. Data represent means ± standard deviation in %. All treatments were n = 9. Treatment effect was assessed by one-way ANOVA with a Tukey's multiple comparison test.

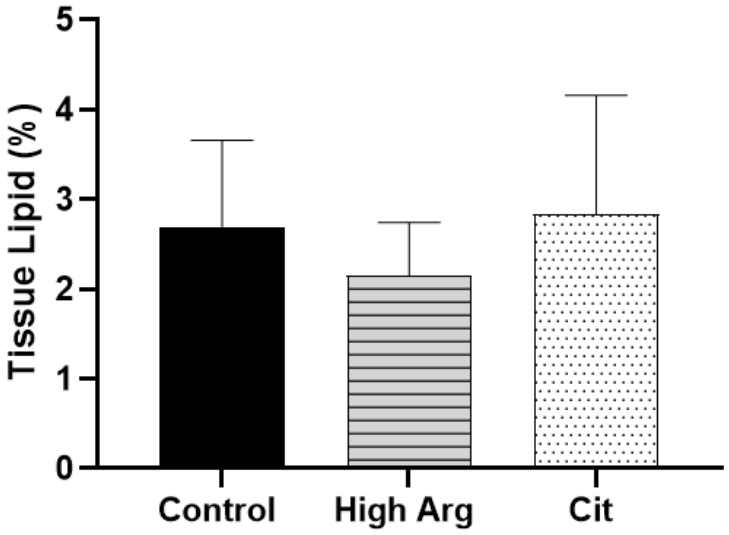

Figure 3.6: Renal lipid composition in percentage of total right kidney weight. Data represent means ± standard deviation in %. All treatments were n = 9. Treatment effect was assessed by one-way ANOVA with a Tukey's multiple comparisons test.

3.2.2 Small Intestine Morphology and Jejunal Histology

Supplemental arginine or citrulline did not elicit a treatment effect on small intestine length, jejunal circumference, mucosal weight or mucosal weight per surface area of the small intestine (Figures 3.7-3.10). Interestingly, piglets fed the parenteral diet containing additional arginine had greater duodenal villus height compared to the control ($P = 0.003$). However, supplemental citrulline did not elicit a similar treatment effect when compared to the control or high arginine treatments (Figure 3.11).

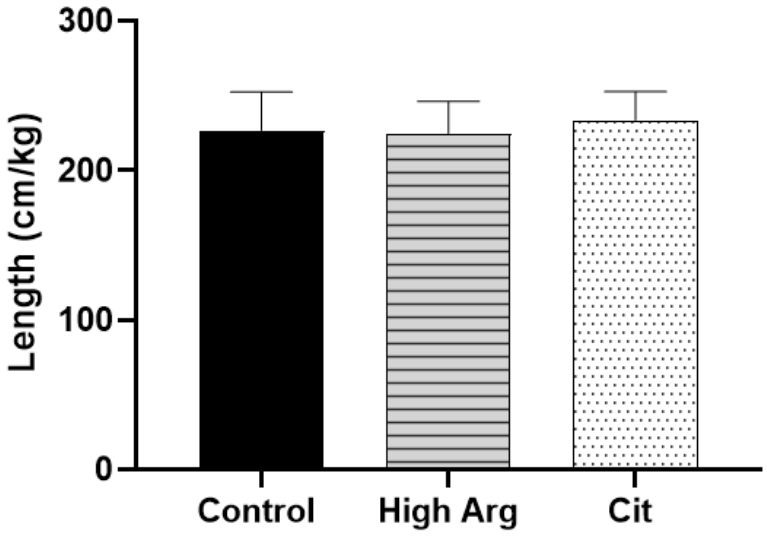

Figure 3.7: Whole small intestine length. Corrected for terminal piglet body weight**.** Data represent means ± standard deviation in cm/kg. All treatments were n = 9. Treatment effect was assessed by one-way ANOVA with a Tukey's multiple comparisons test.

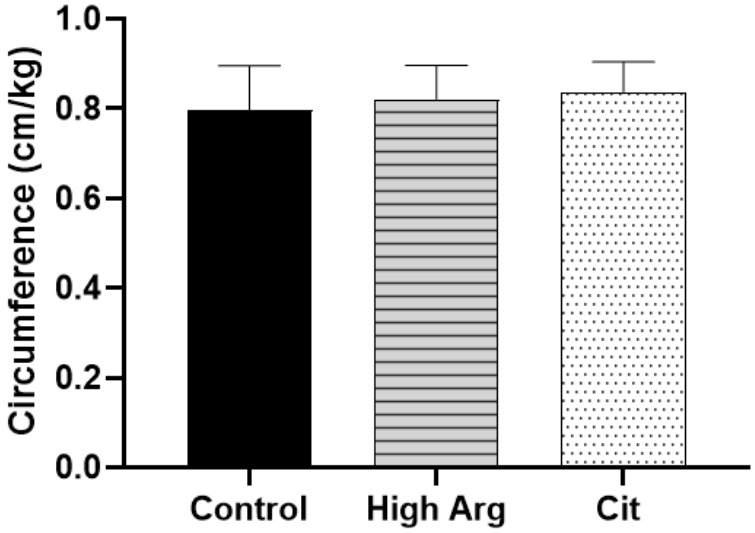

Figure 3.8: Jejunal circumference. Data represent means ± standard deviation in cm/kg. All treatments were n = 9. Treatment effect was assessed by one-way ANOVA with a Tukey's multiple comparisons test

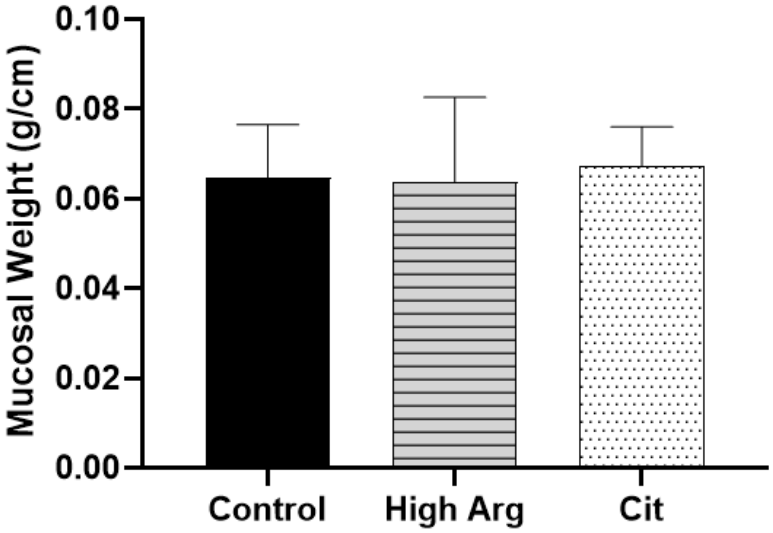

Figure 3.9: Jejunal mucosa weight. Data represent means ± standard deviation in g/cm. Mucosa weight is corrected per cm of small intestine. All treatments were n = 9. Treatment effect was assessed by one-way ANOVA with a Tukey's multiple comparisons test.

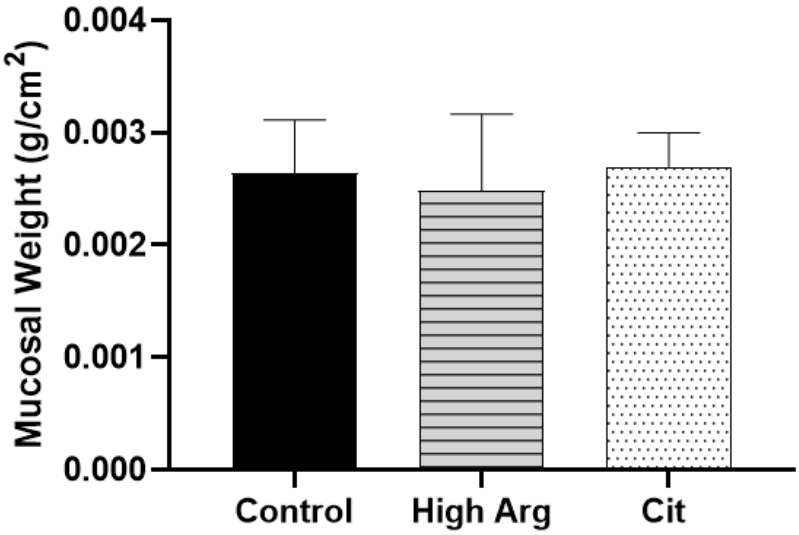

Figure 3.10: Jejunal mucosa weight per surface area. Data represent means ± standard deviation in g/cm^2. All treatments were n = 9. Treatment effect was assessed by one-way ANOVA with a Tukey's multiple comparisons test.

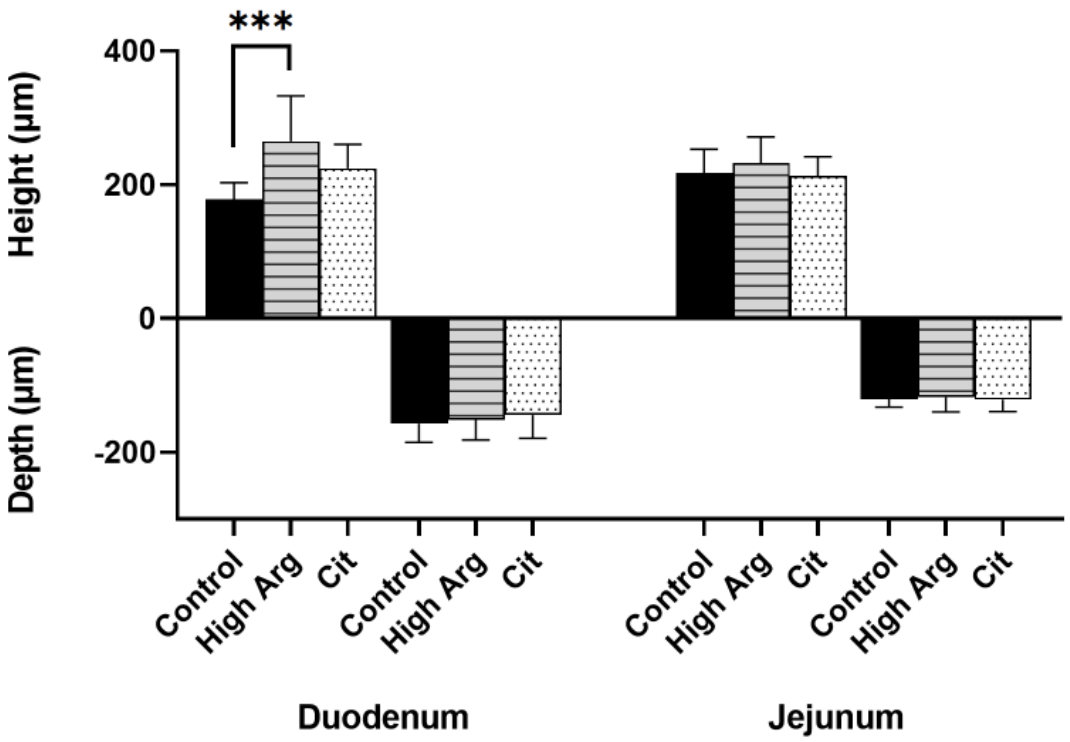

Figure 3.11: Duodenal and jejunal crypt depth and villus height after parenteral feeding. Data represent means ± standard deviation in µm. All treatments were n = 9. Treatment effect was assessed by one-way ANOVA with a Tukey's multiple comparisons test.

3.3 Superior Mesenteric Arterial (SMA) Blood Flow and Terminal Organ Blood Flow Parameters

Supplemental arginine or citrulline did not elicit a treatment effect on blood flow throughout the study nor on terminal blood flow to the gut, kidney or brain (Figure 3.12). However, there was a decrease in blood flow to the gut with parenteral feeding, which was evidenced over the course of the study (Figure 3.13). Parenteral feeding resulted in an approximately 38% reduction in blood flow to the gut, albeit not different among treatments (Figure 3.14).

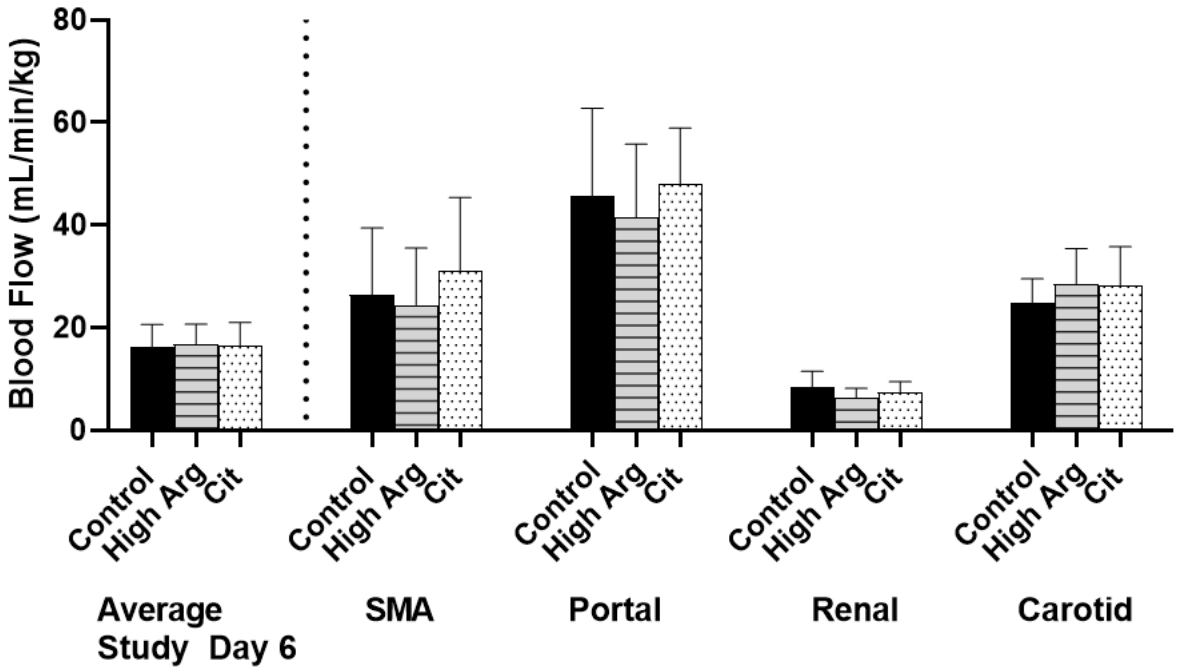

Figure 3.12: Terminal and organ blood flow values. Corrected for terminal piglet body weight. Data represent means ± standard deviation in mL/min/kg. All treatments were n = 9. Treatment effect was assessed by one-way ANOVA with a Tukey's multiple comparisons test.

