Plasma Citrulline Utility and Arginine Synthesis in a Neonatal Short Bowel Syndrome piglet model

by

MARIHAN LANSING

A thesis submitted in partial fulfilment of the requirements for the degree of

Master of Science

MEDICAL SCIENCES- PAEDIATRICS

UNIVERSITY OF ALBERTA

Abstract

Premature infants are at increased risk of short bowel syndrome (SBS), which leads to dependence on parenteral nutrition (PN). While lifesaving, PN support has multiple complications, such as increased risk of sepsis and liver disease. Autonomy from PN is dependent on adaptation of the remnant intestine. Because citrulline (Cit) is synthesized but not utilized in the intestine, measurement in blood has been proposed as a biomarker of intestinal adaptation. However, the neonatal intestine lacks the enzyme *arginase* and has more *arginosuccinase synthase* than later in life, thus the neonatal intestine preferably exports arginine (Arg) rather than Cit. Arg is a semi essential amino acid with critical metabolic roles in nitric oxide production and ammonia detoxification. Previous studies have shown that the intestine is responsible for 60% of Arg synthesis. Thus, our aim was to evaluate the utility of Cit as a biomarker of intestinal adaptation and assess the effect of intestinal resection on Arg synthesis in the neonate.

We used a validated neonatal SBS piglet model, with 75% intestinal resection. In particular, given ileal resection, this model mimics the most common anatomical form of neonatal intestinal failure and has dependency on PN. 80-100% PN was provided to meet piglet nutrient requirements and 20 or 40% enteral nutrition was provided as necessary for a trophic effect to enhance adaptation. In all piglets, intestinal length was measured, plasma or serum amino acids profiles were determined at initiation and termination of the trial and measured using liquid chromatography–mass spectrometry. For assessment of Arg synthesis intragastric stable isotope labelled proline (Pro) and Arg tracers were infused and the plasma isotope enrichments were measured using API 4000 triple quadrupole mass spectrometry;

hence, flux and synthesis were calculated. At termination, jejunal tissue samples were taken to histologically assess microscopic adaptive changes, such as villus height and crypt depth.

In Chapter 3, we determined that plasma Cit levels discriminate between large differences in gut length, hence are able to identify SBS versus the non-resected sham, but do not differentiate adaptation within the anatomical subtypes of SBS studied, with and without ileum, that varied in their adaptive response. Therefore, citrulline does not appear to be a useful as a sensitive biomarker of intestinal adaptation in the neonate.

In Chapter 4, we concluded that SBS piglets without an ileum did not have differences in whole body Arg synthesis from Pro, compared to the non-resected sham. This was despite the sham having 3x longer intestinal length. When correcting the synthesis of Arg from Pro for intestinal length it appears that SBS piglets may be able to upregulate synthesis of Arg in the intestine. This was supported by an increase in the expression of the enzyme *arginosuccinase synthase* in the SBS versus sham piglets.

This research is the first study of Arg synthesis in a neonatal model of SBS, while undergoing PN and partial EN. Preterm neonates are uniquely at risk of SBS and prolonged PN, as it may take months to years for enteral autonomy. This occurs at a time when the enzyme expression within the intestine is geared to participate significantly in whole body Arg metabolism. Hence, we find that assumptions made about the utility of plasma Cit at other ages do not apply to the neonate; and that the intestine may adapt to support whole body Arg synthesis in the SBS neonate. These findings require further confirmation, both in other neonatal experimental models and in neonatal humans. Furthermore, the potential benefits of supplementation of dietary precursors for Arg synthesis in the intestine (both by enteral and parenteral routes) warrant further investigation.

Preface

This thesis is the work of Marihan Lansing, which received ethical approval # AUP00000155 from University of Alberta Faculty of Agriculture, Life and Environmental Sciences Animal Policy and Welfare Committee.

"Nothing in life is to be feared, it is only to be understood.

Now is the time to understand more, so that we may fear less." – Marie Curie

Acknowledgements

I would like to thank my amazing mentors, Dr. Justine Turner, Dr. Paul Wales and Dr. Ron O. Ball, for their continuous guidance and support during my MSc program. Words cannot describe how grateful I am for all their encouraging words, kindness, and patience. They provided a great environment to excel and prosper. Their kindness and passion for research comes through in every encounter. While writing a thesis may be difficult, their intellectual discussions, ability to teach and abundance of knowledge made it simple to learn and understand even the hardest of topics. In their own unique way, they each made achieving this dream a little easier and more enjoyable. I will never forget Dr. Wales famous encouraging cheers: "fantastic!!" Or "like butter", or Dr. Ball's words in frustrating times: "how do you eat an elephant?", reminding me to take it one step at the time. Last but not least, Dr. Turner, always available when I need something, reminding me of the importance of enjoying life by exploring and traveling, continuous guidance, and wonderful encouragement, she truly goes above and beyond. I would also like to thank Dr. Sujata Persad for her guidance as graduate coordinator and for agreeing to Chair the defense examination. I would also like to thank Dr. Doug Korver for partaking as an examiner in my defense.

Of course, a huge thank you to the amazing Pam Wizzard for her help in teaching me all the lab work and assisting whenever possible. Thank you for the many laughs, patience to answer all my questions and always coming to work with a smile, even on weekends. I would like to also acknowledge all the students who assisted in the piglets' around the clock care: David Lim, George Slim, Xi Chen, Petro George, Celeste Lavelle, Mitsuru Muto and Chi Tran. I am also grateful to Dr. Nation for his assistance with piglets when sick and for his expert histology analysis. Also, a big thank you to Charlane Gorsak for the long hours and help during

surgeries, we greatly appreciate it. I would like to also thank all those who helped from the Swine Research and Technology Center, Dr Leanna Grenwich, Jay Willis, Sheila Schaller and others I may have not met for their pre-trial piglet care.

I would like to also acknowledge our collaborators Dr. Paul Pencharz and Dr. Todd Alexander for their help. I would also thank their respective lab associates or students who may have assisted in any way. A special thanks to Mahroukh Raffi for their help in analyzing and understanding the stable tracer data; and Megan Beggs and Justin Lee for their help with qPCR analysis. A big thanks to the pediatrics gastroenterology department for always providing great feedback and guidance for conference presentations: Drs. Mathew Carroll, Jason Silverman, Hien Huynh, Rabin Persad, Eytan Wine, and Jason Yap. Your time and help are greatly appreciated.

I would also like to thank Canadian Institute of Health Research (CIHR) for funding the research, and other institutions who supported me doing my research, including: The Medical Science Graduate Program Scholarship (MSGP) program, the Women and Children's Health Research Institute (WCHRI) and the Alberta Graduate Student Scholarship program. I would like to express my gratitude to University of Alberta, it is an honor to study for an MSc in this great institution. A thank you to Trish Kryzanowski and Mikhaila Skehor, graduate studies administrators in the Department of Pediatrics, who answered all the questions to make this process go smoothly.

Finally, but in no way the least my amazing family who was always there to support me no matter how tough things get. My supportive parents Laila Lansing and Nashaat Lansing, my amazing husband George Slim and his patience and support and as well as that of his family. My wonderful sisters and brothers-in-law Sara and Cheston Darius, and Sandra and Mina Ibrahim.

Finally, it is the smiles from these little guys that make the hard days seem worth it: Gabriel, Thomas, Benjamin, and Anthony.

Table of Contents

Abstract ii
Preface iv
Dedication v
Acknowledgements vi
Table of Contents ix
List of Tables xi
List of Figures and Illustrations xii

CHAPTER ONE: LITERATURE REVIEW 1
1.1 Introduction 1
1.2 Short Bowel Syndrome 3
1.2.1 Causes of SBS in infants: 4
1.2.2 Complications of SBS 4
1.2.3 Management of SBS 6
1.2.4 Outcomes and prognosis 7
1.2.5 Structural Adaptation: 8
1.2.6 Functional Adaptation: 10
1.2.7 Methods used to assess adaptation in human infants 12
1.3 Citrulline as a Biomarker of Intestinal Adaptation 16
1.4 Arginine and Citrulline Metabolism in the Intestine and Beyond 19
1.5 Arginine and Citrulline Metabolism following Intestinal Resection 20
1.6 The piglet models of short bowel syndrome and nutrition 21
1.7 Summary 22

CHAPTER TWO: THE RATIONALE FOR PROPOSED RESEARCH 24
2.1 Objectives 25
2.2 Null Hypotheses 26

CHAPTER THREE: ASSESSMENT OF PLASMA CITRULLINE AS A NON-INVASIVE BIOMARKER FOR INTESTINAL ADAPTATION IN SHORT BOWEL SYNDROME PIGLETS: A MODEL FOR HUMAN NEONATES 27
3.1 Introduction 27
3.2 Methods 28
3.2.1 Parenteral and enteral nutrition (EN) 29
3.2.2 Plasma citrulline 29
3.2.3 Small intestine parameters 29
3.3 Statistics 30
3.4 Results 30
3.4.1 Intestinal Adaptation in SBS piglets 30
3.4.2 Associations between Plasma Citrulline level and Intestinal Adaptation 32
3.5 Discussion 34

CHAPTER FOUR: THE EFFECT OF INTESTINAL RESECTION ON WHOLE BODY ARGININE SYNTHESIS 41
4.1 Introduction 41

4.2 Methods 43
4.2.1 Diet 43
4.2.2 Serum amino acids 46
4.2.3 Stable isotope tracers 46
4.2.4 Calculations 47
4.2.5 Quantitative real time polymerase chain reaction (qPCR) 47
4.2.6 Small Intestinal Measurements 48
4.3 Statistics 48
4.4 Results 48
4.4.1 Piglet 48
4.4.2 Serum Amino Acids 51
4.4.3 Tracers 53
4.4.4 Quantitative PCR 55
4.5 Discussion 56

CHAPTER FIVE: CONCLUSION AND FUTURE DIRECTIONS 61

REFERENCES 68

APPENDIX 83

List of Tables

Table 1: Piglet outcomes data 31

Table 2: Arginine deficient enteral diet composition 45

Table 3: Piglet outcome data 50

Table 4: Day 7 Serum amino acid data of piglets fed arginine deficient diet 52

Table 5: Change in serum amino acids of piglets fed arginine deficient diet 52

Table 6: Whole-body arginine synthesis data 54

Table 7: Quantitative PCR analysis of jejunum tissue 55

Table 8: qPCR enzymes primer and probes sequences 83

Table 9: Day 0 Serum amino acid data in piglets fed arginine deficient diet (μM) 84

Table 10: Day 7 Serum amino acids in piglets fed arginine deficient diet (μM) 85

List of Figures and Illustrations

Figure 1: Arginine and Citrulline metabolism in perinatal mammalian gut (1) 18

Figure 2: Correlation between plasma citrulline (μM) and length (cm) on day 7 (D7). 33

Figure 3: Asymmetric dimethylarginine inhibition on nitric oxide synthase (NOS)..... 67

Chapter One: **Literature Review**

1.1 Introduction

Intestinal failure (IF) is one of the leading causes of mortality and morbidity in infants, especially preterm infants. IF is defined as the inability of the gut to absorb and digest the required nutrients to sustain life or for growth (2). Short bowel syndrome (SBS) is the most common cause of IF in both infants and adult humans (3). Infants, children, and adults with IF depend on parenteral nutrition (PN) for survival. As many as 22 to 52 % of these children will develop life-threatening complications of this PN therapy, such as sepsis or liver disease (2, 4). The ability of the remnant intestine to adapt is key to wean patients off PN. Unfortunately, not all SBS patients achieve enteral autonomy, and therefore, they require lifelong PN therapy or further management such as intestinal transplantation.

The most common etiology of IF in both adult and pediatric populations is short bowel syndrome (SBS), implying loss of gut length. In adults, it has been defined as malabsorption due to a remnant bowel length of less than 200cm (5). Common causes in adults include: cancer, Crohn's disease, vascular diseases and trauma, or complication of other abdominal surgeries (6). In children, and specifically in neonates, it is defined differently, given the variation in gut length with normal growth. Therefore, a neonatal SBS diagnosis is made when the residual bowel length is less than 25% of what is expected for gestational age (7). This is an appropriate definition as many of the causes of neonatal IF and SBS have been associated with premature birth (8). These include: amongst others, necrotizing enterocolitis, congenital disorders like gastroschisis, atresia's and mid-gut volvulus. In Canada, the incidence of SBS in neonates is 24.5 per 100,000 live births, and each year 1.4% of early childhood deaths under 4 years of age are

due to SBS (4). Many of these deaths are directly due to complications of the PN. Finally, IF can also result from loss of gut function, without loss of length, such as with chronic intestinal pseudo-obstruction or mucosal enteropathies (2).

After resection or loss of a diseased portion of intestine, the remaining remnant or viable portion of intestine undergoes a process called adaptation. This is dramatic structural and functional changes that improve the absorptive capacity of the remnant intestine and hence, may overcome the original loss. This process is the only way in which individuals with IF can avoid lifelong PN dependency. The process has been well described in animal models, and although less well described in humans, it does indeed occur (9). Adaptation was once believed to only occur within the first year post-resection surgery; however, recent studies show that the process is ongoing with median time between 2 to 5 years, depending on remnant small intestine and colon length required to achieve enteral autonomy (10).

Adaptation can be sub-divided into structural or functional adaptation. Structural adaptation increases absorption by increasing the intestinal surface through lengthening, luminal dilation, and, most importantly, mucosal cell hyperplasia. Functional adaptation increases absorptive capacity by increasing the number of nutrient transporters, enhancing crypt cell differentiation and increasing transit time to allow more absorption (11).