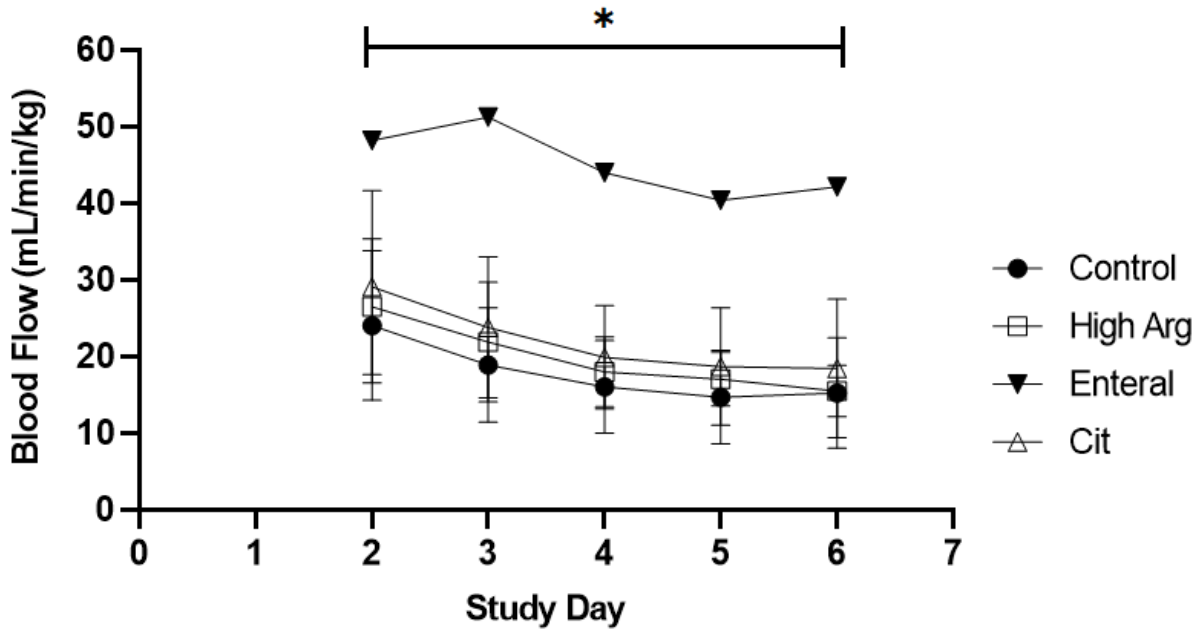

Figure 3.13: Superior mesenteric artery blood flow. Measured over the course of a five-day parenteral feeding regimen. Corrected for terminal piglet body weight. Data represents means ± standard deviation in mL/min/kg. All treatments were n = 9. Treatment effect was assessed by mixed-effect model to assess interactions between treatment and over time. There were no significant differences among treatments ($P > 0.05$) but there was a significant effect of time and of interaction between superior mesenteric blood flow and time ($p=0.0008$).

Since the current study did not contain an enteral treatment group, an enteral control treatment from similar study conducted in the lab was used as a visual representation only (White, 2022). No statistical analysis was performed between the enteral treatment and the treatment groups from the current study.

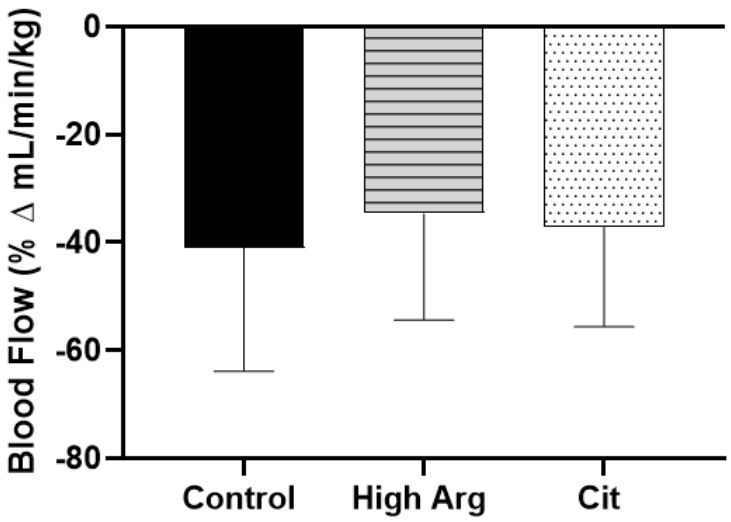

Figure 3.14: Initial and terminal blood flow difference. Measured in the superior mesenteric artery and corrected for terminal piglet body weight. Data represent means ± standard deviation in mL/min/kg. All treatments were n = 9. Treatment effect was assessed by one-way ANOVA with a Tukey's multiple comparisons test.

3.4 Plasma and Tissue Free Metabolites

3.4.1. Central Plasma and Tissue Free Amino Acid Concentrations

Supplemental arginine and citrulline resulted in higher central plasma arginine concentration compared to the control ($P = 0.0006$, $P = 0.017$, respectively) (Table 3.2; Figure 3.15). Supplemental citrulline resulted in increased plasma citrulline concentration compared to control and arginine supplementation in central circulation ($P < 0.0001$ for both comparisons) (Table 3.2; Figure 3.16). Further, plasma ornithine was elevated with supplemental arginine and citrulline compared to control ($P < 0.0001$, $P = 0.0005$, respectively) (Table 3.2).

With regards to free amino acid concentrations in the liver, there was a treatment effect among all three treatments. Supplemental citrulline had the highest amount of free citrulline in the liver, while supplemental arginine increased citrulline above control but less than citrulline supplementation ($P < 0.0001$ Cit vs High Arg, $P < 0.0001$ Cit vs Control, $P = 0.005$ High Arg vs Control) (Table 3.3). Further, high arginine increased liver free ornithine to the greatest extent compared to the Control ($P = 0.023$) (Table 3.3). Interestingly, supplemental citrulline led to higher levels of free histidine, threonine and isoleucine in the liver compared to control and high arginine (overall $P = 0.0051$, $P < 0.0001$, $P = 0.0015$, respectively) (Table 3.3).

Table 3.2: Plasma amino acid concentration after 5 days of treatment diets

	Control	High Arginine	Citrulline	P-Value
Arginine-related Amino Acids (µmol/L)				
Arginine	**155 ± 42[b]**	**340 ± 78[a]**	**281 ± 127[a]**	**P = 0.0007**
Citrulline	**70 ± 13[b]**	**83 ± 16[b]**	**420 ± 141[a]**	**P < 0.0001**
Glutamic Acid	211 ± 96	239 ± 83	205 ± 101	P > 0.05
Glutamine	279 ± 51	261 ± 54	272 ± 10	P > 0.05
Proline	595 ± 120	772 ± 315	866 ± 525	P > 0.05
Ornithine	**271 ± 57[b]**	**549 ± 90[a]**	**472 ± 147[a]**	**P < 0.0001**
Essential Amino Acids (µmol/L)				
Histidine	70 ± 16	70 ± 38	83 ± 53	P > 0.05
Threonine	237 ± 61	276 ± 241	504 ± 589	P > 0.05
Valine	458 ± 73	412 ± 54	421 ± 189	P > 0.05
Methionine	74 ± 29	67 ± 25	75 ± 56	P > 0.05
Isoleucine	557 ± 50	261 ± 146	247 ± 138	P > 0.05
Leucine	564 ± 125	518 ± 171	551 ± 303	P > 0.05
Phenylalanine	1759 ± 202	1965 ± 1061	2697 ± 2125	P > 0.05
Lysine	735 ± 242	714 ± 210	664 ± 375	P > 0.05
Non-essential Amino Acids (µmol/L)				
Aspartic Acid	65 ± 24	66 ± 33	46 ± 26	P > 0.05
Hydroxyproline	61 ± 8	77 ± 36	80 ± 44	P > 0.05
Serine	772 ± 118	682 ± 108	670 ± 415	P > 0.05
Glycine	996 ± 128	852 ± 126	973 ± 435	P > 0.05
Taurine	238 ± 54	274 ± 102	286 ± 121	P > 0.05
Alanine	810 ± 170	692 ± 160	680 ± 469	P > 0.05
Tyrosine	79 ± 35	135 ± 191	105 ± 68	P > 0.05

Data represent means ± standard deviation in µmol/L. All treatments were n = 9. Reported P-value represents overall treatment effect by one-way ANOVA and a Tukey's post-hoc test (superscript). P-value was deemed significant if $P < 0.05$. Means within rows not sharing a superscript letter represent a significant treatment effect.

Table 3.3: Free amino acid concentrations in liver

	Control	High Arginine	Citrulline	P-Value
Arginine-related Amino Acids (µmol/g)				
Arginine	0.63 ± 0.46	0.50 ± 0.32	0.50 ± 0.26	P > 0.05
Citrulline	**0.30 ± 0.22^{c}**	**0.84 ± 0.41^{b}**	**1.93 ± 0.37^{a}**	**P < 0.0001**
Glutamic Acid	0.70 ± 0.32	0.73 ± 0.33	1.10 ± 0.43	P > 0.05
Glutamine	1.13 ± 0.46	1.38 ± 0.45	1.38 ± 0.43	P > 0.05
Proline	1.63 ± 0.40	2.35 ± 1.27	2.59 ± 0.80	P > 0.05
Ornithine	**0.43 ± 0.13^{b}**	**1.25 ± 1.02^{a}**	**0.96 ± 0.26^{b}**	**P = 0.0003**
Essential Amino Acids (µmol/g)				
Histidine	**0.17 ± 0.05^{b}**	**0.17 ± 0.08^{b}**	**0.34 ± 0.15^{a}**	**P = 0.0051**
Threonine	**0.39 ± 0.37^{b}**	**0.52 ± 0.23^{ab}**	**0.78 ± 0.25^{a}**	**P < 0.0001**
Valine	0.46 ± 0.08	0.45 ± 0.12	0.52 ± 0.12	P > 0.05
Methionine	**0.071 ± 0.02^{b}**	**0.072 ± 0.02^{b}**	**0.108 ± 0.02^{a}**	P > 0.05
Isoleucine	**0.24 ± 0.04^{b}**	**0.29 ± 0.08^{b}**	**0.48 ± 0.21^{a}**	**P = 0.0015**
Leucine	0.79 ± 0.12	0.84 ± 0.20	0.96 ± 0.18	P > 0.05
Phenylalanine	2.00 ± 0.40	1.72 ± 0.37	2.91 ± 2.50	P > 0.05
Tryptophan	1.42 ± 0.44	1.67 ± 0.31	1.74 ± 0.45	P > 0.05
Lysine	2.61 ± 0.58	2.63 ± 0.26	2.82 ± 0.51	P > 0.05
Non-essential Amino Acids (µmol/g)				
Aspartic Acid	1.20 ± 0.94	1.04 ± 0.27	1.02 ± 0.41	P > 0.05
Hydroxyproline	0.23 ± 0.05	0.29 ± 0.07	0.22 ± 0.05	P > 0.05
Serine	2.74 ± 0.75	3.07 ± 1.12	3.76 ± 1.20	P > 0.05
Glycine	2.86 ± 0.61	3.07 ± 0.56	3.17 ± 0.86	P > 0.05
Taurine	5.15 ± 1.61	5.76 ± 2.08	3.64 ± 2.10	P > 0.05
Alanine	1.23 ± 0.60	1.76 ± 0.61	1.57 ± 0.43	P > 0.05
Tyrosine	0.19 ± 0.06	0.22 ± 0.09	0.22 ± 0.11	P > 0.05

Data represent means ± standard deviation in µmol/L. All treatments were n = 9. Reported P-value represents overall treatment effect by one-way ANOVA and a Tukey's post-hoc test (superscript). P-value was deemed significant if $P < 0.05$. Means within rows not sharing a superscript letter represent a significant treatment effect.

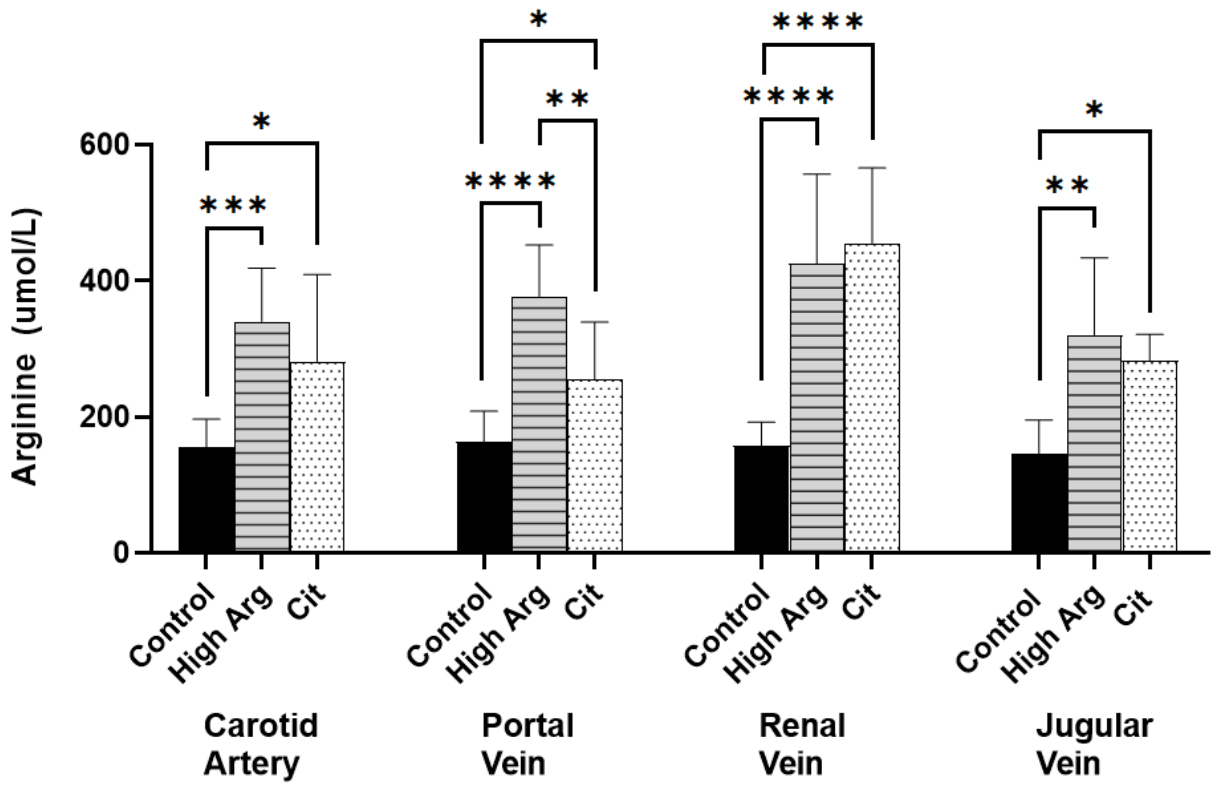

Figure 3.15: Arginine concentration in arterial and venous plasma. Measured after 5 days of treatment diets. Data represent means ± standard deviation in μmol/L. All treatments were n = 9 for carotid artery, portal and renal vein. All treatments were n = 5 for jugular vein. P-value represents overall treatment effect by one-way ANOVA with a Tukey's post-hoc test. P-value was deemed significant if $P < 0.05$. Significance denoted by P = 0.05-0.01 (*), P = 0.01-0.002 (**), P = 0.001-0.0002 (***), $P < 0.0001$ (****).