Currently, the underlying mechanisms and regulators of adaptation that have been studied include; enteral nutrition, gut secretions, hormonal factors, and many others (12-14). One important factor in adaptation is the site of resection and thus remnant intestine length. The presence of the ileum and ileo-cecal valve may enhance adaptation and weaning from PN.

While adaptation is critical to the survival of babies with IF, it is very difficult to measure. What we know about adaptation has primarily been obtained from studies of animals that

included removal of tissues for histological assessment (15, 16). Such an invasive approach is not reasonable for human infants with IF. The question of whether a baby is able to adapt is critical for management of infants with IF, because early listing for intestinal transplantation is lifesaving (2, 17). Therefore, to guide the care of these babies and to determine individual prognosis development, an *in vivo* a non-invasive assessment of adaptation is required. Such a tool would also be useful for clinical trials of new therapies to promote adaptation.

Citrulline (Cit) has been proposed as a biomarker of the adaptation that occurs following intestine resection. Since Cit, a non-essential amino acid, is synthesised solely within the intestine, hence the plasma or serum level is considered to directly reflect the intestinal mucosal mass. However, enzymatic expression relevant to Cit synthesis within the intestine differs with age (1). In neonates, the lack of *arginase* and increased expression of *arginosuccinase synthase* as compared to adults allows for conversion of Cit to arginine (Arg). Arg is a non-essential amino acid that can be synthesized in the body, however in critically ill or premature infants it is conditionally essential, and supplementation is required. The purpose of this thesis was to assess and discuss the limitations of Cit as non-invasive biomarker for the assessment of adaptation following intestinal resection and to specifically assess the intestinal metabolism of both Arg and Cit as it occurs in the setting of SBS. This was undertaken using stable isotope methodology with labelled dietary precursors delivered into the intestine. The possibility of using this methodology as an alternative to assess adaptation was also considered and evaluated.

1.2 Short Bowel Syndrome

Short bowel syndrome (SBS) or loss of gut length can lead to intestinal failure (IF), which is the inability of the gut to absorb and digest the required nutrients to sustain life or growth (2).

The most common etiology of IF is SBS, however, IF can also be a result of loss of intestinal function without loss of length, for example; loss of intestinal function due to mucosal enteropathies, which are a group of diseases that affect the absorptive functional capacity of the intestine (2). Many of these causes of IF and SBS occur predominantly in neonates following premature birth. In Canada, the incidence of SBS among neonates is 24.5 per 100 000 live births (4).

1.2.1 Causes of SBS in infants:

Thirty five percent of neonatal-onset SBS cases are caused by necrotizing enterocolitis (NEC), which is primarily a disease of prematurity and is defined as tissue death in areas of the intestine (7). A multi-centered study in Canada and the United States reported an incidence of NEC of 7% in infants <1,500g with a mortality around 15-30% (18). Other prevalent causes of SBS include: intestinal atresia (congenital absence of areas in the intestine), gastroschisis (congenital abdominal wall defect, where the intestine is protruding outside the body, which is often complicated by NEC and atresia's) or midgut volvulus (twisting of portion of the intestine, which causes strangulation).

1.2.2 Complications of SBS

Complications of SBS adversely impact outcomes, therefore their prevention and management are the cornerstone of SBS treatment. SBS complications can be malabsorption, surgical adverse events, or therapeutic complications of the parenteral nutrition (PN).

Malabsorption is the hallmark of SBS. The initial presentation is usually electrolyte and fluid imbalance and further nutritional deficiencies can occur. Proximal stomas can lead to high fluid and electrolyte loss resulting in electrolyte imbalances, which can be life threatening. Malabsorption of fluids and electrolytes can lead to a total body sodium deficit that impairs

growth (19). Intestine resections also affect growth through protein, carbohydrate, and fat malabsorption as well as micronutrients (iron, zinc) and vitamin (B12, fat soluble) malabsorption. Clinically, diarrhea is observed and this is often associated with troublesome excoriation of the peristomal or perianal area (19). Long term deficiencies of vitamin D, calcium and phosphate may be complicated by altered bone mineralization resulting in osteopenia or osteoporosis (20).

Surgical complications in SBS include anastomotic site stenosis or ulceration. While not a direct surgical complication, the process of adaptation that leads to gut growth in length also leads to dilatation of the gut and this can be complicated by bacterial overgrowth. Ultimately, this exacerbates failure to thrive due to further disruption of fat absorption. Breakdown of malabsorbed carbohydrate by bacteria also occurs. Altogether, this presents with worsening pain, bloating, steatorrhea and ultimately postponing PN weaning (21).

Some other important and life-threatening complications can occur due to therapeutic regimes, includes parenteral nutrition associated liver disease (PNALD). This cholestatic liver disease has been shown to increase mortality risk by 20%. The use of mixed lipids and fish oil has resulted in reduction in mortality from this cause (22). The PN composition has been shown to take part in the pathogenesis of PNALD. Contributors include amino acid excess that lead to liver toxicity, soy lipids rich in omega 6 fatty acids that hinder hepato-biliary transport, and excess carbohydrates which increase insulin levels, thus limiting fatty acid oxidation causing liver steatosis (23). Other major complications of PN include line occlusions, thrombosis, central line infections, and sepsis.

1.2.3 Management of SBS

Following intestinal resection, the management of children with SBS includes three main aims: correction of electrolyte hemostasis and micro and macronutrient deficiencies, enhancement of the potential for intestinal adaptation, and prevention of complications (21). Children with SBS have symptoms of malnutrition, dehydration, and growth failure. Currently a cornerstone of management includes: hydration, parenteral (PN) and enteral nutrition (EN). This ensures early correction of electrolyte balance, and macro/micronutrient status and preservation of growth and development. Current American Society of Parenteral and Enteral Nutrition guidelines recommend initiation of PN within a week if unable to maintain enteral feeds and a minimum of 1.5 g/kg/d protein intake to maintain positive nitrogen balance (24). PN should be customized to patient's needs for maintenance of health, growth, and physical activity. Estimated total PN requirements for infants (in the absence of some oral or enteral nutrition) include: 80 - 110 kcal/kg/, 1g/kg/d lipids, 1.5g/kg/d amino acids to max or 3.5g/kg/d (25).

Enhancing adaptation is crucial for improving prognosis of SBS, as weaning patients off PN is based on the ability of the gut to grow and compensate for the massive functional deficits. Initiation of enteral feeds alone most enhances adaptation by the direct effect of luminal nutrient stimulation (26). Oral or enteral feeds (EN), even if minimal, have been shown to increase adaptation more than PN alone, which in fact can lead to mucosal atrophy. A diet content high in short chain fatty acids or long chain triglycerides also enhances adaptation (27).

A recent therapeutic advance has been the use of exogenous trophic peptides, notably Teduglutide®, a glucagon like peptide-2 (GLP-2) analogue, to promote intestinal adaptation. GLP-2 enhances crypt cell proliferation and inhibits cell death and as such enhances absorption (28, 29). Teduglutide® is approved in Europe in SBS children more than one year old and in

Canada and United States for adult patients (21). Unfortunately, some studies show that the adaptive growth that occurs is reversible if the trophic medication is stopped (30). A large multi center trial of children with SBS between 12 months and 17 years, concluded that Teduglutide® was well tolerated and decreased the patients' requirement for PN (28).

In most patients with SBS the ileum and ileo-cecal valve (ICV) are resected. With loss of the ICV, a barrier that helps prevent translocation of colonic bacteria into the ileum, small bowel bacterial overgrowth can be promoted (21). Currently bacterial overgrowth is treated by empiric use of oral cyclic antibiotics such as metronidazole, clindamycin, ciprofloxacin and neomycin (21)

Given the complexity of SBS and IF, patients are usually managed with a multidisciplinary team approach. Such teams usually include a dietitian or nutrition specialist, social worker, representatives from gastroenterology, neonatology, surgery, transplant, nursing, palliative care, and pharmacy. Multidisciplinary teams for intestinal rehabilitation have been shown to improve morbidity and mortality for children with IF as they allow for early assessments, and intervention (22, 31).

1.2.4 Outcomes and prognosis

Previously, SBS mortality was very significant and up to 50 % of children died from the complications. However, mortality has declined greatly with recent advances in management. Major advances that have reduced mortality are the introduction of omega 3 fatty lipids, which reduced PNALD, and the effective care of multidisciplinary teams to reduce sepsis and improve communication and referral (22). More recently, survival has increased, and mortality is currently reported between 6.7 to 37% (32).

Adaptive growth improves gut function and allows for enteral autonomy. which is the overarching goal of treatment, and recent data shows that up to 70% of infants will demonstrate some adaptation in response to resection (10). Clinically, there are predictors of adaptation that help improve prognosis. Studies have shown enteral autonomy is achieved more often if the underlying aetiology of IF is necrotizing enterocolitis as compared to other aetiologies (10). Other positive predictors of enteral autonomy, or intestinal adaptation, are a longer residual small bowel length, longer colon length and the presence of the ICV. Specifically infants with >50% of small bowel length that is expected for age, regardless of colon length, were able to achieve 80 to 100% enteral autonomy within 2 years (10). Patients that do not achieve enteral autonomy and experience severe complications of IF require referral for intestinal transplantation. Unfortunately, the 5 year survival post-transplant is 58% and an average waiting list time of 59 years (33).

1.2.5 Structural Adaptation:

Structural adaptation includes both macroscopic and microscopic changes. Increasing the length of bowel and its circumference are the macroscopic changes of adaptation; however, the greatest increase in absorptive surface can be viewed microscopically (11). The intestines absorptive power is enhanced by mucosal folds or villi, which increase the surface area available for interaction with digested nutrients. The adaptive process includes lengthening of villi, crypt hyperplasia, gut lengthening, and dilatation (11).

Within the small intestine, the ileum shows the greatest capacity for adaptation following resection. A study performed by Dowling and Booth on rats showed that the ileum mucosa has increased capacity for structural and functional adaptation after 70% resection compared to jejunum (34). In the ileum villus height increase by 53% while it was only 14% in the jejunum

(17,18). Retaining an ileum has also been shown to be important for adaptation in many ways. It is the site for secretion of GLP-2 which enhances adaptation (11, 35, 36). It is therefore important to note that the site of resection (presence or absence of ileum) is as important as the extent of length loss.

Intestinal adaptation following resection is mostly studied using animal models due to the limitations and inconvenience of directly visualizing structural adaptation in humans. Having said that, a limited number of human research studies have been done and found similar changes to animal models. McDuffie et al. observed adaptation in infants by using biopsy sampling during resection surgery and compared them to biopsy sampling during ostomy reversal surgery (37). They concluded that the increase of villus height and crypt depth were the mirror image to that observed in animal models. This conclusion validates the use of animal models to study adaptation further (37). The methods used in this study, such as radiological testing and intestinal biopsy, have multiple disadvantages and limitations for routine clinical use in infants.

In adults, similar conclusions were reached by Buchman et al. when the authors studied the effects of PN on intestinal structural and functional changes (9). In this study, eight healthy adults were hospitalized for three weeks, with total parenteral nutrition (TPN) for 14 days followed by enteral feeding for 5 days. During that time jejunal biopsies where taken from individuals before and after TPN, and after commencing enteral feeds. They observed that following exclusive PN the intestinal mucosa decreased in thickness, cell count per villus decreased, and cellular edema was evident. The biopsies taken following commencement of enteral feeding showed increased cell count/villus and significantly increased villus height. In the study's conclusion, the authors mentioned that the intestinal changes they viewed in previous animal model studies were similar but more noticeable than in this human study, which again

validates the use of animal models for understanding structural and functional intestinal adaptation as a step before human validation (9).

The mucosal hyperplasia observed with intestinal adaptation may not only be a proliferative phenomenon but may also be supported by a decrease in cell death, as has been observed in animal studies (5). However, the findings are inconsistent. For example, Helmrath et al. compared small bowel resected (SBR) mice to sham surgical mice and actually found an increase in apoptotic activity in the remnant ileum. They concluded this was the result of increased cellular turnover and maximum replication (38). Finally, such marked mucosal hyperplasia also requires a significant increase in angiogenesis, or ingrowth of new blood vessels, to support oxygenation of the growing tissues (11, 39).

1.2.6 Functional Adaptation:

The small intestine has many functions that occur across its entire length to a varied degree. Its primary function is the absorption of nutrients, beginning with intraluminal digestion. It also forms a barrier that limits bacteria or gut microflora, which are predominant in the large intestine, from invasion into the body. Finally, the intestine functions to move nutrients along its length, which is essential to the processes of digestion and for site-specific absorption.