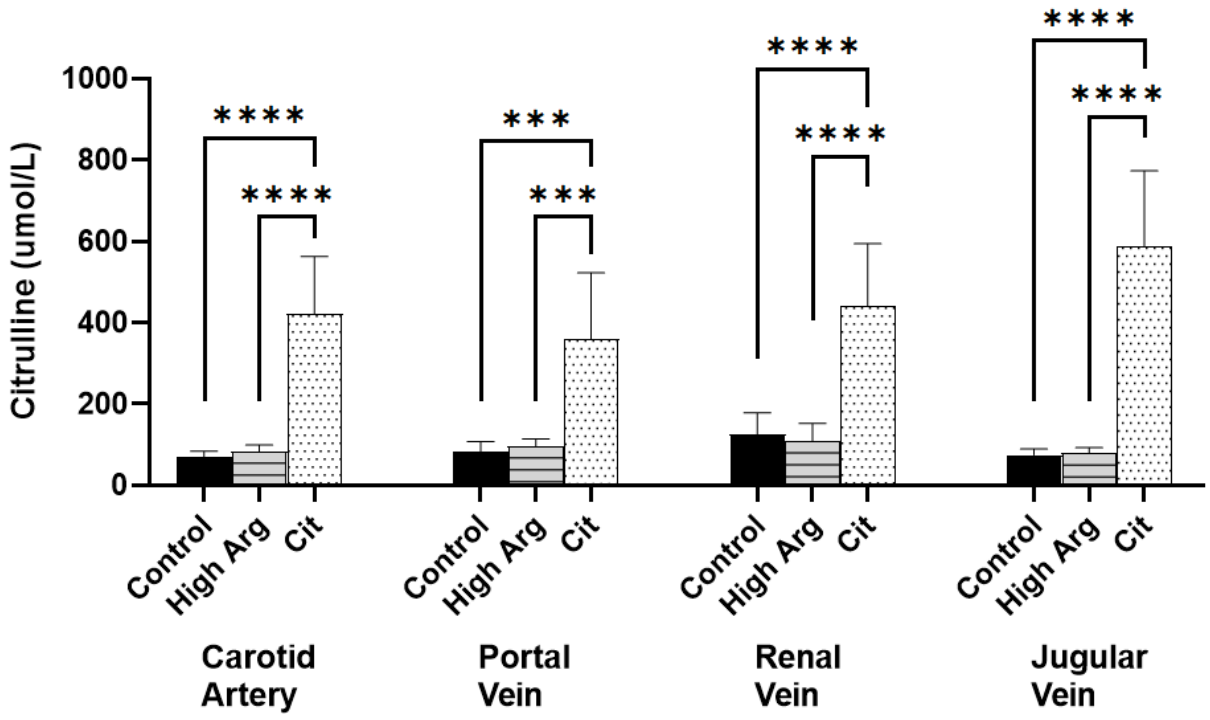

Figure 3.16: Citrulline concentration in arterial and venous plasma. Measured after 5 days of treatment diets. Data represent means ± standard deviation in μmol/L. All treatments were n = 9 for carotid artery, portal and renal vein. All treatments were n = 5 for jugular vein. P-value represents overall treatment effect by one-way ANOVA with a Tukey's post-hoc test. P-value was deemed significant if $P < 0.05$. Significance denoted by P = 0.05-0.01 (*), P = 0.01-0.002 (**), P = 0.001-0.0002 (***), $P < 0.0001$ (****).

3.4.2 Net Organ Balance of Arginine and Citrulline

A treatment effect was evident with regard to arginine balance across the kidney but not the gut or brain. Arginine and citrulline supplementation led to significant arginine release from the kidney, compared to zero (P = 0.023, P = 0.021, respectively) (Figure 3.17). Further, citrulline supplementation increased arginine release from the kidney to a greater extent than arginine supplementation alone and compared to control (P = 0.012, P = 0.012, respectively). Furthermore, there was a treatment effect on arginine balance among organs; citrulline supplementation increased arginine release from the kidneys to a greater extent than from the gut or brain (P = 0.015, P = 0.015 respectively) (Figure 3.17).

No treatment effect regarding citrulline balance was evident across the gut or kidneys. However, citrulline supplementation increased citrulline release from the brain compared to control and supplemental arginine (P = 0.023, P = 0.016, respectively) (Figure 3.18). Citrulline supplementation also resulted in significantly higher citrulline release from the brain compared to the gut (P = 0.006) (Figure 3.18)

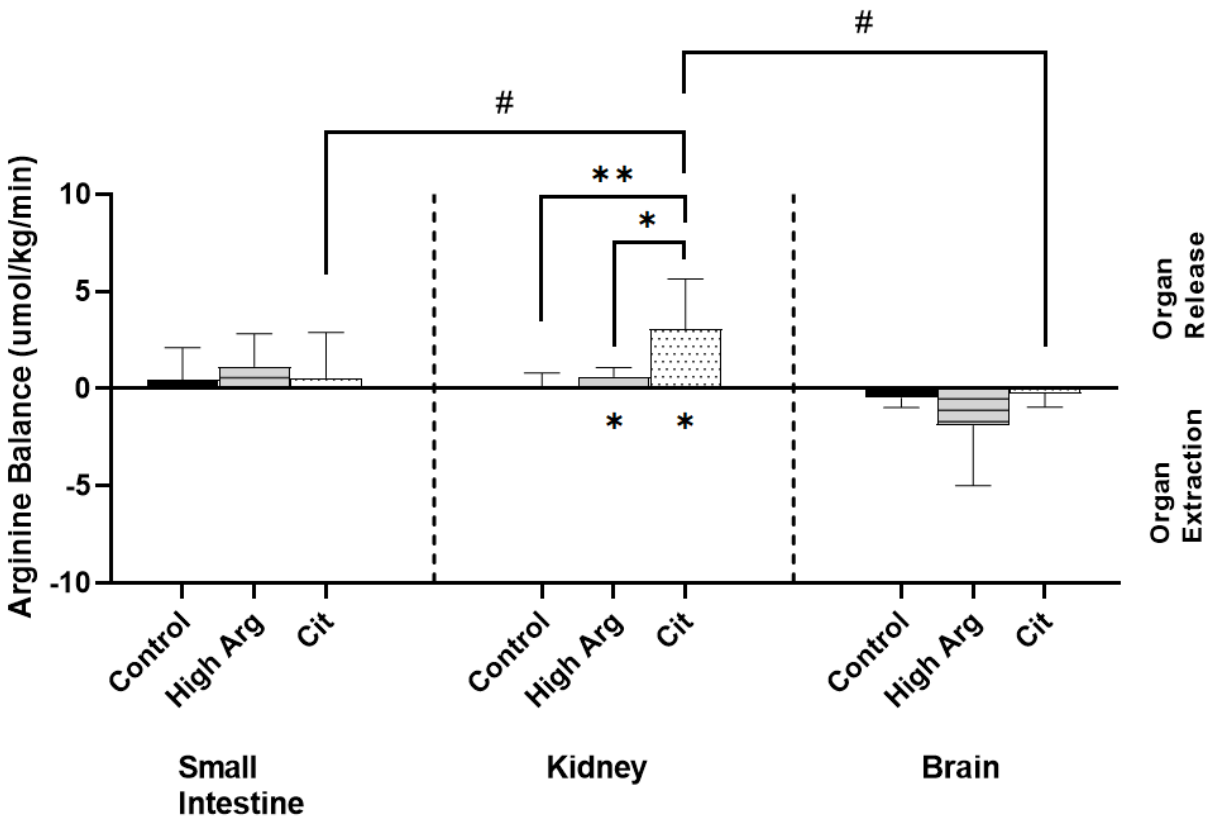

Figure 3.17: Arginine balance across the small intestine, kidney and brain. Data represent means ± standard deviation, corrected for terminal piglet body weight, in µmol/kg/min. Small intestine and kidney balance were n = 9 for all treatments, brain balance was n = 6 for high arginine and citrulline and n = 5 for control treatment. Data were assessed by two-way ANOVA, with organs (P = 0.015), experimental treatment groups (P = 0.0024) and their interaction (P > 0.05) as variables, with a Tukey's multiple comparisons test. Significant treatment effects within organs are denoted by comparator lines and *, while significant treatment effect among organs within a treatment group are denoted by comparison lines and #. * without comparator lines represent a significant net change from zero as was determined by 1-sample *t* test. P-value was deemed significant if P < 0.05

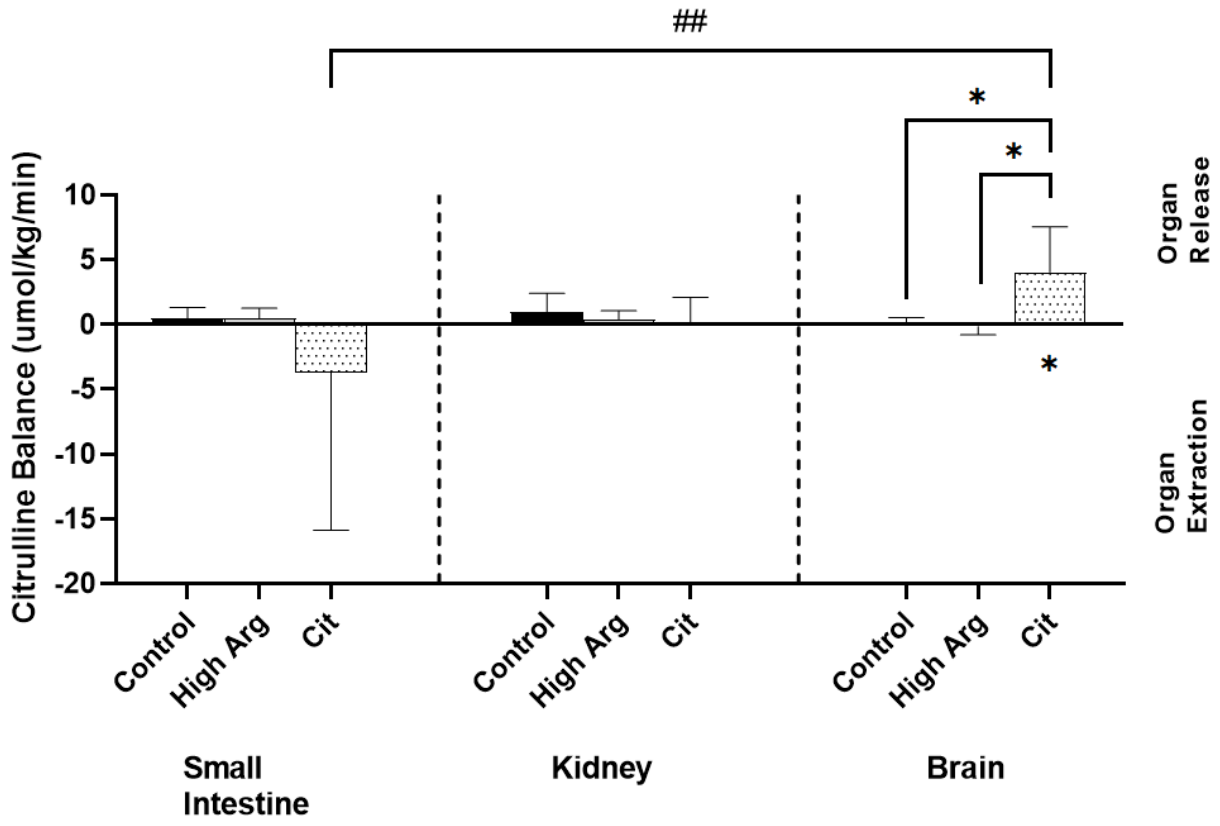

Figure 3.18: Citrulline balance across the small intestine, kidney and brain. Data represent means ± standard deviation, corrected for terminal piglet body weight, in μmol/kg/min. Small intestine and kidney balance were n = 9 for all treatments, brain balance was n = 6 for high arginine and citrulline and n = 5 for control treatment. Data were assessed by two-way ANOVA, with organs (P = 0.014), experimental treatment groups (P = 0.012) and their interaction as variables (P > 0.05), with a Tukey's multiple comparisons test. Significant treatment effects within organs are denoted by comparator lines and *, while significant treatment effect among organ within a treatment group are denoted by comparison lines and #. * without comparator lines represent a significant net change from zero as was determined by 1-sample *t* test.

3.4.3 Plasma and Tissue Free Creatine Concentrations

Regarding plasma creatine concentration, a treatment effect was evident in the supplemental arginine group in the portal vein. Interestingly, portal vein creatine concentration was increased by supplemental arginine compared to the control ($P = 0.014$) (Figure 3.19). Carotid artery, renal and jugular vein plasma creatine concentration did not differ by treatment.

There was also a treatment effect in liver free creatine concentration. However, there was no significant interaction between treatment and gut, brain, or muscle creatine stores. Supplemental arginine increased liver tissue creatine stores more than the control and citrulline treatments ($P = 0.0006$, $P = 0.011$, respectively).

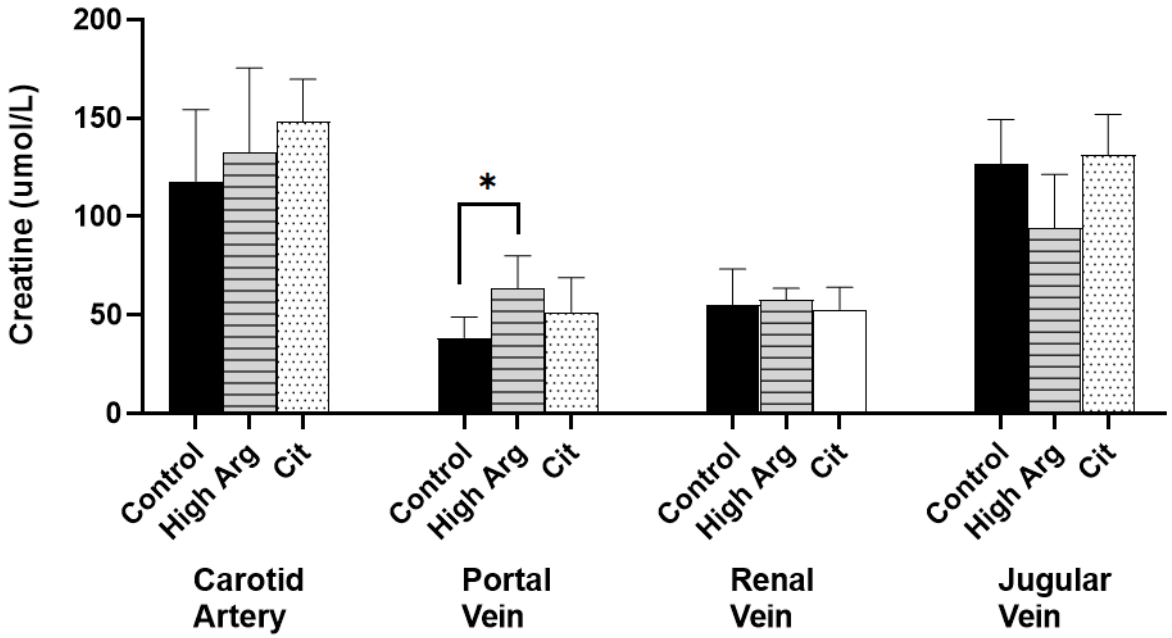

Figure 3.19: Plasma creatine concentration in arterial and venous plasma. Measured after 5 days of treatment diets**.** Data represent means ± standard deviation in μmol/L. Portal and renal vein concentrations were n = 9 for all treatments, jugular vein concentration was n = 6 for high arginine and citrulline and n = 5 for control treatment. Treatment effect was assessed by one-way ANOVA with a Tukey's multiple comparisons test P-value was deemed significant if $P < 0.05$. Significance denoted by P = 0.05-0.01 (*)

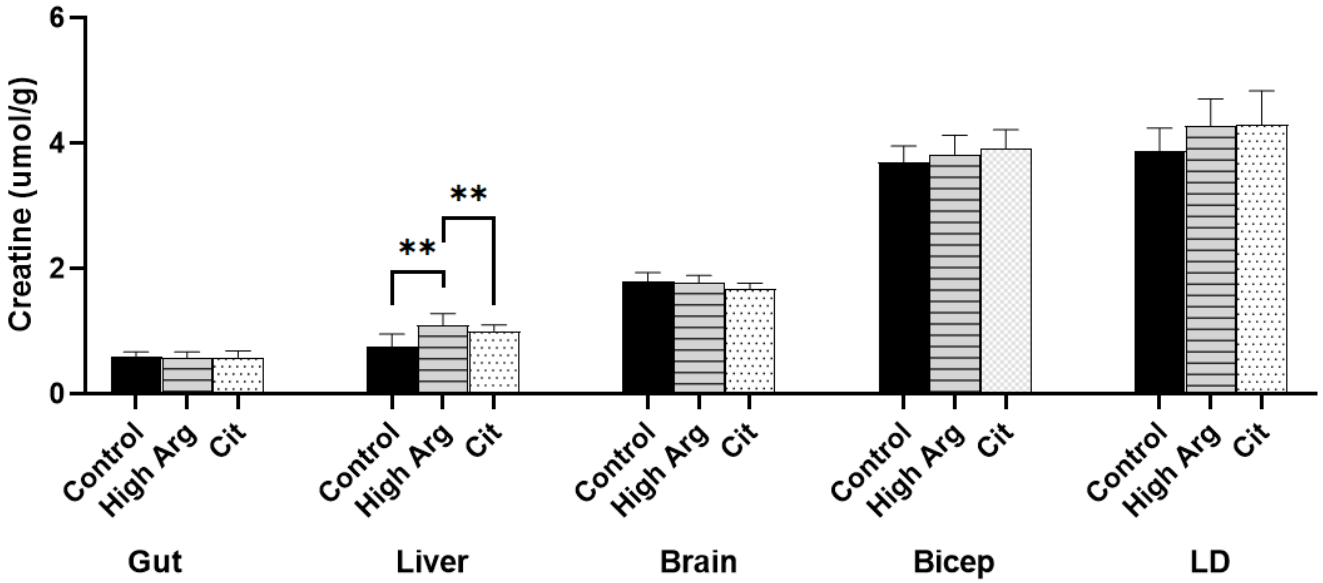

Figure 3.20: Tissue creatine concentration. Data represent means ± standard deviation in µmol/g tissue. All treatment had a n = 9. Treatment effect was assessed by one-way ANOVA with a Tukey's multiple comparisons test. P-value was deemed significant if $P < 0.05$. Significance denoted by P = 0.05-0.01 (*), P = 0.01-0.002 (**)

3.4.4 Net Organ Balance of Creatine, Blood flow and AGAT activity

Creatine balance across the gut differed from zero in all treatment groups. All treatments evidenced a net extraction by gut tissue ($P = 0.015$ Control, $P = 0.021$ High Arg, $P < 0.0001$ Cit) (Figure 3.21) with citrulline supplementation yielding the greatest extraction compared to the control ($P = 0.012$) (Figure 3.21). Creatine balance across the kidney also differed from zero with all treatments ($P = 0.0027$ Control, $P = 0.013$ High Arg, $P < 0.0001$) (Figure 3.21); however, it did not differ among treatments. Creatine balance across the brain differed from zero in only the supplemented arginine treatment. Supplemental arginine significantly increased brain extraction of creatine compared to zero and to control ($P = 0.014$, $P = 0.016$, respectively) (Figure 3.21).

When creatine balance was assessed among organs within a treatment, a greater uptake of creatine was evident in the gut compared to the brain in the control treatment ($P = 0.0059$). Greater uptake of creatine was also evident in the gut compared to the kidneys in both the supplemented arginine and citrulline treatments ($P = 0.019$, $P = 0.0007$, respectively) (Figure 3.21). Lastly, net uptake of creatine in the gut was significantly increased compared to the brain in the citrulline-supplemented treatment ($P = 0.0003$) (Figure 3.21)

Arterial and venous plasma creatine concentration was not correlated to blood flow from the carotid artery, portal and renal vein (Figures 3.22-3.24)

There was no treatment effect with regards to AGAT activity within the kidney (Figure 3.25)

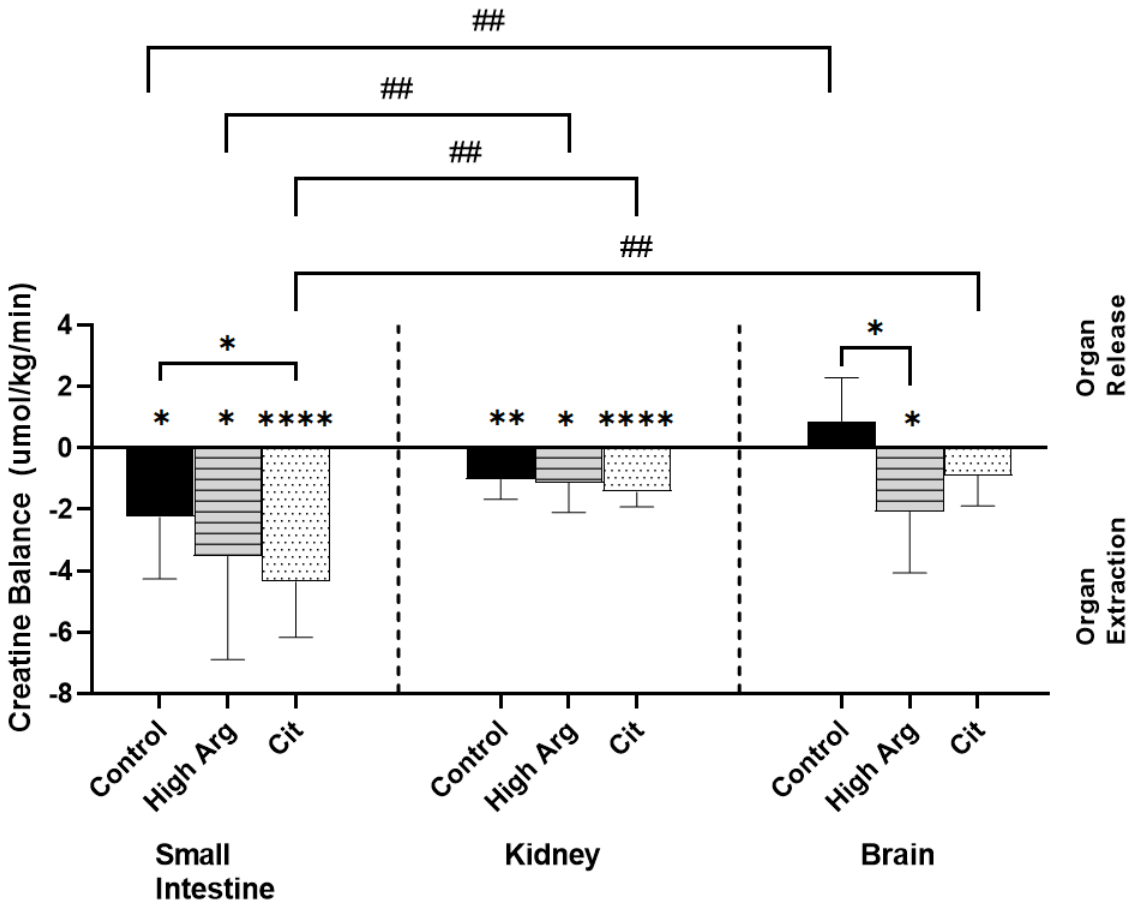

Figure 3.21: Creatine balance across the small intestine, kidney and brain. Data represent means ± standard deviation, corrected for terminal piglet body weight, in µmol/kg/min. Small intestine and kidney balance were n = 9 for all treatments, brain balance was n = 6 for high arginine and citrulline and n = 5 for control treatment. Data were assessed by two-way ANOVA, with organs ($P < 0.0001$), experimental treatment groups ($P = 0.0073$) and their interaction ($P > 0.05$) as variables, with a Tukey's multiple comparisons test. Significant treatment effects within organs are denoted by comparator lines and *, while significant treatment effects among organ within a treatment group are denoted by comparison lines and #. * without comparator lines represent a significant net change from zero as was determined by 1-sample *t* test. Significance denoted by P = 0.05-0.01 (*), P = 0.01-0.002 (**), P = 0.001-0.0002 (***), $P < 0.0001$ (****)

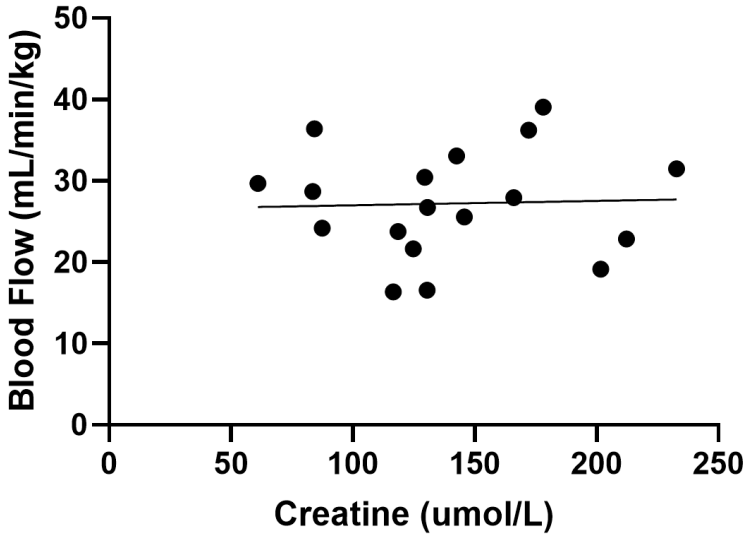

Figure 3.22: Carotid arterial plasma creatine concentration versus blood flow going to the brain. A Pearson correlation was performed between plasma creatine concentration and blood flow. There was no significant correlation between the two variables. r = -0.091, P = 0.72

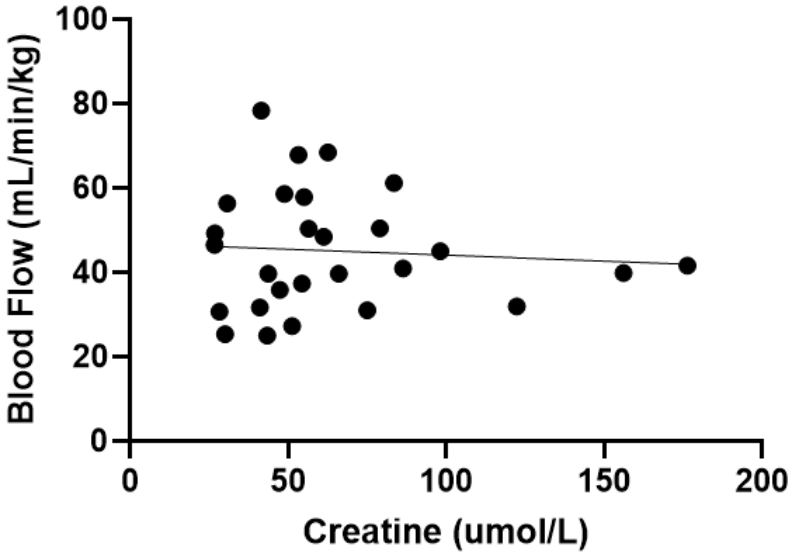

Figure 3.23: Portal vein plasma creatine concentration versus blood flow going to the liver from the gut. A Pearson correlation was performed between plasma creatine concentration and blood flow. There was no significant correlation between the two variables. r = -0.075, P = 0.71

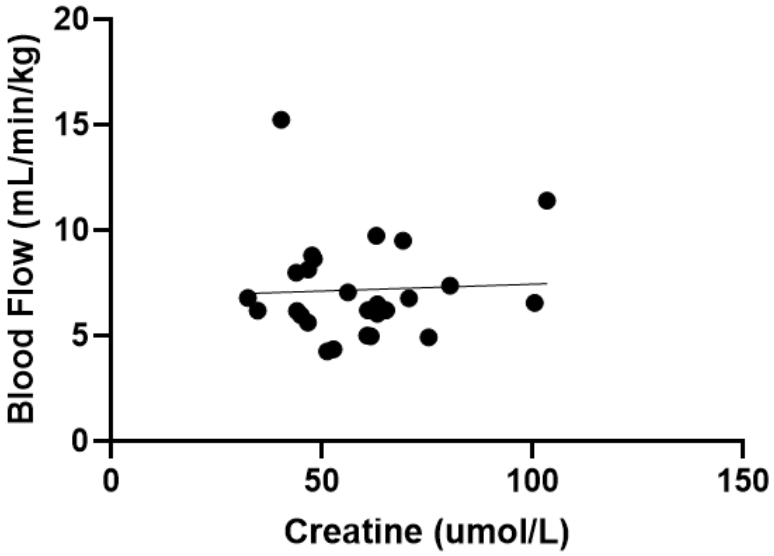

Figure 3.24: Renal vein plasma creatine concentration versus blood flow from the kidney. A Pearson correlation was performed between plasma creatine concentration and blood flow. There was no significant correlation between the two variables. r = 0.048, P = 0.82

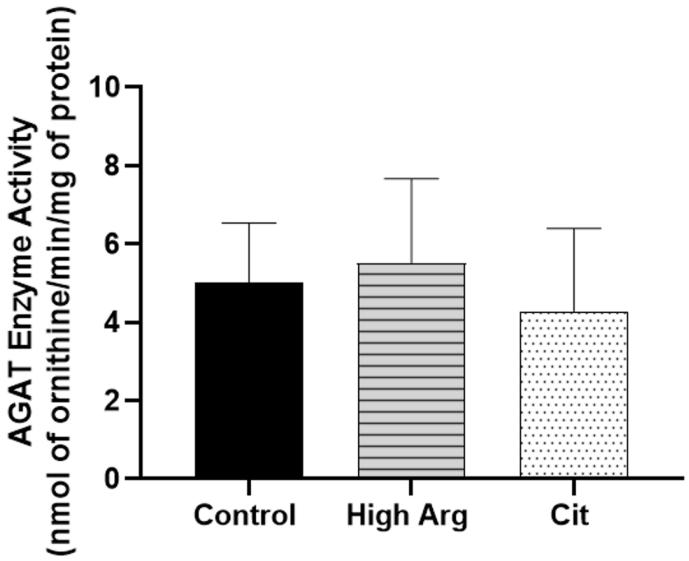

Figure 3.25: AGAT enzyme activity in the kidney. Data represent means ± standard deviation in nmol/min/mg of protein. All treatments were n = 9. Treatment effect was assessed by one-way ANOVA with a Tukey's multiple comparisons test.

3.5 Whole-body Tracer-Tracee Isotope Kinetics

3.5.1 Isotope Kinetics of Key Metabolites Involved in Arginine Metabolism

Supplemental arginine and citrulline lowered isotopic percent enrichment of arginine (M+10) compared to control ($P < 0.0001$, $P = 0.0002$, respectively) (Table 3.4). Additionally, the metabolic flux of arginine (M+10) was significantly higher with arginine and citrulline supplementation, compared to the control ($P < 0.0001$, $P = 0.0015$, respectively) (Table 3.4).