The small intestine is anatomically divided into the duodenum, jejunum, and ileum with each segment having special characteristics. The most proximal segment is the duodenum and it contains the duodenal papilla, which is where the pancreaticobiliary secretions are released into the small intestinal lumen. These pancreatic and biliary secretions are essential for digestion. Pancreatic digestive enzymes, such as proteases, amylase, cholesterol esterase and lipases, digest protein, carbohydrates, and fats. Bile salts in contrast are necessary for fat absorption. Following

the action of pancreatic enzymes on nutrients, other enzymes present in the brush border of intestine further break down nutrients. These digested molecules are then taken up by transporters. For example, amylase divides carbohydrates into maltose, which is further hydrolysed in the intestine into glucose that can then be taken up by a Na/ Glucose transporter across intestine epithelium (40-42). It appears these intestinal secretions are also essential for adaptation because animal studies have shown transposition of duodenal papilla distally to the ileum significantly increases its adaptive potential (43-46)

The jejunum, the longest part of the small intestine, has the longest villi which are rich in digestive enzymes and nutrient transporters, and thus, the jejunum has the greatest capacity for absorption (16). The jejunum absorbs nutrients such as carbohydrate, water soluble vitamins and amino acids. In contrast, the ileum has more specialized functions and absorbs bile acids, fat, fat soluble vitamins and vitamin B_{12}. Furthermore, the ileum contains tighter intercellular junctions providing a barrier function that is greater than in the remaining small intestine. This barrier function is enhanced by the ileo-cecal valve, which prevents reflux of bacteria from the colon back into the small intestine (16). The increased rates of sepsis in neonates with SBS suggests that the intestinal permeability increases after resection and causes failure of gut barrier function (5). Following resection, the intestine has slower transit time to improve contact time and thus enhance absorptive capacity (11).

After resection of the intestine the body's absorptive and digestive capacity is greatly disturbed and the body tries to functionally compensate, primarily by increasing nutrient transporters. For example, in animal models it has been well characterized that there are increases in specific nutrient transporters with adaptation, such as the Na/glucose transporter, and Na/H+ exchanger (NHE). The Na/H+ exchanger generates a suitable alkaline environment for

DNA replication, as well as vital sodium and water absorption to limit diarrhea (47, 48). The hydrogen from NHE transporter is also utilized by PepT1 transporter, which transports di and tri-peptides to be digested inside the cell and is then transported to the blood by amino acid transporters (41). Ziegler et al. studied colon biopsies in adult SBS subjects and using similar molecular techniques and demonstrated fivefold increases of peptide transporters in the human subjects (49); however, this approach to understanding functional adaptation is humans has rarely been applied for obvious reasons.

1.2.7 Methods used to assess adaptation in human infants

Given that intestinal adaptation is such a critical process that impacts outcomes following intestinal resection it is important that we are able to measure the process in SBS patients. That being said, practical bed-side methods to assess adaptation are significantly lacking. Most methods to assess adaptation in humans are inconvenient, uncomfortable, or highly invasive. Pediatric studies for intestinal adaptation in particular are limited for that reason. McDuffie et al. performed a study where they were able to obtain biopsy samples from infants and analyse them using H&E stain before and after resection to prove ongoing intestinal adaptation (37). Currently the gold standard for assessing adaptation is intestinal biopsy because it allows for direct observation of the microscopic changes of functional adaptation. However, due to the invasiveness of the procedure, risks outweigh the benefits of repeated biopsies in clinical practice. Intestinal biopsy carries a risk of bleeding or perforation and the risk is increased given the requirement for multiple sampling times. In infants and children this also requires repeated general anaesthetics. Measuring the length or caliber of bowel can often be done during an operation in patients, but again only if further surgeries are required and as such this is not a practical way to follow adaptation. McDuffie used biopsies taken during the initial SBR and

after reversal of an ostomy that was initially created (37). Again, this is a limited approach only for patients with an ostomy who are going back for a second surgery.

Another method currently used in clinical practice is monitoring serial radiographic changes. Radiographic monitoring includes X-ray, computed tomography scan (CT), magnetic resonance imaging (MRI), small bowel follow-through and barium enema. While these methods are currently very useful in diagnosing and managing various diseases and surgical complications of SBS, their disadvantages make them very difficult to use to follow intestinal adaptation. Some of the disadvantages include: radiation exposure, motion artifacts and the high cost, amongst others (50). The radiation exposure carries a risk of cancer development and this may be greater for younger children, particularly when the exposure load of repeated tests is increased over their lifetime (51). A study in the United States concluded that medical CT radiation alone was responsible for 1.5-2 % of cancer cases (51). A single abdominal CT exposes the patient to 3.48 -10mSv of radiation and studies show that cumulative exposure of more than 50mSv increases the risk of cancer development (50-54). For this reason, repeated radiography is particularly inappropriate for pediatric patients. Radiation also carries the risk of fetal malformation due to ionizing radiation exposure and is contraindicated to pregnant women. The more commonly used radiological method for patients with SBS are barium studies. Barium follow-through studies are useful for evaluation of the length and potentially the caliber of the intestine. However, they require preparation for 24 hours in advance and also carry an average of 1.8-2.2 mSv radiation exposure (53, 54).

While it is very difficult to monitor structural adaptation in patients, there are some less invasive ways in which we can assess functional adaptation. The simplest is clinical assessment of the PN required over time to be hydrated, to maintain weight or in children to grow and

increase weight. A more specific method to assess functional absorption is the measurement of fat absorption using a 72- hour fecal fat test (55). To measure fat absorption two variables must be known: the first is fat intake and the second is the fat excreted (or malabsorbed). Fecal samples are collected for 48-72 hours and fat content can be measured using multiple techniques, for example, gravimetric, titration, or newer methods, such as CEM SmartTrac® technology (17). Fecal fat output is measured, and fat intake is calculated based on the known fat concentration in the enteral nutrition received. The amount of fat absorption can be determined using simple calculations of the fat intake minus the output over the time (15). Unfortunately, the 72-hour fecal fat absorption test has significant limitations and the inconvenience of this technique cannot be over looked. This method requires accurate stool collection, which is problematic in infants with diarrhea who are not toilet trained or for older individuals with fecal incontinence (56, 57). The dietary fat intake has to be absolutely accurate and studies have shown that laboratories fail to adjust for incomplete samples (56).

In understanding how difficult it is to monitor and assess intestinal structural adaptation in humans, it becomes apparent why animal models are so commonly used to study the adaptation process. Most of our understanding of the physiological and anatomical changes occurring after small bowel resection has come from animal research. As previously discussed, the intestine undergoes both structural and functional adaptation following resection. These changes can be studied using different techniques. In animals, structural macroscopic changes such as length and weight are usually measured during resection surgery and compared to measurements taken at the end of the study when the animals are euthanized (58). For microscopic structural changes there are a variety of methods to study adaptation. The most commonly used method is specimen sampling and observation under the microscope using

hematoxylin and eosin (H&E) stain. This method allows for observation of change in villi and crypt length, as well as morphological apoptotic changes if different staining and preparation of the specimen is done (38, 58). Staining can also be used to identify specific markers such as Ki-67, which is marker of cell replication, or caspase-3, a marker for cell death or apoptosis (58). Helmrath et al used a different method to view proliferation and apoptosis of cells (38). For proliferation he injected the animals with 5-bromo-deoxyuridine (BrdU-labeling agent) 1 hour before being euthanized. After specimen preparation for routine histology, the replicating cells, which took up the BrdU, could be detected using an anti- BrdU system (38, 59, 60).

Absorption can be measured in animal and human studies using some of the same methods, like fecal fat collection; however, in animal models' absorptive capacity can be further assessed by molecular techniques that are not available in humans without intestinal specimens. This includes looking for the increased expression of ion transporters. One of the most studied transporters is the NHE and researchers use methods such as labeling an anti-NHE with [^{32}P] deoxynucleotide using labeling kits (47, 61). Chaves et al. used intestine specimens, frozen and prepared to view enzyme activity using a colour reaction technique, microdensitometry (62).

Finally, measuring intestinal permeability can be achieved much more readily in animal studies than in humans. Permeability can also be assessed by radiolabeling polyethylene glycol (PEG) and measuring the amount absorbed by the intestine (63, 64). The permeability of a substance depends on molecular weight, for example small molecular weight substances can readily pass through the intercellular tight junctions. In contrast, larger molecules require enzymes and transporters in order to be absorbed. Sigalet, Martin, and Pool used sugars such as mannitol and lactulose that are passively absorbed through the intercellular junctions to assess permeability (65). They looked at SBS animals and showed that as adaptation occurred,

increasing surface area, mannitol absorption increased, while lactulose remained stable. A lactulose/ mannitol ratio portraying intestinal permeability decrease as intestinal adaptation improved. Therefore, they concluded that mannitol and lactulose absorption can be used as a biomarker to assess permeability following resection (65). Intestinal permeability probes such as lactulose and mannitol are more commonly used for research purposes than in clinical care due to the unpleasant taste and worsening of diarrhea (66).

1.3 Citrulline as a Biomarker of Intestinal Adaptation

Clearly, the need for non-invasive markers of intestinal adaptation would benefit the SBS population greatly and improve overall outcomes. Cit is an amino acid which is synthesized from glutamine only in the enterocytes, the functional unit of the intestine. It has been proposed as a biomarker for intestinal adaptation because some researchers found correlation with intestine mass and absorptive power as the intestine is the main source of Cit production (67).

Cit is synthesized from glutamine by the enzyme ornithine aminotransferase or from Arg by arginase. It is then exported to the kidney where it is converted to Arg. Because Cit is not metabolized by the liver or the intestine, it therefore can be seen as a way of protecting Arg from liver degradation and is a potential marker of the total intestinal mass that participates in amino acid metabolism. While this metabolism is true in adults, pre-weaning neonates lack the enzyme *arginase*, so the intestine preferentially exports Arg instead (68).

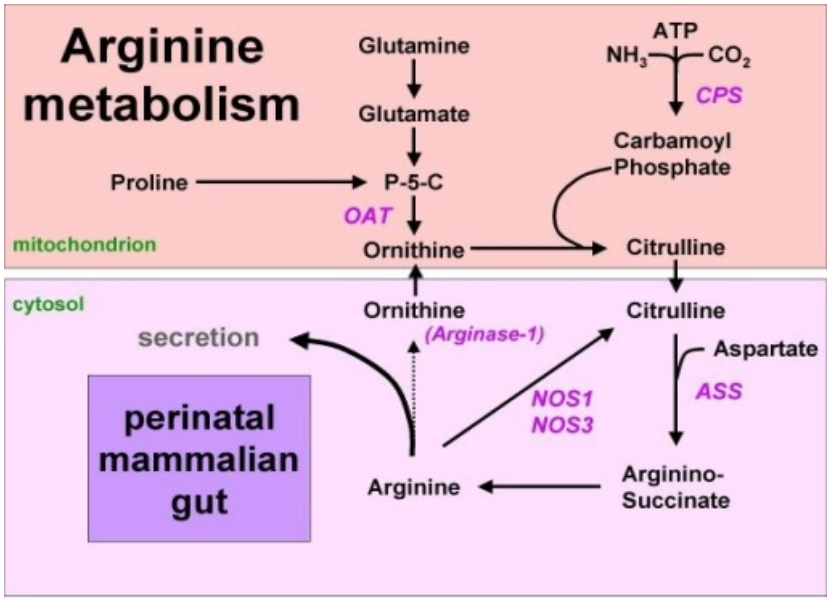
Currently, there are contradicting theories about the usefulness of Cit as biomarker for intestinal adaptation. Cit is reported to be closely associated with absorption capacity and intestinal length in both adult and pediatric patients. Fitzgibbons et al. retrospectively studied pediatric patients (median age 2.4 years) in intestinal rehabilitation clinic between January 2005 and December 2007 (69). The study showed the potential for Cit to be a predictor for PN

dependence and bowel length. They concluded that a level of 15 μmol/L was a good prognostic factor for PN weaning; levels less than 12 μmol/L were a poor prognostic factor for achieving enteral autonomy. They founded no correlation between Cit and bowel length (69). Retrospective studies, while useful, are subject to bias and missing data. In this study, the authors mentioned missing bowel length measurements in some patients. Also, important to note is the median age of 2.4 years (interquartile range of 0.6 and 6 .7 y) for the first Cit level assessment. Kohler et al have studied intestinal enzymatic levels within different age groups and determined that infants between 3-5 years old are devoid of the enzyme arginosuccinase synthase, which is key in Arg synthesis from Cit (1), and from that age the intestine appears to secrete Cit for conversion to Arg in the kidney. Due to a large variability in age inclusion in the study, it is therefore possible that the study findings were more age driven because those younger than 3 years old would have been expected to have a lower Cit level, regardless of percentage of enteral feeds and bowel length. The authors did not report the Cit levels according to the age group.

In contrast, Luo et al. studied 24 adults with stable SBS for 24 weeks in a randomized double-blind study (70). This study found no correlation between Cit levels and intestine absorption function or Cit as a predictor of weaning of PN; however, the study did find correlation between plasma Cit and intestinal length in adult patients (70). The author did not account for the small sample size or a short follow up period of 6 months, which leads us to question the validity of the study. Peter et al's study also came to the same conclusions that plasma Cit levels do not correctly depict intestinal absorption capacity in patients with enterocyte injury (71, 72). This study did not perform biopsies but used absorptiometry to assess cell damage and only studied single Cit measurements compared to more reliable serial measurements in other studies (71).

Cit is metabolized in the kidney; therefore, another disadvantage is the inability to use it in patients where kidney function is compromised (66). A recent study by Poole et al. published in 2015 looked prospectively at the plasma Cit in healthy adults in comparison to critically ill individuals. This study concluded that plasma Cit does not relate to absorption capacity in critically ill patients (73). This study's sample size was limited to 20 critically ill patients and the author discussed that their results could be affected by lack of diet control before initiation of the study.

Figure 1: Arginine and Citrulline metabolism in perinatal mammalian gut (1)



1.4 Arginine and Citrulline Metabolism in the Intestine and Beyond

A discussion of Cit as a biomarker of adaptation raises the question of what is actually known about Cit metabolism in IF and SBS. This should more correctly be a discussion of both Arg and Cit metabolism, given they are intimately related.