The whole-body plasma pool of GAA did not differ between treatments. However, arginine and citrulline supplementation enhanced the fractional conversion of arginine (M+10) to its product GAA (M+2), compared to the control ($P = 0.0086$, $P = 0.015$, respectively). Additionally, *de novo* synthesis of GAA was enhanced with arginine and citrulline supplementation, compared to control ($P = 0.0013$, $P = 0.015$, respectively) (Table 3.4).

Table 3.4: Isotope and product enrichments, fluxes and fractional conversions of arginine, guanidinoacetate and creatine after 5 days of parenteral feeding

	Control	High Arginine	Citrulline	P-Value
Isotope Enrichment (%)				
Arginine (M+10)	**3.44 ± 0.32[a]**	**2.38 ± 0.30[b]**	**2.63 ± 0.37[b]**	**P < 0.0001**
Guanidinoacetate (M+2)	4.20 ± 1.05	4.41 ± 1.60	4.81 ± 1.27	P > 0.05
Citrulline (M+4)*	1.13 ± 0.22	0.574 ± 0.11	0.593 ± 0.12	-
Product Enrichment (%)				
Guanidinoacetate (M+3)	0.172 ± 0.16	0.290 ± 0.17	0.264 ± 0.14	P > 0.05
Creatine (M+3)	0.251 ± 0.20	0.335 ± 0.49	0.524 ± 0.57	P > 0.05
Citrulline (M+9)*	172.2 ± 14.9	117.3 ± 14.7	127.3 ± 17.0	-
Metabolic Flux (µmol/kg/h)				
Arginine (M+10)	**435 ± 39.4[b]**	**640 ± 75.5[a]**	**581 ± 90.9[a]**	**P < 0.0001**
Guanidinoacetate (M+2)	40.2 ± 9.22	39.6 ± 10.6	35.7 ± 11.1	P > 0.05
Citrulline (M+4)*	305.9 ± 52.0	613.2 ± 134.9	589.8 ± 108.1	-
Fractional Conversion (%)				
Arginine to protein	277.1 ± 5.66	276.0 ± 8.31	279.6 ± 6.91	P > 0.05
Arginine (M+10) to GAA (M+3)	**3.38 ± 1.22[b]**	**12.0 ± 7.02[a]**	**11.6 ± 4.75[a]**	**P = 0.0057**
GAA (M+2) to Creatine (M+2)	82.6 ± 17.7	79.5 ± 20.3	71.5 ± 20.7	P > 0.05
Percent Change from Control (%)				
Arginine to Nitric Oxide*	-	96.4	84.6	-

Data represent means ± standard deviation. Control and citrulline were n = 8, arginine was n = 9. Reported P-value represents overall treatment effect by one-way ANOVA and a Tukey's post-hoc test (superscript). P-value was deemed significant if P < 0.05. Means within rows not sharing a superscript letter represent a significant treatment effect.

* Analytic error during quantification of the citrulline M+4 and M+9 isotopes, discussed further in discussion section "*4.6.1 Whole-body Arginine, citrulline and NO metabolism"*.

Table 3.5: Isotope and product enrichments, fluxes and fractional conversions of phenylalanine and tyrosine after 5 days of parenteral feeding

	Control	High Arginine	Citrulline	P-Value
Isotope Enrichment (%)				
L-Phenylalanine (ring- $^{2}H_5$) (M+5)	8.47 ± 1.26	9.17 ± 1.08	8.35 ± 1.26	P > 0.05
L-Tyrosine (ring-d_4) (M+4)	1.32 ± 0.25	1.47 ± 0.50	1.11 ± 0.33	P > 0.05
L-Tyrosine (ring-3,5-d_2) (M+2)	8.54 ± 1.42	8.29 ± 1.43	8.33 ± 1.30	P > 0.05
Metabolic Flux (µmol/kg/h)				
Phenylalanine (M+5)	220 ± 31.6	200 ± 25.6	224 ± 35.7	P > 0.05
Tyrosine (M+2)	83.5 ± 15.7	86.6 ± 18.9	85.7 ± 16.7	P > 0.05
Phenylalanine Kinetics (µmol/kg/h)				
Phe to Tyr conversion (oxidation)	13.1 ± 3.41	13.7 ± 5.04	11.6 ± 4.22	P > 0.05
Phe to protein synthesis	207 ± 31.4	187 ± 28.4	212 ± 32.01	P > 0.05
Phe from protein breakdown	39.04 ± 31.6	19.6 ± 25.6	42.97 ± 35.7	P > 0.05
Phe balance to protein (accretion)	167.9 ± 3.43	167.3 ± 5.04	169.5 ± 4.22	P > 0.05

Data represent means ± standard deviation. Control and citrulline were n = 8, arginine was n = 9. Reported P-value represents overall treatment effect by one-way ANOVA and a Tukey's post-hoc (superscript). P-value was deemed significant if $P < 0.05$.

Table 3.6: Metabolite fate in whole-body pool in piglets fed experimental parenteral diets for 5 days

	Control	High Arginine	Citrulline	P-Value
Dietary Intake (µmol/kg/h)				
Arginine	210	419	210	-
Citrulline	0	0	280	-
Creatine	0	0	0	-
De novo synthesis (µmol/kg/h)				
Arginine	**161.5 ± 45.1^{b}**	**181.6 ± 52.8^{b}**	**301.4 ± 105.1^{a}**	P = 0.0018
Guanidinoacetate	**1.44 ± 0.54^{b}**	**3.81 ± 1.44^{a}**	**3.20 ± 1.06^{a}**	P = 0.0014
Release from protein break-down (µmol/kg/h)				
Arginine	65.4 ± 52.9	37.3 ± 43.5	71.8 ± 59.6	P > 0.05

Data represent means ± standard deviation. Control and citrulline were n = 8, arginine was n = 9. Reported P-value represents overall treatment effect by one-way ANOVA, and a Tukey's post-hoc test (superscript). P-value was deemed significant if $P < 0.05$. Means within rows not sharing a superscript letter represent a significant treatment effect.

3.5.2 Whole-body Protein Metabolism

Whole-body protein synthesis did not differ among treatments (Figure 3.26-3.28) However, tissue-specific protein synthesis differed by treatment in an organ-dependent manner. Supplemental citrulline resulted in greater gut and liver protein synthesis compared to the control and high arginine treatments ($P = 0.0207$, $P = 0.0085$ respectively for gut, $P = 0.006$, $P = 0.0347$ respectively for liver). Further, citrulline supplementation increased tissue-specific protein synthesis in the kidney, compared to control ($P = 0.0133$) (Table 3.5 & Figure 3.29)

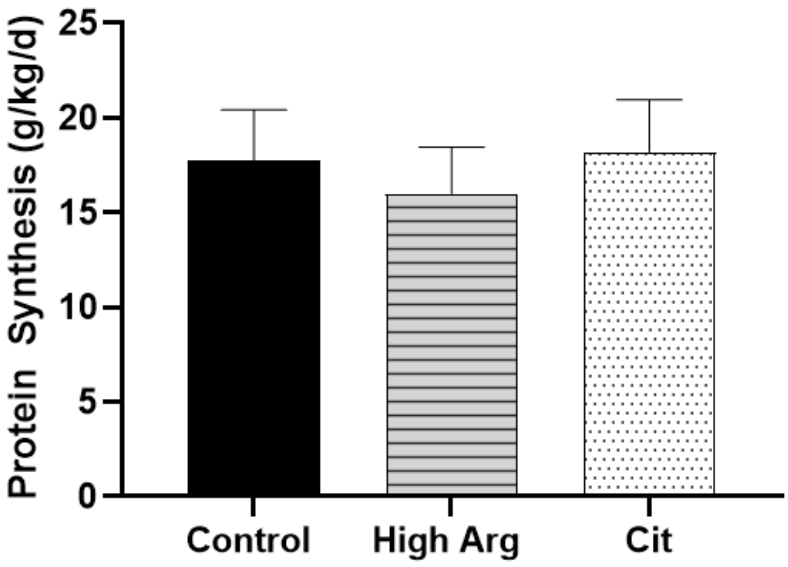

Figure 3.26: Whole-body protein synthesis rates. Established using stable phenylalanine and tyrosine isotope kinetics. Data represent means ± standard deviation in g/kg/d. Control and citrulline were n = 8, arginine was n = 9. Treatment effect was assessed by one-way ANOVA with a Tukey's multiple comparisons test.

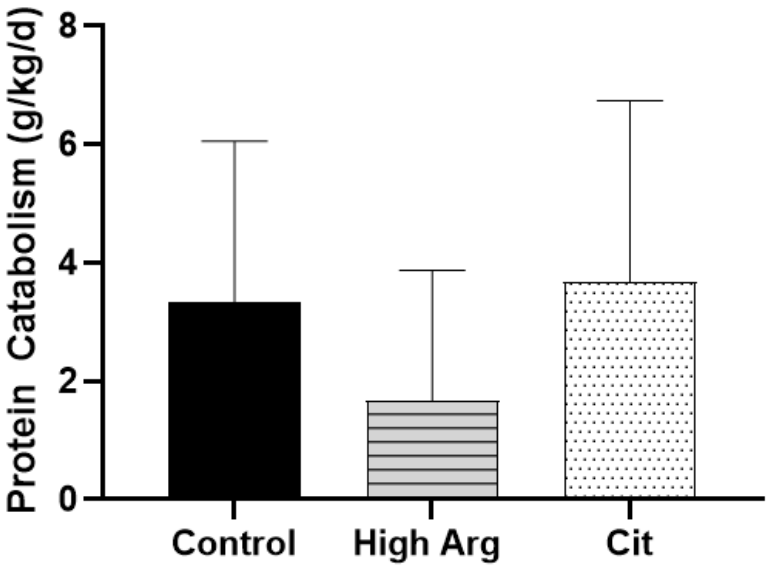

Figure 3.27: Whole-body protein catabolism. Established using stable phenylalanine and tyrosine isotope kinetics. Data represent means ± standard deviation in g/kg/d. Control and citrulline were n = 8, arginine was n = 9. Treatment effect was assessed by one-way ANOVA with a Tukey's multiple comparisons test.

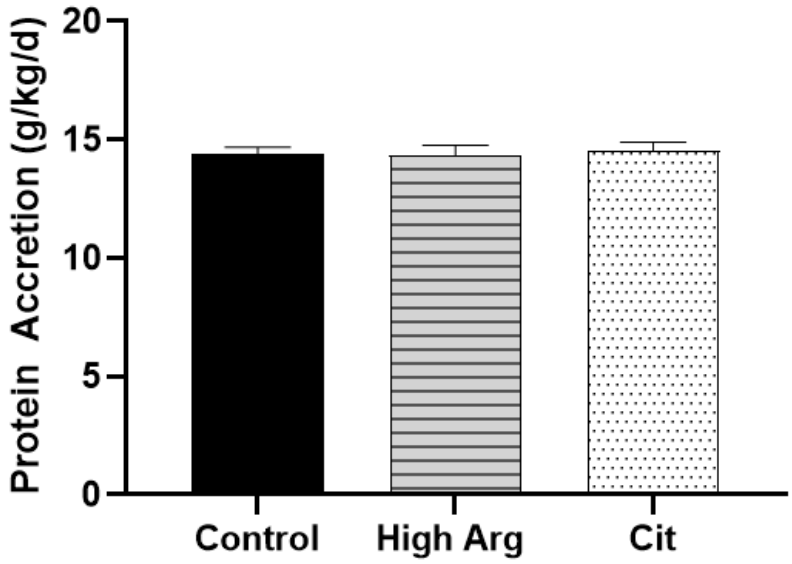

Figure 3.28: Whole-body protein accretion. Established using stable phenylalanine and tyrosine isotope kinetics. Data represent means ± standard deviation in g/kg/d. Control and citrulline were n = 8, arginine was n = 9. Treatment effect was assessed by one-way ANOVA with a Tukey's multiple comparisons test.

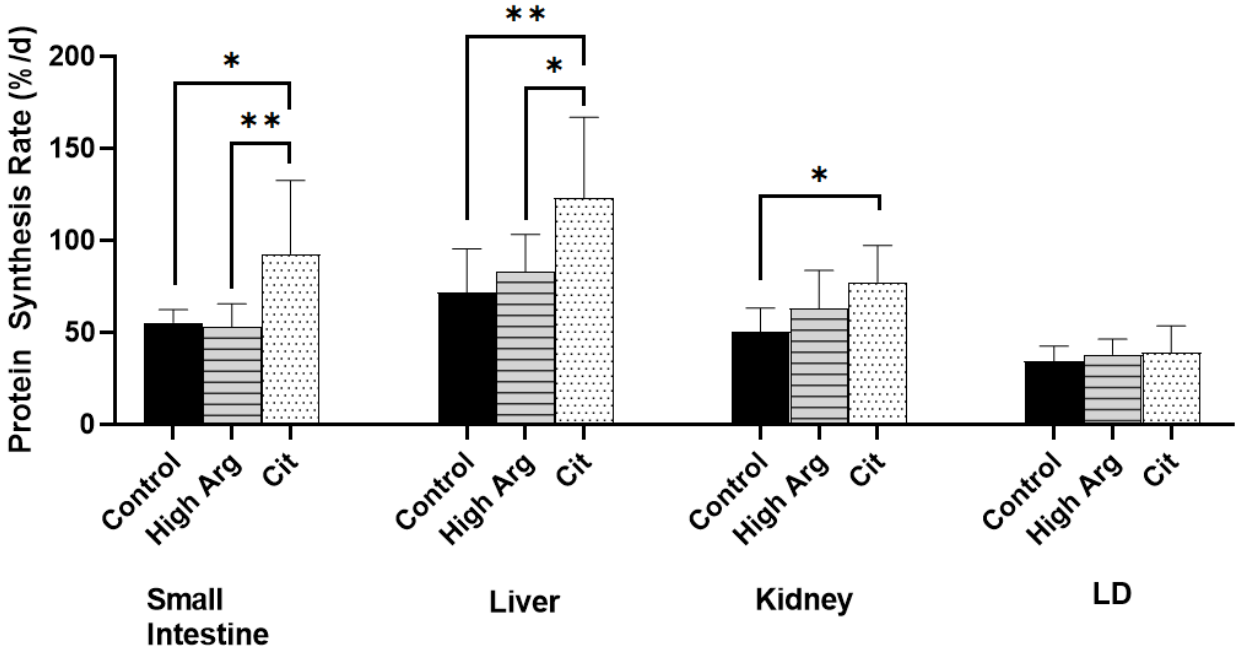

Figure 3.29: Tissue-specific protein synthesis rate. Established using flooding dose phenylalanine isotope kinetics. Data represent means ± standard deviation in % / d. Control and arginine were n = 9, citrulline was n = 8. Treatment effect was assessed by one-way ANOVA with a Tukey's multiple comparisons test. P-value was deemed significant if $P < 0.05$. Significance denoted by P = 0.05-0.01 (*), P = 0.01-0.002 (**)

4.0 Discussion

Parenteral feeding has become essential to managing premature and newborn infants; however, nutrient requirements to optimize growth and development during this sensitive period are relatively unknown. In neonates, exclusive parenteral nutrition results in functional and morphological changes in the gut, leading to a hindered ability to digest and absorb required nutrients and a compromised ability to synthesize arginine (Bertolo et al., 2003). In the current study, we describe metabolic and physiological responses to additional arginine or the addition of citrulline, an arginine precursor, to parenteral nutrition to enhance arginine availability and potentially mitigate some of the adverse consequences of parenteral feeding. The rationale for the study was that if additional arginine and citrulline could enhance arginine availability, the extra arginine would be readily available for nitric oxide, protein, and creatine synthesis. The increased nitric oxide availability may increase blood flow and tissue protein synthesis in the gut, thereby maintaining the physiological and metabolic functioning of the gut and reducing PN-induced gut atrophy. Further, creatine and phosphocreatine are critical for cellular energetics in many tissues, specifically muscles and the brain, and are required for normal neurological development in neonates (Blüml et al., 2013, 2014; Wyss & Kaddurah-Daouk, 2000). Neonatal creatine deficiency or inadequacy in creatine stores have been shown to result in severely impaired psychomotor and neurological functions; thus, sustaining ideal creatine accretion is crucial for neonatal development (Battini et al., 2006; Rostami et al., 2020). Increasing arginine via supplemental arginine or citrulline into parenteral nutrition would maximize arginine availability for creatine synthesis, thereby enhancing creatine availability in parenterally fed piglets. I hypothesized that supplemental arginine and/or citrulline would enhance arginine availability for nitric oxide, protein and creatine synthesis. However, because supplemental arginine is rapidly extracted by the liver

for the urea cycle, whereas citrulline is not, supplementing citrulline as an arginine precursor may prove to be a better agent for increasing systemic arginine availability than additional arginine alone.