Arg is an amino acid that is non-essential in adults but is considered conditionally essential for neonates and growing animals. It is a precursor to many key anti-inflammatory mediators, nitric oxide (NO), and creatinine. In the intestine, glutamine, and proline (Pro) serve as precursors of Arg synthesis ***(Figure 1).*** Pro is then converted in the intestine to proline-5-carboxylic acid by *proline oxidase*, which is converted to ornithine when combined with glutamine by *ornithine aminotransferase* (OAT). Within the intestine the previous synthesis occurs within the mitochondria in the enterocyte. After ornithine is converted to Cit, the latter is exported from the mitochondria to the cytosol, where it undergoes two steps to Arg by enzymes *arginosuccinate synthase* (ASS) and *arginosuccinate lyase* (ASL). ASS, *nitric oxide synthase* (NOS) and *arginase* (ARG I and II) serve as rate limiting enzymes in Arg metabolism.

This pathway is altered by factors such as age, diet composition and feeding routes. The enzymes activities that are involved in Arg synthesis are affected by weaning in both piglets and humans. Köhler et al studied human intestine in vitro from premature neonates to adults 80 years old for expression of those enzymes involved in Arg synthesis (1). These enzymes included but were not limited to ARG I and II, NOS and ASS. As mentioned, ASS, NOS, ARG I and II are rate-limiting and differ with age, highlighting different intestinal Arg metabolism occurs between neonates and adults. Neonates, prior to weaning lack the enzyme ARG I and have gradually increased expression of ARG II, as opposed to ASS, which is high during the suckling period

and subsequently decreased and was absent by 3-5 years of age (1). Over all, these changes allow the intestine to predominantly export Arg pre-weaning and then Cit post-weaning.

Another important determinant of the amino acid pathways and enzyme activities is diet composition. If the diet is sufficient in Arg, its synthesis in the intestine would be expected to decrease. Similarly, if the diet is Arg deficient then the synthesis would increase and we expect a decreased intestinal conversion to Cit to compensate (74). A minimum intake of 0.60g/Kg/d of Arg has been proven to maximize intestinal Arg synthesis in piglets without reaching a hyper-ammonemic state, which would indicate deficiency (75). Other amino acid levels in the diet affect Arg synthesis as well. For example, lysine acts as an antagonist to Arg metabolism and vice versa (76). In this context, another important dietary factor will be the availability of dietary precursors for Arg synthesis. Recent studies have shown that Pro and not glutamine is the main dietary precursor of Arg in the neonatal intestine (77). In one-week-old pigs, an Arg-deficient diet rich in Pro and not glutamine, maintained a hyperammonemia free state when enterally fed (78). Dietary Cit intake and synthesis also creates a sufficient source of Arg and limits Arg synthesis from its other precursors (79).

Route of nutrient intake, whether intravenous or gastric, also plays a key aspect in synthesis of Arg and Cit, as PN bypasses both first pass splanchnic and hepatic Arg metabolism (80). The intestine is responsible for up to 60% of whole body Arg synthesis. When precursors are provided intravenously they bypass the intestines first pass metabolism and ultimately this can lead to Arg deficiency (78, 80, 81).

1.5 Arginine and Citrulline Metabolism following Intestinal Resection

Hence, the intestine is a key organ in Cit and Arg metabolism in neonates. However, there are limited data on the effect of intestinal resection on the metabolism of Arg and Cit. There are

some studies using rat models of SBS. The first was published in 1994, concluding that Arg becomes an essential amino acid following intestine resections in rats (82). Dejong et al studied the change that occurs in intestine-renal metabolism of Arg following a 75% intestine resection that caused a reduction in circulation Cit levels in a fasting rat model of SBS. They showed that while there was a reduction of Arg renal synthesis by 10%, whole body Arg synthesis was not dramatically altered (83). The rat has been widely used for gastrointestinal research, however there are many differences that make it a suboptimal model. Unlike the human neonate, the rodent is born with an immature intestine that cannot digest milk and other carbohydrates. Other differences include life span, intestinal flora, and anatomy. Also their adaptive response is markedly different as they have the remarkable capability to adapt within days following a 90% resection (16). To our knowledge there are no other studies of Arg and Cit metabolism following resection in piglet models.

1.6 The piglet models of short bowel syndrome and nutrition

In comparison to rodents, the piglet is a recognized model for nutrition research for translation to human infants, including amino acid metabolism. A new born piglet represents a 34-week gestation neonate, which is the population at risk for developing SBS (84). There are multiple similarities between neonates and piglets at both the structural and functional levels of the intestine. The piglet is also a medium size animal model that is amenable to SBS surgery and can be used to further understand therapeutic regimens and other aspects of care to improve overall outcomes in SBS with IF.

Structurally, the lengths of the small and large intestine are comparable in newly born piglets and neonates, both ranging from 200-300 for the small intestine and 50 to 70 cm for the large intestine (16, 85). The piglet undergoes the same adaptation process that occurs in

neonates following small intestine resection surgery (16, 37). However, the ultimate benefit is the accelerated growth occurring in piglets. Piglets double their weight by 7 days and the intestinal length by 10 days following birth. This allows for an accelerated assessment of neonatal intestinal adaptation (15, 16, 85-87).

Functionally, similar transporters and amino acid metabolism are present in both piglets and human neonates. Transporters for leucine and glucose are seen in piglets at 40% gestation while in humans at 30% gestation (16). Both also have similar intestine motility and transit time for absorption (16). Also, lipid digestion and absorption are limited in both preterm infants and neonatal piglets, as they have limited amount of functioning pancreatic digestive enzymes and inadequate circulating bile acids, hence limiting the ability to absorb long chain saturated fatty acids (15). PN support has also been studied in piglets in a similar manner to the clinical use in human neonates (88). For all these reasons the piglet is used commonly for research in pediatric nutrition and gastroenterological diseases, like SBS.

While there are many similarities that make piglets a great pre-clinical model, some differences also exist. Piglets have a spiral colon, humans possess a square colon. Also, piglet's bile and pancreatic ducts enter the duodenum from separate areas while humans are combined in the common bile duct (15). Obviously, the overall nutrient requirements are markedly greater for the rapidly growing piglet than the human infant. However, overall the similarities and the rapid growth rate of the piglets make them an excellent model for intestinal research, especially intestinal growth, and adaptation.

1.7 Summary

SBS is the leading cause of IF. SBS children require huge amounts of resources, including a multidisciplinary team, to provide optimal care and improve overall prognosis. Following resection, management includes provision of nutrients though PN to allow normal growth and

development and minimize morbidity. In order limit complications of PN and SBS and to achieve enteral autonomy the remnant intestine must adapt to ensure adequate nutrient absorption. This thesis will address the gaps in current understanding of Arg and Cit metabolism in the intestine of piglets with SBS, as a model for human neonates. Specific questions will be answered. Firstly, how useful is serum Cit in assessment of adaptation following resection of the intestine? Secondly, and importantly related to the first question, what is the actual impact of intestinal resection on Cit and Arg metabolism in the piglet intestine? We undertook a stable isotope tracer study in hope of understanding the changes that occur in Arg and Cit metabolism following a 75% intestine resection in a neonatal SBS piglet model. We used a jejuno-colonic anastomosis model that includes a 75% intestine resection. This model is devoid of the highly adaptive ileum and better represents the more common clinical surgical anatomy in human babies with SBS. By using this model, we hope to better understand the impact of common SBS anatomy on the metabolism of Arg and Cit in SBS

Chapter Two: **The rationale for proposed research**

Short bowel syndrome (SBS) is the most common cause of intestinal failure in neonates (89). These patients are dependent on parenteral nutrition, which has a high rate of morbidity and mortality. To overcome intestinal failure, the intestine must adapt. This adaptation allows patients to be weaned from parenteral nutrition, which improves their survival rates.

To wean patients from parenteral nutrition without fear of causing malnutrition, a biomarker of intestinal adaptation is needed. Citrulline (Cit) has been proposed as a biomarker of adaptation (67). In this thesis we first aim to assess the utility of plasma Cit in a piglet model of SBS. A biomarker for intestinal adaptation that is valid for neonates and enables accurate and safe weaning from parenteral nutrition will not only benefit the patient's quality of life but will also decrease costs related with parenteral nutrition and hospital stays.

However, currently there is very limited information about the impact of intestinal resection on the intestinal metabolism of the amino acids in the arginine pathway, including Cit. The only relevant study to date has been done in rats by Dejong et al (83) . The authors identified that following intestinal resection there was a reduction in circulating Cit, but overall whole-body metabolism of arginine (Arg) was not affected by resection. However, this model utilized mature rodents, and thus is not relevant to neonates. Furthermore the rat models of SBS do not show signs of intestinal failure and rats are able to feed orally post massive resections (16). The metabolism of Arg and Cit during the neonatal period is different to that of the adult. It is known that the neonatal intestine lacks *arginase* enzymes that are required for conversion of Arg to Cit and have an abundance of *arginosuccinase synthase* that increase conversion of Cit to Arg. This gradually decreases with age and becomes absent by 3 years of age (1). Furthermore, intestinal metabolism of Arg and Cit is significantly impacted by route of feeding, both enteral and

parenteral, the latter which bypasses the intestine (79, 90, 91). Neonates with short bowel syndrome and intestinal failure often receive combined parenteral and enteral nutrition. In severe intestinal failure the majority of the nutrition is likely to be parenteral. The model used by Dejong et al was enterally fed. Finally, the common anatomy of neonatal short bowel syndrome is a distal resection of the intestine, whereas the anatomy studied by Dejong et al was a proximal resection maintaining the more adaptive ileum (83). In our experience, the distal resection piglet model of SBS has limited potential for adaptation, while piglets with midgut resection do undergo adaptation (92).

Overall differences in the expression of enzymes involved in Arg and Cit metabolism in the neonatal intestine indicates the need to study the utility of Cit as a biomarker for intestinal adaptation in a neonatal animal model of short bowel syndrome with and without adaptation. Furthermore, the impact of intestinal resection with parenteral and enteral nutrition on the metabolism of Cit and Arg in the intestine should be clarified in the same model. Thus, we aim to assess the effect of 75% bowel resection in validated neonatal piglet models on intestinal Arg and Cit metabolism, using stable isotope methodology.

2.1 Objectives

1. In neonatal piglets, with intestinal resection and short bowel syndrome, to determine if plasma Cit is correlated with macroscopic and microscopic intestinal adaptation.
2. To investigate intestinal Arg and Cit metabolism in neonatal piglets with intestinal resection, while undergoing combined parenteral and enteral nutrition, compared to piglets without resection.
3. To determine the expression of the key genes for Arg and Cit metabolism in the intestine of neonatal piglets with and without intestinal resection.

2.2 Null Hypotheses

1. Plasma Cit levels will not be correlated with macroscopic (small intestinal weight and length) and microscopic (villus height and crypt depth) intestinal adaptation in neonatal piglets with short bowel, regardless of anatomy.
2. Arg synthesis from Pro will not be greater in resected piglets than non-resected sham.
3. Arginase expression in the intestine of neonatal piglets with and without intestinal resection will be undetectable.

Chapter Three: **Assessment of plasma citrulline as a non-invasive biomarker for intestinal adaptation in short bowel syndrome piglets: a model for human neonates**

3.1 Introduction

In neonates, short bowel syndrome (SBS) has been defined as occurring at birth or following emergency neonatal surgery, when the residual bowel length is less than 25% of expected for gestational age (93). Children with SBS and intestinal failure are dependent on intravenous parenteral nutrition (PN) for growth and development. Massive resection of the intestine is a trigger for intestinal adaptation; a process that includes both structural and functional changes that improve absorption to meet nutritional needs for growth and survival (94).

PN is a lifesaving nutrition replacement; however, the complications, such as cholestatic liver disease and sepsis, cause significant morbidity and potential mortality (95). Intestinal adaptation is required for patients to reach autonomy from PN. In the current era, new therapies are emerging that may promote intestinal adaptation in children (21). Therefore, an accurate method to measure adaptation would be clinically advantageous. However, in vivo measurement of structural adaptation is limited to gross assessment of small intestinal length or caliber, using fluoroscopy that is inaccurate and hazardous from the point of view of repeated radiation exposure (50). Similarly, investigations to assess functional adaptation, such as 72-hour fecal fat collections, are cumbersome and inconvenient (57).

Citrulline (Cit) is produced exclusively in intestinal enterocytes and plasma levels have been proposed to reflect intestinal production in proportion to enterocyte mass (96, 97) . For this reason, researchers and clinicians have proposed plasma Cit as a biomarker of intestinal

adaptation and bowel length. The role of Cit as a marker of adaptation in neonates is even more controversial, because the neonatal gut has limited expression of the enzyme *arginase* (ARG), while there is greater expression of *arginosuccinase synthase* (ASS) until the age of 3-5 years (1). This enzyme expression pattern suggests that the neonatal intestine is geared to release arginine (Arg) into the plasma amino acid pool, rather than Cit. This would limit the utility of Cit as a marker of enterocyte mass in neonates. Therefore, the aim of this study was to determine the utility of Cit in predicting histological adaptation and intestinal lengthening in a neonatal piglet model of SBS. We used our neonatal piglet models of SBS with and without total ileal resection, given that the two anatomical subtypes have markedly different potential for intestinal adaptation (92). Specifically, we have consistently only observed adaptation in piglets with a residual ileum. Hence, if Cit is useful it should be able to discriminate between these two models.