4.1 Body Weight and Growth Parameters

Body weight and growth parameters were assessed to determine if potentially increasing arginine availability via supplemental arginine or citrulline was sufficient to impact overall growth. The difference in body weight from study day 0 (surgery day) to study day 6 (necropsy day) was calculated and divided by the total number of study days to determine the daily growth rate. Surgery day and necropsy day weights were also assessed to see if there was any overall weight difference between treatment groups at the beginning and end of the study period. Supplemental arginine and citrulline to the parenteral feeding regimens did not have a significant effect on piglet body weight throughout the course of the study. Although the daily growth rate did not differ among treatments, mean piglet body weight significantly increased throughout the study ($p = 0.0008$). All treatments had a combined average body weight increase of 0.786 ± 0.13 (SD) kg.

4.2 Organ Morphology

Supplementing additional arginine or citrulline to parenteral feeding regimens did not elicit a treatment effect on gross organ weight. However, when gross organ weight was corrected for terminal piglet body weight, a significant treatment effect was evidenced in the kidney and spleen. Piglets receiving supplemental citrulline had significantly heavier kidneys than control and high arginine treatments ($P = 0.038$, $P > 0.0001$, respectively). Renal tissue composition was assessed via tissue-specific protein synthesis, hydration, and lipid composition. Citrulline supplementation

led to greater protein synthesis in the kidneys compared to the control (P = 0.013) but not compared to the high arginine treatment, whereas renal hydration and lipid composition did not differ among treatments. The effects of citrulline on kidney weight were not expected and warrant further evaluation.

Additionally, supplemental arginine reduced overall spleen weight compared to control and citrulline (p = 0.017, p = 0.024, respectively). The spleen is an immunologically important organ responsible for initiating and modulating the innate and adaptive immune system. Thus, the spleen plays a vital role in balancing pro- and anti-inflammatory factors within the body. However, the immunological role of arginine was not assessed within the scope of the study. Supplemental arginine has been shown to increase NOS via iNOS for detoxification of reactive oxygen species (ROS) in inflammatory states, such as sepsis, thus playing a role in improving inflammatory states (Wijnands et al., 2015). Further, splenomegaly can be an indication of increased immune cell production, such as in the case of inflammatory states (Pozo et al., 2009) Hypothetically, since parenteral nutrition alone can upregulate immune cells and produce a proinflammatory state, lower spleen weight in the supplemental arginine treatment may be indicative of an improved inflammatory state compared to the control and citrulline treatments. The role of supplemental arginine in initiating and modulating the immune response in parenterally fed piglets warrants further investigation in future studies.

Regarding gut morphology and functional capacity, additional arginine in parenteral nutrition led to greater villus height but not the crypt depth. In the case of PN-induced gut atrophy, longer villi indicate either increased intestinal recovery or prevention of atrophy via increased blood flow and protein synthesis in the intestines. However, mesenteric blood flow throughout the study and mucosal protein synthesis did not differ in the piglets fed additional arginine or the

control treatment; thus, the data suggest an alternate mechanism may be responsible for the longer villi in the arginine-supplemented piglets. Since the isotope infusion was performed on study day 6 and protein synthesis was measured in % / day it is possible that a period of accelerated protein synthesis occurred prior to the isotope infusion and was therefore not captured within the study window. Further, arginine is also a precursor for polyamines, which promote gut growth and enterocyte development; therefore, polyamine synthesis warrants further investigations in future studies (McCormack & Johnson, 1991).

4.3 Blood Flow and Parenteral Nutrition

When blood flow to the gut was assessed and standardized to body weight, there was no effect of treatment either chronically or acutely, indicating that additional arginine and supplemental citrulline did not increase arginine availability for vasodilation via nitric oxide synthesis. More accurately, supplements did not mitigate the significant reduction in blood flow due to parenteral feeding.

However, overall parenteral feeding resulted in an approximately 38% decrease in blood flow to the gut over the course of the study. The degree of decline in SMA blood flow with parenteral feeding is similar to that published by Niinikoski et al. (2004). Moreover, Burrin et al. found that administering parenteral nutrition chronically for six days reduced blood flow to the portal vein compared to enterally fed piglets (Burrin et al., 2003). The reduction in blood flow further illustrates the detrimental effect of parenteral nutrition on blood flow to and from the gut in the acute and chronic administration periods. Furthermore, treatments did not affect blood flow to the kidneys or brain, as measured at the end of the experiment. The lack of effect of arginine and citrulline on blood perfusion of the gut contrasted with our hypothesis. Unfortunately, there

was an analytical processing error with the citrulline isotope when undergoing quantification at Sickkids. The citrulline isotope enrichments were 100-fold higher than expected, making direct quantification and statistical analysis of arginine M+10 conversion to citrulline M+9 via NOS unreliable. Due to the analytical error the isotope consentrations provided were physiologically implausible and due to time restraints for the study period, the error was not resolved with Sickkids at this time. However, since the data was 100-fold greater for all treatments, the percent change from control was still assessed in the high arginine and citrulline treatment groups. Even though statistical analysis was not performed among treatment groups, supplemental arginine increased NO synthesis by approximately 96.4%, while citrulline increased synthesis by 84.6% from control. The increase in NO by supplemental arginine and citrulline is comparable to results by (Schwedhelm et al., 2008). Schewedhelm et al. noted an increase in NO production indirectly by increasing arginine production, improving the arginine/ADMA ratio and resulting in improved overall endothelial vasodilator function.

Furthermore, in rodent models of hypertension, citrulline supplementation increased the arginine:ADMA ratio and promoted improved endothelial function (Chien et al., 2014; Tain et al., 2015). Unfortunately, ADMA was not measured in the current study; thus, the arginine/ADMA ratio could not be determined, and further investigations are warranted as it may help further understand the current research findings. Even with the apparent increase in NO production via NO synthase, the lack of change in blood flow among treatments was interesting. Since NO has many physiological functions, these results may suggest that the apparent increased NO production with arginine and citrulline supplementation is being utilized for other means. Parenteral nutrition has been shown to promote a systemic pro-oxidant and proinflammatory state; thus, in this study, NO may be shunted and utilized for the detoxification of oxidants as opposed to being utilized as

a vasodilator. As mentioned earlier in the discussion, the role of arginine and citrulline in NO production in inflammatory states warrants further investigations in follow-up studies.

4.4 Plasma and Tissue Free Metabolites

As hypothesized, citrulline supplementation increased plasma citrulline levels above the control and high arginine treatments ($P < 0.0001$, $P < 0.0001$, respectively). Citrulline supplementation also increased plasma arginine concentration to the same extent as arginine supplementation, when compared to the control ($P = 0.017$, $P = 0.0006$, respectively). These data indicated that citrulline, as a precursor for arginine, is an alternative means for increasing systemic arginine availability.

Additionally, plasma ornithine was elevated with supplemental arginine and citrulline compared to the control (plasma $P < 0.0001$, $P = 0.0005$, respectively). However, supplemental arginine elevated free ornithine in the liver (overall $P = 0.0003$)., whereas citrulline supplementation showed similar levels to that of the control treatment. Regulation of OTC (ornithine transcarbamylase) in the liver is dependent on two substrates, ornithine (produced from arginine via arginase) and carbamyl phosphate (produced from ammonia via carbamoyl phosphate synthase-1) (Figure 1.1) (Couchet et al., 2021). However, the rate limiting step in the urea cycle is the production of carbamoyl phosphate via carbamoyl phosphate synthase I (CPS1) (Nitzahn & Lipshutz, 2020). Thus theoretically, if carbamoyl phosphate production rate is held constant, ornithine will accumulate when arginine is supplemented. Interestingly, liver free arginine concentration did not differ among treatments. Since ornithine is an important intermediate in arginine-citrulline metabolism, specifically via arginase flux in the urea cycle, these results indicate that a large proportion of supplemental arginine is being shunted to the liver and broken

down via the urea cycle. In contrast, arginine synthesized from citrulline was not extracted to the same extent for the urea cycle.

Lastly, liver free citrulline differed among all three treatments (overall $P < 0.0001$). Citrulline supplementation led to the greatest liver free citrulline concentration, suggesting that citrulline is not efficiently converted to arginine or utilized by the urea cycle in the liver, thus effectively bypassing liver metabolism. In contrast, arginine supplementation led to the second-highest liver free citrulline, suggesting that supplemented arginine is being converted to citrulline via the urea cycle or NOS in the liver. Because we could not catheterize the hepatic vein, balance of citrulline across the liver was not determined; thus, there is not sufficient evidence to conclude if the citrulline produced in the liver from supplemented arginine is being released into the systemic circulation.

Interestingly, citrulline supplementation yielded the greatest net release of arginine from the kidney compared to control and supplemental arginine ($P = 0.0021$, $P = 0.0124$, respectively). When arginine balance was assessed between organs, citrulline supplementation led to greater arginine release from the kidney compared to the gut and the brain ($P = 0.0145$, $P = 0.0145$, respectively), suggesting that citrulline was not converted to arginine in the gut or brain but was transported to the kidney for conversion to arginine. Although arginine supplementation did increase arginine release from the kidneys to a smaller degree, supplemental citrulline effectively bypassed the liver and was converted to arginine in the kidneys and posed as a more effective precursor for elevating systemic arginine. Furthermore, the lack of local conversion of citrulline to arginine in the gut in the study was interesting and unexpected. ASS and ASL are present in enterocytes of neonates, however, they are less abundant in adults, thus will utilize citrulline to produce arginine in neonates but not adults (Marini et al. 2017). Moreover, Marini et al. (2017)

also demonstrated that the gut-renal axis for citrulline conversion to arginine is intact in young piglets. In the current study, the arginine balance results suggest that arginine synthesis in the neonate follows the adult pattern of inter-organ synthesis, whereby the kidneys synthesized and released arginine into systemic circulation. Thus, PN-induced gut atrophy may further hinders the gut's ability to efficiently convert supplemented citrulline locally to meet whole body arginine availability in neonatal piglets.

Citrulline balance across the gut and kidneys did not differ among treatments. However, citrulline release from the brain was higher compared to the control and supplemental arginine groups ($P = 0.023$, $P = 0.016$, respectively). The greater release of citrulline from the brain, in combination with the higher plasma citrulline concentration in the carotid artery supplying the brain, and lack of arginine extraction or release from the brain with citrulline supplementation, together suggest that citrulline was not utilized as an arginine precursor in the brain and was instead released back into the systemic circulation.

Lastly, although caution must be applied when assessing free amino acid differences in isolation when relating differences to changes in whole body amino acid metabolism, citrulline supplementation resulted in higher free histidine, threonine and isoleucine concentrations in the liver, key amino acids in carnosine, protein, and muscle metabolism (overall $P = 0.0018$ histidine, $P = 0.0145$ threonine, $P = 0.0015$ isoleucine). Citrullines' role in histidine, threonine, and isoleucine metabolism warrants further study.

4.5 Creatine Metabolism and Parenteral Nutrition

As discussed thus far, supplemental citrulline seems to be an effective alternative for increasing systemic arginine, thereby augmenting arginine availability for the plethora of metabolic pathways in which arginine is involved. One such critically important pathway is creatine synthesis. Arterial plasma creatine concentration did not differ among treatments. However, supplemental arginine resulted in greater plasma creatine concentration in the portal vein compared to the control ($P = 0.0135$), which may have led to higher liver creatine concentrations compared to the control and citrulline groups ($P = 0.0006$, $P = 0.0111$, respectively). Together, these data suggest that the amount of arginine supplemented in the high arginine treatment group may have been sufficient to meet the demands of both the urea cycle and creatine synthesis in the liver. At the same time, it suggests that the control level of arginine in PN is not sufficient to maintain creatine synthesis for the piglet. Liver creatine balance could not be assessed in this model; however, such balance data could have helped to understand whether the higher liver creatine was being released into the systemic circulation or utilized within the liver.

Creatine balance across the gut differed from zero in all treatment groups. All treatments evidenced a net extraction by the gut ($P = 0.0153$ Control, $P = 0.0207$ High Arg, $P < 0.0001$ Cit), with citrulline supplementation yielding the greatest creatine extraction, compared to the control ($P = 0.0121$). Dinesh et al. demonstrated that in an acute setting, piglets receiving acute intravenous infusions of arginine had the capacity to synthesize sufficient GAA in the gut, which in turn was converted to creatine and resulted in a significant net release of creatine from the gut (Dinesh et al., 2020). Interestingly, the current study demonstrated the opposite result with regards to creatine balance across the gut, suggesting that PN-induced gut atrophy in our model diminished the gut metabolic capacity to synthesize GAA and creatine, resulting in an increased demand for systemic

creatine supply to meet the metabolic needs of the gut. Since citrulline resulted in even greater gut extraction of creatine, these results may indicate that citrulline is a better precursor for creatine synthesis and availability than arginine supplementation alone. However, analysis of the plasma, tissue, and organ balance of GAA would help to understand the consequences of PN-induced gut atrophy on creatine metabolism in the gut further and, therefore, warrants further investigation.

Creatine balance across the kidney also differed from zero with all treatments ($P = 0.0027$ Control, $P = 0.0126$ High Arg, $P < 0.0001$); however, the creatine extraction by the kidney did not differ among treatments. Similar to the gut, in parenterally fed piglets, the kidneys relied on systemic creatine supply to meet the metabolic demands within the tissue. Additionally, Dinesh et al. illustrated that citrulline is a better precursor for GAA synthesis in the kidneys compared to arginine in an acute setting (Dinesh et al., 2020). In theory, the GAA synthesized in the kidneys via AGAT is then exported to various tissues containing GAMT, primarily the liver, where GAA is converted to creatine. Therefore, theoretically, citrulline may act as a better precursor for creatine synthesis in the liver. However, as mentioned earlier, liver free creatine was higher with arginine supplementation compared to control and citrulline supplementation, and AGAT activity did not differ by treatment, indicating that under chronic conditions, in the current study, arginine may prove to be a better precursor for overall creatine synthesis. Furthermore, supplemental arginine led to increased brain creatine extraction ($P = 0.014$) thus suggesting that arginine may also be a better precursor to supply the brain with sufficient creatine to meet the metabolic needs during a critical point in neonatal development and thereby maintain proper neurodevelopment. When creatine balance was assessed among organs within a treatment, the net uptake of creatine was greater in the gut compared to the brain in the citrulline treatment ($P = 0.0059$). Overall, these data suggest that the creatine produced from arginine is being used by the gut, kidneys, and brain;

however, the creatine produced from citrulline is being shunted only to the gut and kidneys and was independent of blood flow in the vasculature supplying the organ. The implications of arginine-citrulline metabolism with regard to brain creatine status are interesting and warrant further investigations in future studies.

Although plasma, tissue and organ balance of GAA was not analyzed for this thesis, whole-body GAA and creatine synthesis was assessed using isotope kinetics and is discussed further in section "4.6 Whole-body tracer-tracee isotope kinetics".

4.6 Whole-body Tracer-Tracee Isotope Kinetics

By using the difference in the tracer, the labelled isotope (arginine, citrulline, GAA), and tracee, the product produced from the metabolic conversion of the tracer, we were able to calculate the metabolic flux of arginine, citrulline and GAA. Whole-body metabolic flux is the movement of a metabolite in and out of the plasma pool; whereas isotope enrichment is the abundance of isotope within the plasma pool and is affected by the concentration of naturally occurring metabolite within the pool (i.e. more metabolite, less enrichment and vice versa)

4.6.1 Whole-body Arginine, Citrulline and NO Metabolism

The isotopic enrichment of arginine was lower with arginine and citrulline supplementation compared to the control ($P < 0.0001$, $P = 0.0002$, respectively). Conversely, the metabolic flux of arginine (M+10) was increased with arginine and citrulline supplementation, compared to the control ($P < 0.0001$, $P = 0.0015$, respectively). The higher metabolic flux and lower isotope enrichment of arginine indicate that arginine supply was enhanced by both arginine and citrulline supplementation. Further, citrulline supplementation increased *de novo* synthesis of arginine to the

greatest extent, compared to arginine supplementation and control (P = 0.0152, P = 0.002, respectively), demonstrating that citrulline was an effective alternative means for increasing systemic arginine availability.

Due to an analytic error during quantification of the citrulline (M+4) and (M+9) isotopes, the isotopic abundance of citrulline was approximately 100 fold higher than expected; therefore, statistical analysis of the arginine to citrulline isotopic conversion was not performed. However, relative percent change from baseline (control) was used as a proxy to assess indirect NO synthesis. Supplemental arginine and citrulline resulted in a 96.4 and 84.6% increase in NO synthesis compared to control, suggesting that both arginine and citrulline increase arginine availability for NO synthesis via NOS. Although arginine is the predominant precursor for NO production via NOS, Flam et al. demonstrated that citrulline can also be utilized as a precursor for NOS (Flam et al., 2007). Therefore, reassessment of the citrulline isotopic abundance is warranted in future studies to describe the effects of citrulline on NO production more accurately and to draw statistical conclusions. Moreover, the apparent increase in NO production with arginine and citrulline supplementation in conjunction with the lack of treatment effect on blood flow suggests that NO is being utilized in other metabolic pathways.

4.6.2 Whole-body Creatine Synthesis

Isotopic enrichment and metabolic flux of GAA did not differ among treatments, indicating that the whole-body plasma pool of GAA was largely unaffected by arginine and citrulline supplementation. However, interestingly, fractional conversion of arginine to GAA was similarly increased with arginine and citrulline supplementation, compared to control (P = 0.0086, P = 0.0150 respectively) as was *de novo* synthesis of GAA (P = 0.0013, P = 00.0148, respectively).

The lack of treatment effect on plasma GAA pool in conjunction with increased fractional conversion and *de novo* synthesis of GAA with arginine and citrulline suggest that arginine utilization for GAA synthesis was enhanced. Because GAMT is not feedback regulated, it is likely that this higher GAA synthesis led to greater creatine synthesis as well. However, there were insufficient data in the current study to draw any absolute conclusions regarding the effect of arginine and citrulline supplementation in parenteral nutrition on direct whole-body and tissue-specific creatine synthesis, as our isotope label dose was not sufficient to be detected in creatine.

4.6.3 Whole-body Protein Synthesis and Tissue-Specific Protein Synthesis

Whole-body protein synthesis did not differ between treatments but instead differed by treatment in an organ-dependent manner. Supplemental citrulline resulted in greater gut and liver protein synthesis compared to the control and high arginine treatments ($P = 0.0207$, $P = 0.0085$ respectively for gut, $P = 0.006$, $P = 0.0347$ respectively for liver). As opposed to the effects of arginine, citrulline may be maintaining protein synthesis rates (via arginine synthesis) in the gut and liver, thus mitigating some of the repercussions of the use of parenteral nutrition, such as gut atrophy and liver disease. Furthermore, citrulline supplementation increases tissue-specific protein synthesis in the kidney, compared to control ($P = 0.0133$). The increase in protein synthesis in conjunction with the increase in net kidney weight, in theory, may indicate that the kidneys increased in size to compensate for the increased metabolic demand required to convert supplemental citrulline to arginine; however, there are insufficient data to draw absolute conclusions based on the current study.

Lastly, citrulline supplementation led to higher free methionine in liver tissue, compared to the control and supplemental arginine ($P = 0.0037$, $P = 0.0044$, respectively). Methionine is the

only essential sulphur-containing amino acid and plays a crucial role as the methyl donor in transmethylation reactions (Stead et al., 2006). One such transmethylation reactions is methylation of GAA to produce creatine. Further, methionine is also essential for protein synthesis, and adequate methionine is required to maintain adequate protein synthesis. Since citrulline supplementation did not appear to enhance liver tissue creatine compared to arginine supplementation, but increased tissue-specific protein synthesis in the liver, the data suggest a precursor-specific metabolic portioning of arginine. Therefore, the role of citrulline and arginine supplementation on methionine warrants further investigation.

5.0 Conclusion and Future Directions

While parenteral nutrition is life-saving, its impact on growth, organ morphology, blood flow, and metabolic pathways necessitates a deeper understanding for optimization. The current study suggests that supplemental arginine and citrulline to a typical parenteral solution did increase systemic arginine availability. Citrulline supplementation emerged as a particularly intriguing precursor, bypassing liver extraction and increasing arginine synthesis and release by the kidney. Citrulline, as an arginine precursor, thus was an effective alternative means for enhanced systemic arginine availability.

Furthermore, supplemental arginine and citrulline appeared to influence various metabolic pathways differently. Arginine and citrulline appeared to increase arginine availability for nitric oxide (NO). However, their effects on blood flow were not as pronounced as hypothesized, suggesting alternative metabolic fates for the increased NO produced via NOS. Although there was no treatment effect on whole-body protein synthesis, tissue-specific protein synthesis in the gut, liver, and kidneys was increased with citrulline supplementation only, further suggesting that citrulline may be a better mechanism to increase arginine availability. Although the direct effect of NO on blood flow was not evidenced, augmenting systemic NO production may play a role in immune modulation. Benefits may also be more apparent when the gut is more challenged with an inflammatory or bacterial load, such as in necrotizing enterocolitis, whereby the gut becomes inflamed, leading to bacterial invasion, cellular damage and necrosis. In theory, providing disease-specific individualized PN regimens could further augment treatment regimens and the quality of outcomes in neonates.

Creatine metabolism, vital for neurological development, was also influenced by supplementation, with arginine and citrulline showing differential effects on creatine synthesis and

utilization. While arginine seemed to enhance liver creatine synthesis, citrulline showed promise in maintaining gut and kidney function, potentially due to its role in sustaining protein synthesis rates, compared to arginine supplementation, suggesting precursor-specific arginine partitioning within the arginine metabolic cycle.

In conclusion, citrulline proved to be an effective means for increasing systemic arginine availability; however, there was evidence of precursor-specific arginine partitioning within the arginine cycle. Since the increase in NO evidenced with arginine and citrulline did not influence blood flow, future research is needed to fully understand the role of arginine, citrulline and NO regarding the immune response elicited with parenteral feeding. Additionally, due to the complex regulation of inter-organ creatine synthesis, more research is needed to fully understand the implications of arginine and citrulline on systemic creatine production and turnover in parenteral feeding.

6.0 References

Alderton, W. K., Cooper, C. E., & Knowles, R. G. (2001). Nitric oxide synthases: structure, function and inhibition. *The Biochemical Journal*, *357*(Pt 3), 593–615.

Antoniades, C., Demosthenous, M., Tousoulis, D., Antonopoulos, A. S., Vlachopoulos, C., Toutouza, M., Marinou, K., Bakogiannis, C., Mavragani, K., Lazaros, G., Koumallos, N., Triantafyllou, C., Lymperiadis, D., Koutsilieris, M., & Stefanadis, C. (2011). Role of Asymmetrical Dimethylarginine in Inflammation-Induced Endothelial Dysfunction in Human Atherosclerosis. *Hypertension*, *58*(1), 93–98.

Battini, R., Alessandrì, M. G., Leuzzi, V., Moro, F., Tosetti, M., Bianchi, M. C., & Cioni, G. (2006). Arginine:glycine amidinotransferase (AGAT) deficiency in a newborn: early treatment can prevent phenotypic expression of the disease. *The Journal of Pediatrics*, *148*(6), 828–830.

Battini, R., Leuzzi, V., Carducci, C., Tosetti, M., Bianchi, M. C., Item, C. B., Stöckler-Ipsiroglu, S., & Cioni, G. (2002). Creatine depletion in a new case with AGAT deficiency: clinical and genetic study in a large pedigree. *Molecular Genetics and Metabolism*, *77*(4), 326–331.

Bertolo, R. F., & Burrin, D. G. (2008). Comparative Aspects of Tissue Glutamine and Proline Metabolism. *The Journal of Nutrition*, *138*(10), 2032S-2039S.

Bertolo, R. F. P., Brunton, J. A., Pencharz, P. B., & Ball, R. O. (2003). Arginine, ornithine, and proline interconversion is dependent on small intestinal metabolism in neonatal pigs. *American Journal of Physiology. Endocrinology and Metabolism*, *284*(5), E915-22.

Bertolo, R. F. P., Chen, C. Z. L., Pencharz, P. B., & Ball, R. O. (1999). 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. *The Journal of Nutrition*, *129*(5), 1045–1052.

Bidlingmeyer, B. A., Cohen, S. A., & Tarvin, T. L. (1984). Rapid analysis of amino acids using pre-column derivatization. *Journal of Chromatography B: Biomedical Sciences and Applications*, *336*(1), 93–104.

Blüml, S., Wisnowski, J. L., Nelson, M. D., Paquette, L., Gilles, F. H., Kinney, H. C., & Panigrahy, A. (2013). Metabolic maturation of the human brain from birth through adolescence: insights from in vivo magnetic resonance spectroscopy. *Cerebral Cortex (New York, N.Y. : 1991)*, *23*(12), 2944–2955.

Blüml, S., Wisnowski, J. L., Nelson, M. D., Paquette, L., & Panigrahy, A. (2014). Metabolic Maturation of White Matter Is Altered in Preterm Infants. *PLoS ONE*, *9*(1), e85829.

Bray, S. (2017). *Creatine Synthesis and Methionine Partitioning During TPN in Yucatan Miniature Piglets* [Thesis]. Memorial University.

Brosnan, J. T., & Brosnan, M. E. (2007). Creatine: Endogenous Metabolite, Dietary, and Therapeutic Supplement. *Annual Review of Nutrition*, *27*(1), 241–261.

Brosnan, J. T., da Silva, R. P., & Brosnan, M. E. (2011). The metabolic burden of creatine synthesis. *Amino Acids*, *40*(5), 1325–1331.

Brosnan, J. T., Wijekoon, E. P., Warford-Woolgar, L., Trottier, N. L., Brosnan, M. E., Brunton, J. A., & Bertolo, R. F. P. (2009). Creatine Synthesis Is a Major Metabolic Process in Neonatal Piglets and Has Important Implications for Amino Acid Metabolism and Methyl Balance ,. *The Journal of Nutrition*, *139*(7), 1292–1297.

Brosnan, M. E., & Brosnan, J. T. (2016). The role of dietary creatine. *Amino Acids*, *48*(8), 1785–1791.

Burrin, D. G., Stoll, B., Chang, X., Van Goudoever, J. B., Fujii, H., Hutson, S. M., & Reeds, P. J. (2003). Parenteral nutrition results in impaired lactose digestion and hexose absorption when enteral feeding is initiated in infant pigs. *The American Journal of Clinical Nutrition*, *78*(3), 461–470.

Burrin, D. G., Stoll, B., Jiang, R., Chang, X., Hartmann, B., Holst, J. J., Greeley Jr, G. H., & Reeds, P. J. (2000). Minimal enteral nutrient requirements for intestinal growth in neonatal piglets: how much is enough? *The American Journal of Clinical Nutrition*, *71*(6), 1603–1610.

Castillo, L., Beaumier, L., Ajami, A. M., & Young, V. R. (1996). Whole body nitric oxide synthesis in healthy men determined from [15N] arginine-to-[15N]citrulline labeling. *Proceedings of the National Academy of Sciences of the United States of America*, *93*(21), 11460–11465.

Chapman, K. P., Courtney-Martin, G., Moore, A. M., Ball, R. O., & Pencharz, P. B. (2009). Threonine requirement of parenterally fed postsurgical human neonates. *American Journal of Clinical Nutrition*, *89*(1), 134–141.

Chen, K., Pittman, R. N., & Popel, A. S. (2008). Nitric Oxide in the Vasculature: Where Does It Come From and Where Does It Go? A Quantitative Perspective. *Antioxidants & Redox Signaling*, *10*(7), 1185–1198.

Chien, S.J., Lin, K.M., Kuo, H.C., Huang, C.F., Lin, Y.J., Huang, L.T., & Tain, Y.L. (2014). Two different approaches to restore renal nitric oxide and prevent hypertension in young spontaneously hypertensive rats: l-citrulline and nitrate. *Translational Research : The Journal of Laboratory and Clinical Medicine*, *163*(1), 43–52.

Clarke, H., Kim, D.-H., Meza, C. A., Ormsbee, M. J., & Hickner, R. C. (2020). The Evolving Applications of Creatine Supplementation: Could Creatine Improve Vascular Health? *Nutrients*, *12*(9).

Couchet, M., Breuillard, C., Corne, C., Rendu, J., Morio, B., Schlattner, U., & Moinard, C. (2021). Ornithine Transcarbamylase - From Structure to Metabolism: An Update. *Frontiers in Physiology*, *12*, 748249.