3.2 Methods

The research was approved by the Faculty of Agriculture, Life and Environmental Sciences Animal Policy and Welfare Committee, University of Alberta. Research was conducted in a biosecure swine research facility, according to the guidelines of the Canadian Council of Animal Care.

Piglets studied were male Landrace-Large White cross bred aged 3-5 days. The piglets had been randomly allocated to a mid (jejunoileal-JI, n=5) or distal (jejunucolic-JC, n=5) 75% intestinal resection or sham group (n=4) with no intestinal resection. Operations were performed under a general anesthetic using isoflurane (2-3%; Bensen Medical Industries Inc., Markham, Ontario, Canada). Following a longitudinal abdominal incision, the intestine was measured from ligament of Treitz to the ileocecal valve along the anti-mesenteric border using a 60cm 0-Silk suture. During surgery all piglets had a 5-French jugular central venous catheter (Braintree Scientific Inc., Braintree, MA, USA) placed for parenteral nutrition and a 10-French gastrostomy

tube placed for enteral nutrition (EN). Piglets were housed individually in metabolic cages in a 25-28°C temperature and 12h Light/dark cycled room. To avoid sepsis and minimize pain, piglets were maintained on an antibiotic and analgesia regimen for 3 days post-operative. If presumed clinical sepsis occurred after day 3, additional broad-spectrum antibiotics were added to the regimen. All these methods have been previously published (98).

3.2.1 Parenteral and enteral nutrition (EN)

Formulas were made in our laboratory and infused post-operatively, containing 16g/kg/day amino acids, 10g/kg/d lipid (Intralipid®) and 29g/kg/d glucose. In the EN, glucose was replaced with polycose to prevent osmotic diarrhea. PN was initiated at 50% of nutrient requirements post-operatively and gradually increased over 24 hours to a 100%. EN was initiated and maintained at 20% on day 2 post-operative for all piglets. This amount was provided as trophic nutrition to support intestinal adaptation (36). Treatment piglets were maintained on 100% PN and 20% EN of the daily requirement, to account for the expected diarrheal loss of resected piglets, while Sham piglet PN was decreased to 80% with initiation of EN.

3.2.2 Plasma citrulline

Plasma Cit was assessed at termination (D7) using Waters Empower 3 Chromatography Software (SPARC Biocentre, Hospital of Sick Children, Toronto).

3.2.3 Small intestine parameters

Sample collection and intestinal length re-measurement were performed under general anesthesia (D7). The same technique was used to re-measure bowel length followed by humane euthanasia. The intestine distal to the ligament of Treitz to the colon was resected, the contents were discarded, and the intestine was weighed. A mucosal scraping of a 20 cm jejunal segment was collected, weighed and snap frozen with liquid nitrogen. Mucosal mass was then calculated and adjusted for length and piglet weight (jejunum scraping weight/20 cm x1000/ pig weight;

expressed in mg/cm/kg). Jejunal, and ileal samples were collected and preserved in formaldehyde, for subsequent preparation and H&E staining for histological analysis by a certified veterinary pathologist, blinded to treatment allocation. An average of ten villus height and crypt depth measurements were taken for each piglet and measured to the nearest 0.1mm, using a micrometer eyepiece (Nikon Eclipse 80i).

3.3 Statistics

Comparisons between piglets allocated to each surgical group were made using independent Mann Whitney U test or Kruskal-Wallis as appropriate. Linear regression was used to assess relationship between adaptive structural changes and plasma citrulline levels. All data was analysed using IBM SPSS 24 statistic data editor and results are presented as median and interquartile range (25^{th}-75^{th} percentiles). Results with $P < 0.05$ were considered significantly different.

3.4 Results

3.4.1 Intestinal Adaptation in SBS piglets

JC animals demonstrated less potential for intestinal lengthening and adaptation ***(Table 1***). Specifically, on day 7, significant differences were observed in small bowel length between JC 125.0 cm (118.2-128.6); and JI 173.0 cm (157.0-189.2) (P=0.009), mucosal mass JC 43.1 mg/cm/kg (34.6-45.8); and JI 51.3 mg/cm/kg (47.6-59.1) (P=0.028) and small bowel weight JC 7.2 g/kg (6.5-7.9); and JI 14.23 g/kg (11.5-14.3) (P= 0.014). Jejunum villus height was not significantly different between JC 7.1 per 0.1 mm (6.2-7.6); and JI 7.8 per 0.1 mm (6.9-11.0) (P= 0.25)

Table 1: Piglet outcomes data

	Group	Median	25th percentile	75th percentile	P value
Day 0 Weight (Kg)	JC	2.32	2.29	2.44	0.60
	JI	2.20	2.16	2.43	
	SHAM	2.41	2.15	2.46	
Day 7 weight (kg)	JC	3.48	3.16	3.83	0.99
	JI	3.50	3.42	3.66	
	SHAM	3.56	3.06	3.88	
Change in weight (Kg)	JC	1.16	0.82	1.44	0.87
	JI	1.26	1.20	1.30	
	SHAM	1.15	.9050	1.43	
Length post-resection (cm)	JC	141.00	136.50	146.50	0.005
	JI	152.00	148.10	163.00	
	SHAM	627.25	558.12	652.13	
Length at Termination (cm) [a]	JC	125.00	118.25	128.60	0.003
	JI	173.00	157.00	189.25	
	SHAM	646.50	576.63	670.62	
Change in length (%) [a]	JC	-12.59	-18.12	-6.36	0.02
	JI	14.76	1.99	19.97	
	SHAM	6.83	-2.34	41.15	
Jejunum Scraping Weight (g/20cm) [a]	JC	2.70	2.46	3.29	0.01
	JI	3.70	3.29	4.22	
	SHAM	2.20	2.03	2.55	
Jejunum Villus height (0.1mm)	JC	7.12	6.21	7.57	0.10
	JI	7.82	6.93	10.96	
	SHAM	6.31	5.39	7.02	
Mucosal mass (mg/cm/kg) [a]	JC	43.11	34.60	45.85	0.008
	JI	51.29	47.63	59.15	
	SHAM	33.46	28.98	34.95	
Small bowel weight (g/kg) [a]	JC	7.27	6.52	7.92	0.005
	JI	14.23	11.54	14.28	
	SHAM	28.85	28.00	32.96	
Day 7 Citrulline (µM)	JC	677.67	550.09	886.70	0.056
	JI	801.04	476.05	945.88	
	SHAM	1,198.32	886.94	1,637.33	
Day 7 Arginine (µM)	JC	222.88	201.37	364.86	0.46
	JI	255.41	235.03	320.32	
	SHAM	279.52	234.09	367.12	

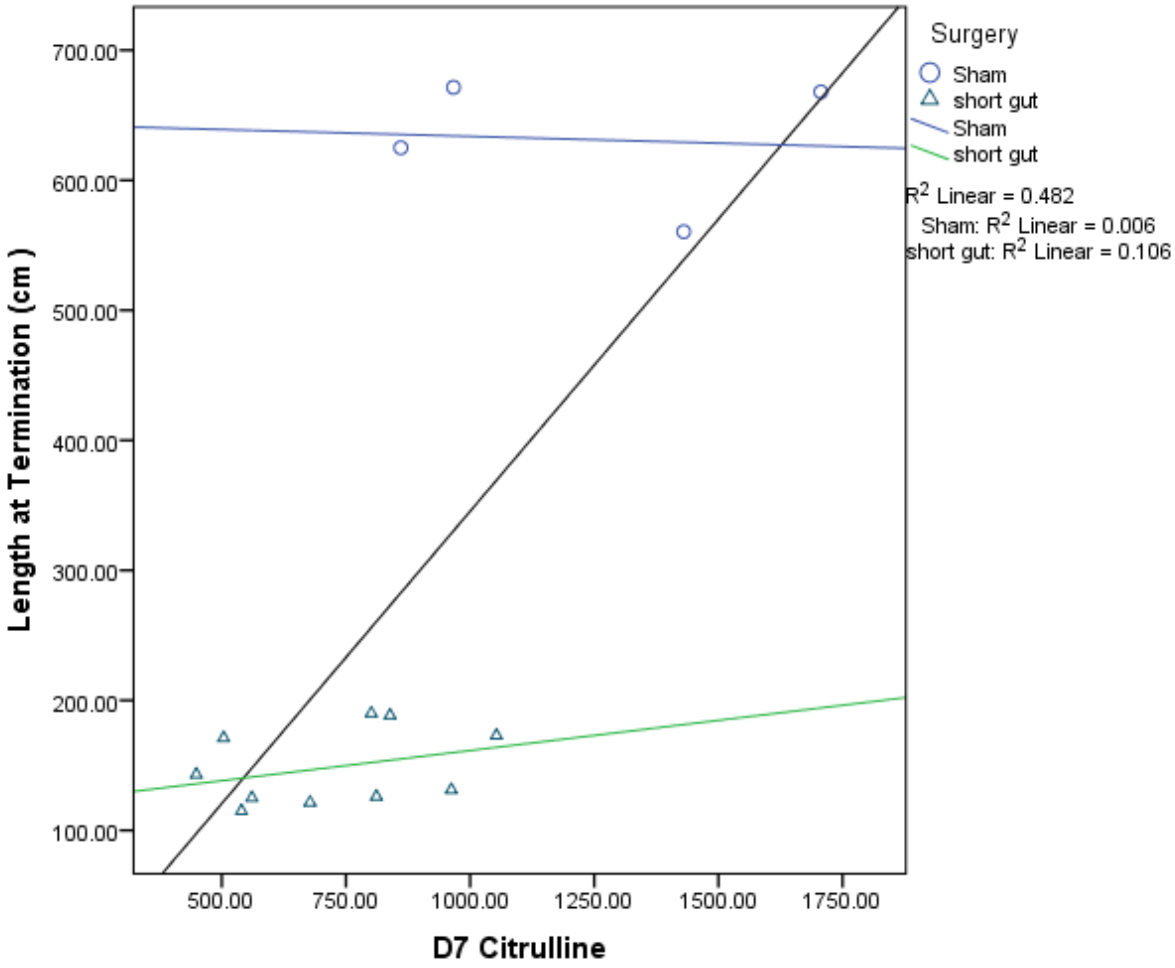
Jejunoileal: JI, n=5; jejunocolic: JC, n=5; sham, n=4

[a] Significant between JI and JC allocation $P<0.05$

3.4.2 Associations between Plasma Citrulline level and Intestinal Adaptation

Despite the differences in structural adaptation between SBS anatomical groups, day 7 plasma Cit levels were not different between JC 677.7µm (550.1-886.7) and JI 801.0 µm (476.0-945.98) (P=0.92). Plasma Cit level was significantly lower for both SBS groups compared to the Sham control 1,198.3 µm (886.9 -1,637.3; P= 0.014). Plasma Cit level was correlated with bowel length at termination (R^2=0.83; P=0.0001) ***(Figure 2)***, when all piglets were included in the analysis; however, when sham piglets were removed there was no correlation between plasma Cit and residual bowel length at termination for JC and JI piglets (P>0.05). Mucosal mass was not correlated with plasma citrulline levels (P=0.25; R^2= 0.11), nor was jejunal villus height (P=0.62; R^2=0.02).

Figure 2: Correlation between plasma citrulline (µM) and length (cm) on day 7 (D7).



Analysis includes Sham, JI and JC: P= 0.006, R^2=0.48; when only short bowel piglets (short gut=JC and JI): P= 0.36, R^2= 0.11.

3.5 Discussion

Based on research indicating that there is exclusive production in intestinal enterocytes, the plasma levels of Cit have been proposed to reflect intestinal production in proportion to enterocyte mass (99, 100). Furthermore, it has been proposed that Cit level can reflect intestinal function (101). This has led to the clinical use of Cit level in short bowel syndrome to monitor adaptation and in conditions associated with mucosal damage, such as radiation enteritis and acute rejection post intestinal transplantation (102). However, most of the literature has been based on adult studies and older children, with the inclusion of small numbers of younger children. Based on the developmental expression of enzymes relevant to the export of citrulline from the intestine we should be cautious about assuming any utility for Cit to indicate intestinal adaptation in neonates and young infants with short bowel syndrome. This study in a neonatal model of SBS does not support the use of Cit as a biomarker for adaptation in neonates.

In the current study, we used our neonatal piglet models of SBS with and without ileum. In SBS, retention of the ileum and ileocecal valve has been associated with the greatest potential for post resection adaptation (10). This was again confirmed in the current study, where the JI median percent change in length was +15%, compared to -13% in the JC piglets (P=0.016). Similarly, mucosal mass was on average 12.1 mg/cm/kg higher in JI than JC piglets despite exactly the same initial intestinal resection. While these are clinically relevant adaptive changes between the two surgical groups, plasma Cit levels failed to differentiate between the two groups. Although day 7 Cit levels correlated with intestinal length, the association was driven solely by the difference between the 2 resection treatments and the sham piglets. Therefore, Cit could potentially identify SBS, as opposed to normal length (sham), but cannot discriminate structural adaptation occurring within the SBS groups.

Similar to our study, in an experimental model of SBS in rodents, Gutierrez et al., examined 50% proximal, 50% distal, or sham surgical models and determined the relationship between serum Cit levels over an 8-week period and small intestinal length and histology (103). Notably, in this rodent model the animals did not develop intestinal failure and were able to be maintained on oral diet alone. Regardless, similar to our own findings, they concluded that Cit level can differentiate between sham and resected rats but was not related to histological adaptation. Also consistent with our studies, the authors showed that the proximal resection model had greater histological adaptation, however the Cit level failed to discriminate these histological differences (103). Therefore, these emerging data from surgical animal models show that the discriminatory power of Cit level for structural adaptation is highly limited.