Curis, E., Nicolis, I., Moinard, C., Osowska, S., Zerrouk, N., Bénazeth, S., & Cynober, L. (2005). Almost all about citrulline in mammals. *Amino Acids*, *29*(3), 177–205.

de Betue, C. T. I., Garcia Casal, X. C., van Waardenburg, D. A., Schexnayder, S. M., Joosten, K. F. M., Deutz, N. E. P., & Engelen, M. P. K. J. (2017). 24-Hour protein, arginine and citrulline metabolism in fed critically ill children - A stable isotope tracer study. *Clinical Nutrition (Edinburgh, Scotland)*, *36*(3), 876–887.

DeGroot, A. A., Braun, U., & Dilger, R. N. (2019). Guanidinoacetic acid is efficacious in improving growth performance and muscle energy homeostasis in broiler chicks fed arginine-deficient or arginine-adequate diets. *Poultry Science*, *98*(7), 2896–2905.

Dinesh, O. C., Brunton, J. A., & Bertolo, R. F. (2020). 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. *Journal of Nutrition*, *150*(3), 443–449.

Dinesh, O. C., Dodge, M. E., Baldwin, M. P., Bertolo, R. F., & Brunton, J. A. (2014). Enteral arginine partially ameliorates parenteral nutrition-induced small intestinal atrophy and stimulates hepatic protein synthesis in neonatal piglets. *Journal of Parenteral and Enteral Nutrition*, *38*(8), 973–981.

Dinesh, O. C., Kankayaliyan, T., Rademacher, M., Tomlinson, C., Bertolo, R. F., & Brunton, J. A. (2021). Neonatal Piglets Can Synthesize Adequate Creatine, but only with Sufficient Dietary Arginine and Methionine, or with Guanidinoacetate and Excess Methionine. *Journal of Nutrition*, *151*(3), 531–539.

Dodge, M. E., Bertolo, R. F., & Brunton, J. A. (2012). Enteral Feeding Induces Early Intestinal Adaptation in a Parenterally Fed Neonatal Piglet Model of Short Bowel Syndrome. *Journal of Parenteral and Enteral Nutrition*, *36*(2), 205–212.

Edison, E. E., Brosnan, M. E., Meyer, C., & Brosnan, J. T. (2007). Creatine synthesis: production of guanidinoacetate by the rat and human kidney in vivo. *American Journal of Physiology-Renal Physiology*, *293*(6), F1799–F1804.

Featherston, W. R., Rogers, Q. R., & Freedland, R. A. (1973). Relative importance of kidney and liver in synthesis of arginine by the rat. *The American Journal of Physiology*, *224*(1), 127–129.

Flam, B. R., Eichler, D. C., & Solomonson, L. P. (2007). Endothelial nitric oxide production is tightly coupled to the citrulline–NO cycle. *Nitric Oxide*, *17*(3–4), 115–121.

Folch, J., Lees, M., Sloane Stanley, G.H. (1957). A simple method for the isolation and purification of total lipides from animal tissues. *The Journal of Biological Chemistry*, *226*(1), 497–509.

Goldstein, R. M., Hebiguchi, T., Luk, G. D., Taqi, F., Guilarte, T. R., Franklin, F. A., Niemiec, P. W., & Dudgeon, D. L. (1985). The effects of total parenteral nutrition on gastrointestinal growth and development. *Journal of Pediatric Surgery*, *20*(6), 785–791.

Hsu, C.-N., & Tain, Y.-L. (2019). Impact of Arginine Nutrition and Metabolism during Pregnancy on Offspring Outcomes. *Nutrients*, *11*(7), 1452.

Humm, A. (1997). Crystal structure and mechanism of human L-arginine:glycine amidinotransferase: a mitochondrial enzyme involved in creatine biosynthesis. *The EMBO Journal*, *16*(12), 3373–3385.

Ichimori, K., Fukahori, M., Nakazawa, H., Okamoto, K., & Nishino, T. (1999). *Inhibition of Xanthine Oxidase and Xanthine Dehydrogenase by Nitric Oxide NITRIC OXIDE CONVERTS REDUCED XANTHINE-OXIDIZING ENZYMES INTO THE DESULFO-TYPE INACTIVE FORM*.*

Johnson, P. J. (2013). Review of Macronutrients in Parenteral Nutrition for Neonatal Intensive Care Population. *Neonatal Network*, *33*(1), 29–34.

Johnson, P. J. (2014). Review of Micronutrients in Parenteral Nutrition for the NICU Population. *Neonatal Network*, *33*(3), 155–161.

Klinger, J. R., Kadowitz, P. J., Siervo, M., Stephan, B. C. M., Feelisch, M., Bluck, L. J. C., Chapman, K. P., Courtney-Martin, G., Moore, A. M., Ball, R. O., Pencharz, P. B., Samuel, T. K., Gitlin, J. D., Ichimori, K., Fukahori, M., Nakazawa, H., Okamoto, K., & Nishino, T. (2009). Copper and nitric oxide meet in the plasma. *Free Radical Biology and Medicine*, *274*(4), S71–S79.

Lamarre, S. G., Edison, E. E., Wijekoon, E. P., Brosnan, M. E., & Brosnan, J. T. (2010). Suckling rat pups accumulate creatine primarily via de novo synthesis rather than from dam milk. *The Journal of Nutrition*, *140*(9), 1570–1573.

Lamarre, S. G., Saulnier, R. J., Blier, P. U., & Driedzic, W. R. (2015). A rapid and convenient method for measuring the fractional rate of protein synthesis in ectothermic animal tissues using a stable isotope tracer. *Comparative Biochemistry and Physiology Part - B: Biochemistry and Molecular Biology*, *182*, 1–5.

Majdeddin, M., Braun, U., Lemme, A., Golian, A., Kermanshahi, H., De Smet, S., & Michiels, J. (2020). Guanidinoacetic acid supplementation improves feed conversion in broilers subjected to heat stress associated with muscle creatine loading and arginine sparing. *Poultry Science*, *99*(9), 4442–4453.

Marini, J. C., Agarwal, U., Robinson, J. L., Yuan, Y., Didelija, I. C., Stoll, B., & Burrin, D. G. (2017). The intestinal-renal axis for arginine synthesis is present and functional in the neonatal pig. *American Journal of Physiology - Endocrinology and Metabolism*, *313*(2), E233–E242.

Martí i Líndez, A.-A., & Reith, W. (2021). Arginine-dependent immune responses. *Cellular and Molecular Life Sciences*, *78*(13), 5303–5324.

McBreairty, L. E., & Bertolo, R. F. (2016). The dynamics of methionine supply and demand during early development. *Applied Physiology, Nutrition, and Metabolism*, *41*(6), 581–587.

McBreairty, L. E., McGowan, R. A., Brunton, J. A., & Bertolo, R. F. (2013). Partitioning of [Methyl-3H]methionine to methylated products and protein is altered during high methyl demand conditions in young yucatan miniature pigs. *Journal of Nutrition*, *143*(6), 804–809.

McCormack, S. A., & Johnson, L. R. (1991). Role of polyamines in gastrointestinal mucosal growth. *American Journal of Physiology-Gastrointestinal and Liver Physiology*, *260*(6), G795–G806.

Mori, M., & Gotoh, T. (2004). Arginine Metabolic Enzymes, Nitric Oxide and Infection. *The Journal of Nutrition*, *134*(10), 2820S-2825S.

Mori, M., Gotoh, T., Nagasaki, A., Takiguchi, M., & Miyanaka, K. (1998). Arginine metabolism and nitric oxide production. *Pathophysiology*, *5*, 60.

Morris, S. M. (2007). Arginine Metabolism: Boundaries of Our Knowledge. *The Journal of Nutrition*, *137*(6), 1602S-1609S.

National Research Council. (2012). *Nutrient Requirements for Swine* (7th ed.). National Academic Press.

Niinikoski, H., Stoll, B., Guan, X., Kansagra, K., Lambert, B. D., Stephens, J., Burrin, D. G., Hartmann, B., & Holst, J. J. (2004). Onset of Small Intestinal Atrophy Is Associated with Reduced Intestinal Blood Flow in TPN-Fed Neonatal Piglets. *The Journal of Nutrition*, *134*(6), 1467–1474.

Nitzahn, M., & Lipshutz, G. S. (2020). CPS1: Looking at an ancient enzyme in a modern light. *Molecular Genetics and Metabolism*, *131*(3), 289–298.

Portocarero, N., & Braun, U. (2021). The physiological role of guanidinoacetic acid and its relationship with arginine in broiler chickens. *Poultry Science*, *100*(7), 101203.

Pozo, A. L., Godfrey, E. M., & Bowles, K. M. (2009). Splenomegaly: Investigation, diagnosis and management. *Blood Reviews*, *23*(3), 105–111.

Robinson, J. L., McBreairty, L. E., Randell, E. W., Brunton, J. A., & Bertolo, R. F. (2016). Restriction of dietary methyl donors limits methionine availability and affects the partitioning of dietary methionine for creatine and phosphatidylcholine synthesis in the neonatal piglet. *Journal of Nutritional Biochemistry*, *35*, 81–86.

Rostami, P., Hosseinpour, S., Ashrafi, M. R., Alizadeh, H., Garshasbi, M., & Tavasoli, A. R. (2020). Primary creatine deficiency syndrome as a potential missed diagnosis in children with psychomotor delay and seizure: case presentation with two novel variants and literature review. *Acta Neurologica Belgica*, *120*(3), 511–516.

Samuel, T. K., & Gitlin, J. D. (2006). Copper and nitric oxide meet in the plasma. *Nature Chemical Biology*, *2*(9), 452–453.

Schutzman, D. L., Porat, R., Salvador, A., & Janeczko, M. (2008a). Neonatal nutrition: a brief review. *World Journal of Pediatrics*, *4*(4), 248–253.

Schwedhelm, E., Maas, R., Freese, R., Jung, D., Lukacs, Z., Jambrecina, A., Spickler, W., Schulze, F., & Böger, R. H. (2008). Pharmacokinetic and pharmacodynamic properties of oral L-citrulline and L-arginine: Impact on nitric oxide metabolism. *British Journal of Clinical Pharmacology*, *65*(1), 51–59.

Shen, L.-J., Beloussow, K., & Shen, W.-C. (2005). Accessibility of endothelial and inducible nitric oxide synthase to the intracellular citrulline–arginine regeneration pathway. *Biochemical Pharmacology*, *69*(1), 97–104.

Shulman, R. J. (1988). Effect of different total parenteral nutrition fuel mixes on small intestinal growth and differentiation in the infant miniature pig. *Gastroenterology*, *95*(1), 85–92.

Shulman, R. J. (1993). The piglet can be used to study the effects of parenteral and enteral nutrition on body composition. *Journal of Nutrition*, *123*(2 SUPPL.), 395–398.

Sibal, L., Agarwal, S. C., Home, P. D., & Boger, R. H. (2010). The Role of Asymmetric Dimethylarginine (ADMA) in Endothelial Dysfunction and Cardiovascular Disease. *Current Cardiology Reviews*, *6*(2), 82–90.

Siervo, M., Stephan, B. C. M., Feelisch, M., & Bluck, L. J. C. (2011a). Measurement of in vivo nitric oxide synthesis in humans using stable isotopic methods: A systematic review. In *Free Radical Biology and Medicine* (Vol. 51, Issue 4, pp. 795–804).

Sorenson, R. L., Stout, L. E., Brelje, T. C., Van Pilsum, J. F., & McGuire, D. M. (1995). Evidence for the Role of Pancreatic Acinar Cells in the Production of Ornithine and Guanidinoacetic Acid by l-Arginine. *Pancreas*, *10*(4), 389–394.

Stead, L. M., Brosnan, J. T., Brosnan, M. E., Vance, D. E., & Jacobs, R. L. (2006). Is it time to reevaluate methyl balance in humans? *The American Journal of Clinical Nutrition*, *83*(1), 5–10.

Tain, Y.-L., Huang, L.-T., Lee, C.-T., Chan, J. Y. H., & Hsu, C.-N. (2015). Maternal Citrulline Supplementation Prevents Prenatal NG-Nitro-l-Arginine-Methyl Ester (l-NAME)-Induced Programmed Hypertension in Rats1. *Biology of Reproduction*, *92*(1).

Thompson, G. N., Pacy, P. J., Merritt, H., Ford, G. C., Read, M. A., Cheng, K. N., & Halliday, D. (1989). Rapid measurement of whole body and forearm protein turnover using a [2H5]phenylalanine model. *The American Journal of Physiology*, *256*(5 Pt 1), E631-9.

Tomlinson, C., Rafii, M., Sgro, M., Ball, R. O., & Pencharz, P. (2011). Arginine is synthesized from proline, not glutamate, in enterally fed human preterm neonates. *Pediatric Research*, *69*(1), 46–50.

Urschel, K. L., Shoveller, A. K., Uwiera, R. R. E., Pencharz, P. B., & Ball, R. O. (2006). Citrulline Is an Effective Arginine Precursor in Enterally Fed Neonatal Piglets1,2. *The Journal of Nutrition*, *136*(7), 1806–1813.

Van Pilsum, J. F., Taylor, D., Zakis, B., & McCormick, P. (1970). Simplified assay for transamidinase activities of rat kidney homogenates. *Analytical Biochemistry*, *35*(1), 277–286.

Wallimann, T., Wyss, M., Brdiczka, D., Nicolay, K., & Eppenberger, H. M. (1992). Intracellular compartmentation, structure and function of creatine kinase isoenzymes in tissues with high and fluctuating energy demands: the 'phosphocreatine circuit' for cellular energy homeostasis. *Biochemical Journal*, *281*(1), 21–40.

White, J. (2022). *The effect of glutathione supplementation on intestinal morphology and liver antioxidant status in parenterally fed piglets* [Thesis]. Memorial University.

Wijnands, K. A. P., Castermans, T. M. R., Hommen, M. P. J., Meesters, D. M., & Poeze, M. (2015). Arginine and citrulline and the immune response in sepsis. *Nutrients*, *7*(3), 1426–1463.

Windmueller, H. G., & Spaeth, A. E. (1981). Source and fate of circulating citrulline. *American Journal of Physiology-Endocrinology and Metabolism*, *241*(6), E473–E480.

Wu, G. (2009). Amino acids: metabolism, functions, and nutrition. *Amino Acids*, *37*(1), 1–17.

Wu, G., Jaeger, L. A., Bazer, F. W., & Rhoads, J. M. (2004). Arginine deficiency in preterm infants: Biochemical mechanisms and nutritional implications. In *Journal of Nutritional Biochemistry* (Vol. 15, Issue 8, pp. 442–451).

Wu, G., & Morris, S. M. (1998). Arginine metabolism: nitric oxide and beyond. *The Biochemical Journal*, *336 (Pt 1)*(Pt 1), 1–17.

Wu, G., Ott, T. L., Knabe, D. A., & Bazer, F. W. (1999). Amino acid composition of the fetal pig. *The Journal of Nutrition*, *129*(5), 1031–1038.

Wykes, L. J., Ball, R. O., & Pencharz, P. B. (1993). Development and Validation of a Total Parenteral Nutrition Model in the Neonatal Piglet. *The Journal of Nutrition*, *123*(7), 1248–1259.

Wyss, M., & Kaddurah-Daouk, R. (2000). Creatine and Creatinine Metabolism. *Physiological Reviews*, *80*(3), 1107–1213.

Yang, H., Feng, Y., Sun, X., & Teitelbaum, D. H. (2009). Enteral versus parenteral nutrition: effect on intestinal barrier function. *Annals of the New York Academy of Sciences*, *1165*, 338–346.