Clinical studies in the pediatric population are similarly not supportive of the utility of Cit level to assess adaptation or predict autonomy from PN. Stultz et al., assessed Cit levels in pediatric patients, aged 1 to 44 months, both with and without intestinal resection, all receiving PN and considered to have intestinal failure (104). They noted an initial decline in Cit level following resection; however, thereafter, the amino acid level plateaued in all resected children. Over the follow up period, EN intake increased, suggesting some degree of gut adaptation was occurring. In contrast, in the non-resected intestinal failure patients, the Cit levels increased following the initial decline (104). During that time, they had a greater increase in EN intake. Some limitations of this study include that the bowel length measurement data was incomplete and the follow up period was short. The authors concluded that monitoring Cit may better reflect intestinal absorptive function than structure; however, no attempt to measure absorption was undertaken (104). The conclusion is poorly supported by the data, as both groups of patients increased their EN intake during the follow up period, but with minimal decrease in PN intake.

For example, there was an increase in the percentage of total calories of +5.8% in the resected patients and +12% in the non-resected groups, yet parenteral non-amino acid calories increased 4% in the resected group and decrease only 1% in non-resected group. Differences in intestinal Arg and Cit metabolism will occur with both age and with the route of dietary amino acid administration, enteral versus parenteral (79, 91). The increase in Cit level might have simply reflected increased EN delivery. The exact relationship to absorptive function and intestinal structure is not clear at all in this small pediatric study.

Clinical studies have assessed Cit as a marker of adaptation in both pediatric and adult populations with conflicting findings. The differences in intestinal enzymatic ontogeny for Arg and Cit metabolism has not been widely considered and these developmental differences may account for the disparate findings in the literature (68, 69, 105, 106). Kohler et al., studied enzymatic levels related to Arg metabolism in the intestinal biopsy tissue of children and adults undergoing intestinal surgery or endoscopy for disparate diseases such as intestinal atresia, meconium ileus and cancer (1). The age range included premature infants to adults 80 years old. They confirmed that children older than 3-5 years do not express the enzyme ASS, pivotal for Arg synthesis from Cit, in the small intestine (1). This means that it is only after this age that the intestine reliably exports Cit for conversion to Arg in the kidney (107). This change in physiology must be considered when interpreting the role of Cit to assess intestinal structure or function, as now discussed.

Bourdon et al., monitored Cit levels in very low birthweight preterm infants (< 32 weeks) (68). These otherwise healthy preterm babies were enterally and parenterally fed due to intestinal immaturity. The authors found a weak correlation between urinary Cit and gestational age plus postnatal age, suggesting there is a relationship between Cit level and maturation (68). Certainly,

this study suggests that in neonates and young infants, Cit is not a reliable marker of intestinal function, based on lack of utility of the amino acid to predict enteral tolerance. Fitzgibbons et al., retrospectively studied pediatric SBS patients (median age 2.4 years; interquartile range 0.6-6.7 years) and concluded that Cit level correlated with intestinal length measured at baseline surgery and with later enteral tolerance (69). They determined that a plasma Cit level ≥ 15 μmol/L was a positive prognostic factor for PN weaning and similarly, levels ≤12 μmol/L were a poor predictor of enteral autonomy (69). However, it is important to note the limited number of young infants in this study (n=27). The findings are likely driven by older children and may not apply to neonates and younger infants. Unfortunately, the authors did not report the Cit levels according to age group. Similarly, Diamanti et al., prospectively assessed children with SBS between 6 months to 5.7 years and measured plasma Cit prospectively for one year (105). They found a modest correlation ($R^2 = 0.22$) between baseline Cit level and small intestinal length measured at initial surgery. At follow up, a correlation ($R^2=0.48$) with percentage enteral calories was found (105). Considering children that achieved autonomy and those that did not (n=14 both groups), both Cit level and percent increase in Cit were lower in those that did not achieve autonomy. However, the main difference between these groups was the intestinal residual length initially post surgery, with a larger number of children with extreme short gut in the group that did not adapt. Therefore, similar to our findings Cit may well be a marker of large differences in small bowel length. This study design does not allow us to identify if the change in Cit is truly related to structural adaptation. Bailly-Botuha et al., also examined Cit prospectively in children with SBS aged 1 month to 15 years (106). They demonstrated that Cit level varied by large differences in bowel length and found only a modest negative correlation between Cit level and need for PN calories at follow up (106). However, when you consider the change in Cit levels there is both

large variability and inconsistency. For example, of 8 children who weaned from PN the Cit level declined or stayed the same in 3, even though they clearly had adapted.

Studies examining the ability of Cit to predict adaptation in adults are generally more positive. In adults with SBS, Cit is expected to be excreted from the intestine to the kidney and it is therefore plausible that that Cit correlates more with gut length at this age. However, even in the adult age group there is some concern over the discriminatory power of Cit to predict adaptation. A study of adult SBS patients over a 24-week period, by Luo et al., was unique for including a metabolic outcome. They found Cit to be a predictor of small bowel length at baseline, but during follow up did not relate to absorptive function (70). Over the duration of the study, oral calorie intake increased (28%) and PN requirement decreased (37%), yet plasma Cit did not significantly change and on average actually declined.

The conflicting findings in both pediatric and adult studies might also be driven by methodological issues. The first is the relationship between timing of Cit measurement and whether a subject is in a fasting state. Fjermestad et al., studied adult patients with SBS with < 200 cm remnant small bowel length compared to healthy controls both fasting and postprandially (108). In both SBS and healthy controls, the timing of Cit level postprandially impacted the measured level. Timing of Cit measurement is inconsistently reported in most studies. Furthermore, Cit levels of PN dependent SBS patients were not significantly different to the controls. In addition, fasting plasma Cit level did not correlate with intestinal absorption (measured using 72-hours metabolic balance studies), with small bowel length (measured during surgery) or with PN volume (108).

A further limitation is related to sample size and adequate statistical power to detect meaningful differences. Short bowel syndrome is a relatively rare condition and most

publications represent single institution studies. Another consideration is the impact of renal dysfunction on Cit levels. Up to 70% of adult intestinal failure patients demonstrate evidence of renal dysfunction over time related to chronic PN exposure (109). Enteral versus parenteral delivery of amino acids is also relevant for measured Cit levels. First pass intestinal metabolism of glutamine improves Cit synthesis more than parenteral glutamine (110). These limitations are all practical issues when working with such a heterogeneous population as intestinal failure patients.

Fragkos and Forbes recently published a meta-analysis exploring the role of Cit as a biomarker for adaptation, to address the limitation of sample size experienced in single centre studies (102). The pooled data was primarily from adult patients. The results demonstrated significant heterogeneity. The authors found plasma Cit level correlated with small bowel length (presumably at baseline). They claimed diagnostic utility in Cit to be able to diagnose SBS versus healthy status and PN dependency vs autonomy; however, these differences were readily discriminated clinically. The ability of Cit to predict small changes over time in intestinal function or structure that might be useful to guide treatment decisions remains to be proven.

Advantages of our experimental study design compared to the current clinical studies include enteral pair feeding, the use of genetically and developmentally homogenous neonatal animals and our ability to measure length at follow up (not simply at baseline as is most often reported in clinical studies). However, our study also had a limited sample size and we did not find the differences in villus height between the JC and JI piglets seen in our previous publications (111), although other adaptive differences were found such as; mucosal mass, small bowel length and weight.

In conclusion, we contend that Cit is not a useful biomarker of intestinal adaptation in neonates. Because Cit metabolism differs with age, it is possible that it would have greater validity as a biomarker of adaptation in older age groups; however how discriminatory it may be as a biomarker remains controversial. To clarify this issue further, studies examining the intestinal metabolism of Arg and Cit in the neonatal intestine following gut resection are required.

Chapter Four: **The effect of intestinal resection on whole body arginine synthesis**

4.1 Introduction

Short bowel syndrome (SBS) is the leading cause of intestinal failure (IF) in children. IF leads to inability to absorb sufficient nutrients for normal growth and survival (112). Preterm infants are the most vulnerable and have an increased risk of developing necrotizing enterocolitis (NEC), the main cause of SBS in this population (112). One accepted definition of SBS comes from the North American Society for Pediatric Gastroenterology, Hepatology and Nutrition, where SBS entails parenteral nutrition requirement for more than 60 days following intestinal resection or when the remnant intestine is less than 25% of what is expected for age (3). However, regardless of any intestinal resection, IF is defined by dependence on parenteral nutrition (PN) for more than 60 days in order to sustain life and for children to grow (3). Following significant intestinal resection, the intestine can undergo structural growth and functional changes that allow for independence from PN, called intestinal adaptation (11). However, in growing neonates and infants it has been shown that this process can take many years (10).

The intestine is an important site for whole body amino acid metabolism, particularly for glutamine, arginine, methionine, glycine, lysine, threonine and citrulline (113). In particular, the small intestine has been shown to contribute to 60% of whole body arginine (Arg) synthesis and in a total PN fed piglet model, mucosal mass and arginine synthesis have been shown to be directly related (80). In preterm infants, growing children and critically ill adults, Arg is considered to be a conditionally essential amino acid (114). It has important roles in immune function, ammonia detoxification, synthesis of polyamines, nitric oxide (NO) production (a

potent vasodilator), and creatine synthesis (115). Preterm infants with NEC have lower plasma Arg and arginine supplementation decreases the incidence of NEC in this population (116, 117).

The dietary precursors for intestinal Arg synthesis include proline (Pro), glutamine (Gln) and citrulline (Cit). Previously, we have shown in piglets that first-pass intestinal metabolism of an enterally-fed diet is responsible for 40-60% of Arg synthesis from Pro (75). The oral route of Gln has also been shown to increase synthesis of Cit and Arg more than the parenteral route (110). When solely parenterally fed, Arg synthesis from Pro is reduced and insufficient to meet whole-body Arg requirements (90). Finally, Arg metabolism in the intestine of neonates and infants up until 3-5 years of age differs from other ages, due to a gradual reduction of the *arginosuccinase synthase* enzyme (ASS) and the loss of *arginase* enzymes, thus favouring the release of Arg from the intestine, rather than conversion to Cit in situ (1).

For all the above reasons it can be hypothesized that neonates with IF and SBS that are dependent on PN, and with decreased metabolically active intestinal mass, will have significantly perturbed whole body Arg metabolism. However, there has been no study of the effect of intestinal failure and SBS on Arg metabolism in this specific population to date. The purpose of this study was to assess the effect of 75% intestinal resection on Arg production from Pro. We used a neonatal piglet model of SBS, undergoing both enteral and PN, and stable isotope tracer methodology to assess whole body Arg synthesis. This situation mimics the common clinical scenario following major neonatal intestinal resection of an infant who is predominantly PN dependent, while receiving trophic amounts of enteral nutrition (EN), then gradually advancing EN prior to PN weaning, dependent on tolerance. A significant number of these infants will continue to have a predominance of PN over EN throughout the time that Arg metabolism remains dependent on the intestinal contribution.

4.2 Methods

The Faculty of Agriculture, Life and Environmental Sciences Animal Policy and Welfare Committee, University of Alberta, approved the research methods. The project was conducted at a bio secure swine research center, conforming to guidelines of the Canadian Council of Animal Care.

Thirty-two male Landrace Large White cross-bred piglets aged 3-5 days old were randomly assigned to receive either 20% or 40% of their total nutrient requirements by the enteral route. They were further allocated to either surgical sham, with no intestinal resection, or a distal intestinal resection (Jejunocolic-JC). Piglets underwent isoflurane general anesthesia for surgical intervention (2-3%; Bensen Medical Industries Inc., Markham, Ontario, Canada). Surgery included a laparotomy incision followed by measurement of the intestine using a 60cm silk suture along the anti-mesenteric border with minimal traction. Only the JC underwent resection of 75% intestine, which included the more adaptive ileum, ileocecal valve and 1cm of colon. In all piglets, a 5-French left jugular catheter (Braintree Scientific Inc., Braintree, MA, USA) was inserted for parenteral nutrition (PN) and a 10-French gastrostomy tube for enteral nutrition (EN). Following recovery from anesthesia, all piglets were housed individually in metabolic cages that were temperature (25-28°C) and light controlled (12h on/off light cycle). Antibiotic prophylaxis and pain medications were initiated and maintained for 3 days post-operative (92). Additional broad-spectrum antibiotics were added if piglets shown signs and symptoms of clinical sepsis as previously published (98).

4.2.1 Diet

All piglets were given PN at 100% of their nutrient requirements, with the addition of 20% on day 2, and further advancement to 40% EN on day 3 for a subgroup, to mimic minimal

enteral and advancing EN. Both formulations were made in our laboratory and provide 16g/kg/day amino acids, 10g/kg/d lipid (Intralipid®) and 29g/kg/d glucose. To prevent osmotic diarrhea, glucose was replaced with polycose in the EN. The formula was previously published and validated (88). Post-operatively, PN was initiated at 50% and gradually increased to 100% by the following morning. An Arg-deficient PN diet was initiated on day 3 (0.6 g/kg/d Arg). To maintain an isonitrogenous solution the concentration of L-Alanine was increased. The EN solution contained adequate Arg (1.2g/kg/d) (for diets see ***Table 2)***

Table 2: Arginine deficient enteral diet composition			
AMINO ACIDS	g/L	**Glucose**	g/L
L-ALANINE	10.39	Glucose (Dextrose)	89.67
L-ARGININE	2.21	**K Phosphates**	g/L
L-ASPARTATE	3.35	K2HPO4	1.57
L-GLUTAMATE	5.81	KH2PO4	1.08
GLYCINE	0.44	**Other minerals**	g/L
L-HISTIDINE	1.73	K acetate	1.46
L-ISOLEUCINE	2.54	NaCl	2.15
L-LEUCINE	5.78	MgSO4	0.78
L-LYSINE-HCL	5.73	ZnSO4	0.09
L-METHIONINE	1.07	MnSO4 (uL)	135
L-PHENYLALANINE	2.24	Ca Gluconate	6.36
L-PROLINE	4.59		
L-SERINE	1.78		
TAURINE	0.25		
L-THREONINE	2.94		
L-TRYPTOPHAN	1.18		
L-TYROSINE	0.43		
L-VALINE	2.94		
GLYCYL-TYROSINE	1.53		
L-CYSTEINE	0.81		

4.2.2 Serum amino acids

Serum amino acid profiles (citrulline, arginine, proline, ornithine, glutamine, methionine, alanine, asparagine, glycine, leucine, lysine, phenylalanine, serine, tyrosine, valine) were obtained at baseline (Day 0) and on day 7 using liquid chromatography–mass spectrometry (LC/MS/MS).

4.2.3 Stable isotope tracers

Tracer studies were conducted on day 6, after 72-hours adaptation to the low Arg PN diet, and were given via the gastric catheter for a constant infusion of 6 hours. All piglets received a primed bolus and constant infusion as follows: Arg m+2 label (^{15}N2 guanido-arginine primed 12 μmol/kg/h, constant 20 μmol/kg/h), and proline m+1 label (^{15}N proline, primed 20 μmol/kg/h, constant 40 μmol/kg/h). Blood sampling was undertaken at -60, -30, 0, 60, 120, 180, 240, 270, 300, 330, and 360 minutes. Plasma samples were deproteinized using methanol (500 μL), which were then centrifuged for 10 min. Supernatants were moved to derivatized vials and were nitrogen dried 37°C. They were also derivatized using 100 μL of 3.0 N HCl-butanol derivative reagent (Regis Technologies Inc, Morton Grove, IL), for 20 minutes at 55°C, and re-dried using nitrogen at 37 °C. Finally, 0.1% formic acid was used to reconstitute the amino acid samples which were then analyzed using API 4000 triple quadrupole mass spectrometer (Applied Biosystems/MDS SCIEX) coupled with HPLC system (Agilent Technologies Canada Inc, Mississauga, Canada) as previously described (74). For analysis only, baseline and plateau points were used (a minimum of 3 plateau points were included).

4.2.4 Calculations

Flux of either Arg, Pro or Cit was determined as follows: where Q is flux, i is infusion rate, Ei is enrichment of the tracer in infusion and APE is atom percent excess of either Arg, Pro or Cit which was calculated from base and plateau values:

$$APE=((plateau-base)/(1+(plateau-base)) \times 100$$

$$Q_{flux} = i[(E_i/APE)-1]$$

The conversion rates of the precursor infused isotope to the product was calculated with the formulas as follows:

$$Q_{precusor-product} = E_{product}/E_{precursor} \times Q_{flux}$$

4.2.5 Quantitative real time polymerase chain reaction (qPCR)

Intestinal *arginase* I and II (ARG I and II), *ornithine decarboxylase* (ODC), *ornithine aminotransferase* (OAT), *pyrroline-5-carboxylate reductase* (P5C reductase) and *arginosuccinase synthase* (ASS) were isolated from jejunum scrapings of intestinal tissue on day 7. TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) was used to isolate mRNA, reversed transcribed to cDNA using probes and primers supplied by Integrated DNA Technologies, Inc. (San Diego, CA USA). Primers and probes were mixed with RNAse free water and a universal qPCR master mix (TaqMan) were further mixed with each respective cDNA. All genes were normalized by housekeeping gene *Gapdh* and to normal control values (same age untreated piglets). ABI Prism 7900 HT Sequence Detection System (Applied Biosystems Inc, Foster City, CA, USA) was used to assess gene expression. Samples were run in triplicates and an average taken, those with cycle thresholds >30 were interpreted as non-detectable and excluded. All primers are shown in ***(Appendix Table 8).***

4.2.6 Small Intestinal Measurements

On day 0 and day 7, while piglets were anesthetized, the small intestinal length was measured as was described above, including post resection in the JC group. On day 7, piglets were euthanized, the intestinal length remeasured and tissue samples were collected from jejunum, ileum, and colon. Mucosal scrapings of a 20-cm jejunum segment were used to measure mucosal mass (jejunum scraping weight/20 cm x1000/ pig weight; expressed in mg/cm/kg). Intestinal samples were collected and preserved in formaldehyde for examination by a certified veterinary pathologist, blinded to treatment group. Routine H&E preparation was done and a Nikon Eclipse 80i was used to measure villus height and crypt depth. Ten standard villus heights and related crypt depths were measured, and the mean was taken.

4.3 Statistics

Data is expressed according to normality as mean and total standard error or median with interquartile range (25th -75th percentiles) dependant, on data distribution. Groups were compared by either ANOVA or Kruskal Wallis tests, with Bonferroni or Mann Whitney tests for post-hoc analysis. Significance was set at $P<0.05$. Sow-fed controls (N=5) are provided as a reference for data, other than isotope measurements, but are not included in the analysis. Quantitative PCR data was excluded if it surpassed ± 2 standard deviations.

4.4 Results

4.4.1 Piglet

Results are inclusive of the following with 20% EN: sham (n=8), JC (n=10), and with 40% EN: sham (n=9) and JC (n=5). Piglets had similar age at trial commencement (20% fed sham: 4.4 d and JC: 4.0 d; 40% fed sham: 4.4 d and JC: 4.2 d; Std. error ±0.10; P=0.32) and gained similar weight over the trial (20% fed sham: 1.6 kg and JC 1.5 kg; 40% fed sham: 1.4 kg

and JC 1.5 kg; Std error± 0.03; P=0.25) (***Table 3).*** Intestinal lengthening was only observed in the sham group (20% fed sham 29.9 cm and JC -10.4 cm; 40% fed sham 41.3 cm and JC -3.9 cm; Std Error ±6.1; P=0.001). Small bowel weight was significantly higher in the sham group than JC regardless of amount of EN (20% sham 24.7 g/kg and JC 7.3 g/kg; 40% fed sham 28.1 g/kg and JC 6.8 g/kg; Std Error ±2.0; P= 0.0001) Mucosal mass was significantly higher in 20% fed JC vs sham but not in those fed 40% diets (20% fed sham 29.2 mg/cm/kg and JC 38.8 mg/cm/kg; 40% fed sham 33.1 mg/cm/kg and JC 35.5 mg/cm/kg; Std Error ±1.1; P=0.007). While jejunum villus height was similar among all groups (20% fed sham 0.68 mm and JC 0.63 mm; 40% fed sham 0.59 mm and JC 0.62; Std Error ±0.2; P=0.63).

Table 3: Piglet outcome data

	20%EN fed		40% EN fed		Total	P-value	Reference
	Sham N=8	JC N=10	Sham N=9	JC N=5			Sow fed N=6
	Mean	Mean	Mean	Mean	Stand. Error		Mean (Min-Max)
Age at surgery (days)	4.43	4.00	4.44	4.20	0.10	0.32	
Weight at surgery (kg)	2.33	2.32	2.41	2.30	0.04	0.74	
Weight at termination (kg)	3.89	3.78	3.81	3.83	0.06	0.88	3.39 (2.9-4.1)
Change in weight (Kg)	1.56	1.46	1.41	1.53	0.03	0.25	
Pre-resection small bowel length (cm)	569.81	591.67	586.89	602.40	7.28	0.55	
Small bowel length at termination (cm)	599.75[a]	137.45[b]	628.22[a]	146.90[b]	40.60	0.0001	688.5 (613.0-801.5)
Change in length (cm)	29.94[ac]	-10.45[b]	41.33[c]	-3.90[ab]	6.06	0.001	
% Change in length	5.20[a]	-7.10[b]	6.93[a]	-2.41[ab]	1.61	0.001	
Jejunal Scraping Weight (g/20cm)	2.27[a]	2.90[b]	2.52[ab]	2.72[ab]	0.07	0.007	2.4 (2.0-3.0)
Jejunum villus height (0.1mm)	6.75	6.30	5.89	6.16	0.24	0.63	
Jejunum Crypt depth (0.1mm)	1.54	1.73	1.43	1.75	0.05	0.07	
Small bowel weight (g/kg)	24.69[a]	7.27[b]	28.11[c]	6.76[b]	2.02	0.0001	39.4 (36.3-48.2)
Mucosal Mass (mg/cm/kg)	29.19[a]	38.82[b]	33.14[ab]	35.54[ab]	1.12	0.007	

Jejunocolic- JC, Superscripts represent differences between treatment groups by Two-way ANOVA

4.4.2 Serum Amino Acids

Serum Arg (20% fed sham 55.1 µM and JC 40.4 µM; 40% fed sham 125.8 µM and JC 79.9 µM; Std. Error ± 7.6; P=0.0001) and Cit (20% fed sham 58.9 µM and JC 50.9 µM; 40% fed sham 114.1 µM and JC 61.8 µM; Std. Error ± 6.8; P=0.001) were significantly higher in 40% fed sham than other groups. The precursors, Pro (20% fed sham 209.0 µM and JC 304.1 µM; 40% fed sham 464.3 µM and JC 401.6 µM; Std. Error ± 23.0; P= 0.0001) and ornithine (Orn) (20% fed sham 41.1 µM and JC 33.1 µM; 40% fed sham 91.6 µM and JC 47.9 µM; Std. Error ± 5.6; P=0.0001) were higher in 40% sham, compared to 20% sham and JC ($P<0.05$). While, Gln was significantly higher in JC vs Sham regardless of % EN diet (20% fed sham 202.3 µM and JC 569.0 µM; 40% 403.3 µM and JC 651.4 µM; Std. Error ±40.9; P= 0.001). To consider only the trial effects on serum amino acids and control for baseline values, we calculated the change that occurred from day 0 to day 7. Notably Arg, Cit, Pro and Orn all declined over the trial period; while Gln declined only in the sham and was increased in JC groups (20% fed sham -344.0 and JC 211.8; 40% fed sham -358.9 and JC 92.4; Std. Error ±77.0; P=0.004). Serum amino acids are reported in ***(Tables 4, 5 and Appendix 9, and 10).***

Table 4: Day 7 Serum amino acid data of piglets fed arginine deficient diet

Serum amino acid (µM)	20% EN fed		40% EN fed		Total	P-Value	Sow fed control	
	Sham	JC	Sham	JC				
	Mean	Mean	Mean	Mean	Std. Error		Mean	Std. Error
Citrulline	58.9^{b}	50.9^{b}	114.1^{a}	61.8^{b}	6.8	0.0001	186.8	22.6
Arginine	55.1^{bc}	40.4^{b}	125.8^{a}	79.9^{c}	7.6	0.0001	368.3	29.1
Proline	209.0^{a}	304.1^{ac}	464.3^{b}	401.6^{bc}	23.0	0.0001	827.2	53.0
Ornithine	41.1^{a}	33.1^{a}	91.6^{b}	47.9^{a}	5.6	0.0001	175.5	12.4
Glutamine	202.3^{a}	569.0^{b}	403.3^{ab}	651.4^{b}	40.9	0.0001	645.7	56.5

Jejunocolic- JC

Superscripts represent differences between treatment groups by Two-way ANOVA

Table 5: Change in serum amino acids of piglets fed arginine deficient diet

Amino acids (µM)	20% EN fed		40% EN fed		Total	P-Value	Sow fed control	
	Sham	JC	Sham	JC				
	Mean	Mean	Mean	Mean	Std. Error		Mean	Std. Error
Citrulline	-110.1	-56.6	-73.7	-134.4	12.8	0.17	-12.0	22.0
Arginine	-112.5	-114.9	-112.8	-122.2	16.1	0.99	67.7	39.2
Proline	-411.3	-239.8	-425.1	-373.2	42.6	0.35	-53.5	80.2
Ornithine	-116.2	-104.7	-121.0	-120.3	16.0	0.98	1.0	13.4
Glutamine	-344.0^{a}	211.8^{b}	-358.9^{a}	92.4^{ab}	77.0	0.004	-96.7	78.1

Jejunocolic- JC

Superscripts represent differences between treatment groups by Two-way ANOVA

4.4.3 Tracers

Arg flux (20% fed sham 1220.3 µmol/kg/h and JC 1311.9 µmol/kg/h; 40% fed sham 1255.9 µmol/kg/h and JC 972.5 µmol/kg/h; Std Error±85.4; P=0.64) and Pro flux (20% fed sham 1823.0 µmol/kg/h and JC 1987.8 µmol/kg/h; 40% fed sham 1698.4 µmol/kg/h and JC 3097.7 µmol/kg/h; Std Error ± 183.4; P=0.07) were not statistically different between groups. Similarly, fractional Pro to Arg conversion (20% fed sham 0.2 µmol/kg/h and JC 0.2 µmol/kg/h; 40% fed sham 0.3 µmol/kg/h and JC 0.5 µmol/kg/h; Std. Error±0.05; P=0.27) and molar conversion of Pro to Arg (Q Pro>Arg) (20% fed sham 240.8 µmol/kg/h and JC 291.1 µmol/kg/h; 40% fed sham 403.9 µmol/kg/h and JC 526.7 µmol/kg/h; Std. Error± 63.8; P= 0.50) appear higher in JC, although this did not reach statistical significance. However, correcting for the differences in intestinal length, Pro to Arg conversion was higher in the JC than the sham group (20% fed sham 0.4 µmol/kg/h/cm and JC 2.2 µmol/kg/h/cm; 40% fed sham 0.7 µmol/kg/h/cm and JC 3.6 µmol/kg/h/cm; Std. Error± 0.4; p=0.019), only 40% fed JC vs 20% sham reached statistical significance (p=0.036).

Table 6: Whole-body arginine synthesis data

	20% EN Fed		40% EN fed		Total	P-Value
	Sham	JC	Sham	JC		
	Mean	Mean	Mean	Mean	Std. Error	
Arginine Flux (µmol/kg/h)	1220.25	1311.88	1255.85	972.53	85.44	0.64
Proline Flux (µmol/kg/h)	1822.97	1987.78	1698.36	3097.65	183.37	0.07
Fractional Pro>Arg (µmol/kg/h)	0.21	0.20	0.32	0.48	0.05	0.27
Q Pro>Arg (µmol/kg/h)	240.76	291.06	403.87	526.72	63.80	0.50
Q Pro>Arg as % of Arg flux	20.99	20.49	32.28	48.35	5.08	0.27
*Q Pro>Arg as% Arg flux /cm gut length	0.04^{ac}	0.15^{b}	0.05^{c}	0.33^{b}	0.03	0.002
*Q Pro > Arg (µmol/kg/h/cm)	0.40^{a}	2.21^{b}	0.66^{a}	3.57^{b}	0.39	0.01

Jejunocolic- JC, Proline (Pro), Arginine (Arg), Citrulline (Cit), Conversion proline to arginine (Q pro>Arg)
Superscripts represent differences between treatment groups
*Analysed with non-parametric test as data is not normally distributed

4.4.4 Quantitative PCR

ARG 1 and ARG 2 were not quantifiable in the neonatal piglet intestine. The key enzymes for intestinal Arg synthesis are shown in (***Table 7)***. Of note, ASS was significantly higher in JC 20% than sham 20% (20% fed sham 0.02 (0.01-0.08) and JC 0.37 (0.08-2.01); p=0.027). A similar trend was seen with the 40% diet; however, this did not reach statistical significance (sham 0.17 (0.07-3.90) and JC 0.56 (0.16-0.74); P=0.84).

Table 7: Quantitative PCR analysis of jejunum tissue

	20% EN fed						40% EN fed						P-Value.	Sow fed		
	Sham			JC			Sham			JC				Reference		
	Median	25	75	Median	25	75	Median	25	75	Median	25	75		Median	25	75
ODC	0.07	0.01	0.19	0.18	0.03	3.47	0.11	0.04	1.49	0.28	0.05	1.33	0.60	0.87	0.80	1.27
P-5-C reductase	0.25	0.06	1.24	0.30	0.04	1.39	0.11	0.01	0.63	0.11	0.08	4.28	0.89	0.77	0.66	1.46
OAT	0.06	0.04	0.21	0.10	0.06	2.83	0.08	0.07	3.80	0.09	0.08	3.61	0.77	0.99	0.90	1.10
ASS	0.02[a]	0.01	0.08	0.37[b]	0.08	2.01	0.17[b]	0.07	3.90	0.56[b]	0.16	0.74	0.059	0.79	0.54	1.57

Jejunocolic- JC, *ornithine decarboxylase* (ODC), *ornithine aminotransferase* (OAT), *Pyrroline-5-carboxylate reductase* (P5C reductase) and *arginosuccinase synthase* (ASS), Enteral nutrition (EN)
Superscripts represent differences between treatment groups

4.5 Discussion

An investigation of whole body amino acid metabolism in neonates with SBS is warranted; firstly, because neonatal SBS is a costly and serious problem (118), and secondly, because we know, based on our studies in piglets, that the intestine meets 60% of whole body Arg synthesis (75). Furthermore, the neonate has insufficient endogenous production of Arg to meet the metabolic demands for response to inflammation, urea clearance and nitric oxide (NO) production (119). Finally, another key difference for neonates is the greater capability of the intestine to produce Arg for entry into the plasma amino acid pool varies than at other ages. Children younger than 3-5 years have all the enzymes required for Arg production solely in the intestine (1).

Despite the above, to date we have little data on the limitations on whole body Arg synthesis if the intestine is compromised. Most studies to date have focused on NEC in premature infants, given the observation of low plasma Arg levels preceding NEC and the reduction in prevalence of NEC with Arg supplementation in this population (117). In a premature piglet intraluminal model of NEC, Lorenzo et al., allocated piglets to receive either Arg as a NO substrate or N-omega-nitro-L-arginine methyl ester (L-NAME) as an inhibitor of NO synthase (120). In this 3h model, the Arg supplemented group showed less transmural damage, while L-NAME had higher grade NEC on histopathology. Similarly, a systemic review of the available clinical literature, with inclusion of 235 neonates, reported a 59%, reduction of NEC with supplementation of Arg (121). Of note, this systemic review only included 2 publications and clearly more studies are required.

Because the intestine plays such an important role in Arg synthesis, it is unknown if significant resection of the intestine will hinder whole body Arg synthesis and contribute to the morbidity, including limited growth, observed in SBS infants. In this study, we chose to use a neonatal piglet model of SBS without ileum, as this better represents clinical SBS in infants, where the ileum is usually lost or resected (21). We have previously shown that this JC model will develop true IF, with a requirement for PN, consistent with what we observe as limited potential for adaptation (10). The majority of infants with IF are provided early trophic nutrition to enhance adaptation and will subsequently wean from PN according to the tolerance for EN. Previously, it has been shown in piglets that 40% of nutrient requirements delivered enterally is the minimal amount necessary to support growth in mucosal mass and this would generally be considered above trophic amounts of nutrition (122). Therefore, we studied both 20% and 40% EN to mimic trophic nutrition and enteral advancement. However, we did not see a major impact of the 40% advancement on mucosal adaptation as jejunal scraping and mucosal mass were not significantly different in 40% EN fed piglets, while 20% EN fed piglets were higher in JC group.

Regardless, when comparing the JC model to the sham we know that the JC had about one quarter of the gut length, both at the end of intestinal resection and also at the end of the trial on day 7. While we had anticipated that this would have a negative impact on whole body Arg synthesis, we found that even with this massive reduction in intestinal length, Arg synthesis was in fact similar between both the JC and sham. Therefore, this would suggest that the JC SBS piglets were able to adapt to massive resection and upregulate intestinal Arg synthesis, so as to maintain similar levels as the non-resected sham. This hypothesis is supported by our molecular data, showing an increase in ASS expression in the jejunal tissue of the JC piglets, at least with 20% minimal trophic nutrition. Furthermore, the plausibility of upregulated synthesis was made

evident when adjusting synthesis from enteral Pro for the piglet intestinal lengths, the JC showed a 5.5-fold increase in Arg synthesis from Pro over the sham, regardless of the amount of enteral diet fed (Q Pro to Arg conversion (μmol/kg/h/cm) 20% sham 0.40 and JC 2.21; 40% sham 0.66 and JC 3.57; Std. Error 0.39; P=0.02). However, we acknowledge that we did not perform portal catheterization and thus strictly speaking have not isolated the intestinal synthesis of Arg. Nevertheless, given we did identify key enzymatic differences within the intestine, it is reasonable to assume these contribute to measured whole body Arg synthesis from the dietary precursor Pro. To reiterate, this was equivalent between both groups, despite large differences in intestinal mass and no other model system differences, including similar PN delivery.

Wilkinson et al., compared Arg-deficient vs Arg-sufficient diets and the different synthesis that occurs using both intragastric and intraportal tracer infusions to isolate the intestinal contribution in neonatal piglets (75). They found that whole body Arg synthesis was upregulated in piglets given the Arg-deficient diet, however, the intestine's contribution was not increased. While they did find that the intestine was responsible for 50-60% of whole body Pro to Arg conversion, it is assumed another site was responsible for the upregulation that occurred, and this cannot be excluded in our own study. Further studies are required to understand the whole-body adaptations in Arg metabolism that occur, given a limited dietary supply of Arg and/or massive intestinal resection in neonates. This study is the first to provide data that the intestine contributes to such adaptation, by increased enzymatic expression of ASS.

A notable finding in our study was the decline in serum Arg in all groups over the trial period. Given equivalent synthesis from dietary Pro, this suggests either increased Arg utilization or insufficient whole-body synthesis to maintain the serum amino acid level with resected intestine. Arg is an important substrate for the production of NO, which modulates intestinal

muscle relaxation, regulates mucosal blood flow and maintains barrier function (121). Other notable metabolic fates of Arg include ammonia detoxification, and creatine and polyamine synthesis. Overall, all amino acids that are utilized by the urea cycle declined equivalently in all our study groups, with the exception of Gln, which interestingly was higher in JC than Sham groups. As the major fuel of the intestine it is possible that the increase in Gln in the JC piglet was driven by decreased utilization in the intestine, because of shorter intestinal length, with increased utilization expected in the sham with the longer length. Perhaps consistent with this theory, there was lower serum Gln in the 40% fed JC than the 20% fed JC, while there was slightly less loss of length at day 7 with the increased EN. Adjusting the change of Gln, from baseline to d7, for intestinal length, the 20% fed JC showed a greater reduction than sham, not seen when EN was increased to 40% (20% fed sham -16.4 μM/cm and JC -122.5 μM/cm; 40% fed sham -8.3 μM/cm and JC 2.03 μM/cm; Std Error ± 26.2; P=0.3). This may suggest that the JC increased the utilization of Gln, either as a nitrogen donor for ammonia detoxification, or for Arg synthesis, or as energy for the intestine to grow accordingly. Further analysis using isotope tracers would be required to clarify these issues.

While this is the first study of the impact of SBS anatomy on Arg metabolism in the piglet, Dejong et al., studied a rat model of SBS with 75% intestine resection to assess the effect of resection on Cit and Arg tissue concentration, and renal uptake and release. Using Cit and Arg radioactive isotope tracers in sham, 75% resected, and fasted control rats, they showed a reduction of Arg renal synthesis by 10%, in the SBS group while whole body Arg synthesis was not dramatically altered (83). Thus, the kidney is not responsible for the upregulation to maintain whole body Arg levels, but rather intestinal upregulation is plausible. However, further assessment of intestinal contribution in SBS and other organ isolation is warranted. Furthermore,

while the rat has been widely used for gastrointestinal research, rodents do not adequately represent human neonates due to key developmental, anatomical, and physiological differences. As opposed to human neonates, rodents are born with an immature intestine that is unable to digest milk and other carbohydrates (84). Also, when mature, their adaptive response is markedly different and they have the capability to adapt within days following a 90% intestinal resection (16). To our knowledge, the current study is the first to assess Arg metabolism following resection in a piglet model of SBS and this is warranted for greater potential to translate to human infants.

A limitation of this study was the small sample size in the 40% fed JC group, which may have limited our ability to identify significant differences from the 20% EN group. A further limitation was the inability to more accurately infer the intestinal contribution in whole body Arg synthesis, given we did not perform portal vein sampling for isotope enrichment studies. However, as aforementioned, we assessed key Arg enzymatic changes in the jejunal tissue of the intestine and our findings suggest that intestine plays an important role in the regulation of whole body Arg synthesis, following massive intestinal resection. Measurement of the protein, rather than merely mRNA expression would have provided additional information.

In conclusion, whole body Arg synthesis is similar in resected and unresected piglets due to upregulation of synthesis possibly by the intestine or other organs. Further assessment should be done to clarify the exact site of upregulation. In addition, as the overall effect of PN was to reduce serum amino acids, further supplementation of these important amino acids could be needed in this vulnerable population to aid normal growth and development.

Chapter Five: **Conclusion and future directions**

The intestine plays a critical role in amino acid metabolism, but minimal data exists on the effect of intestinal resection on arginine (Arg) metabolism, as well as the utility of citrulline (Cit) in these resected infants. These questions were answered in this thesis using our neonatal piglet short bowel syndrome (SBS) model, which represents a 34-week-old preterm infant (84). With regards to Cit, we have shown that as a biomarker, it is only capable of distinguishing large differences in gut length but does not have the discriminatory power to detect smaller adaptive changes that occur following intestinal resection, which is the more clinically relevant outcome. We have also shown in the current work that Arg synthesis is upregulated in SBS piglets so as to maintain similar synthesis as observed in non-resected shams. While whole body synthesis was similar between JC and Sham, when corrected for the intestine length, which is of course markedly different, the JC showed almost 3x more synthesis than the Sham per/cm intestine. Similarly, an unexpected finding was the JC piglets seemed to utilize more glutamine/cm. More research would be required to better understand the importance and role of glutamine (Gln) in SBS, because glutamine is the main source of energy for proliferating intestinal cells (115). Gln supplementation has been shown to improve outcomes in a variety of situations (123, 124) but to the author's knowledge has not been studied in SBS. It may be that additional EN Gln supplementation would increase the rate and extent of intestinal adaptation in SBS infants.

Some limitations of our study included the small sample size in one group (40% fed JC) and that we did not isolate the effect of the intestine through portal catheterization. However, it is notable that we saw increased intestinal expression of *arginosuccinase synthase* (ASS) in jejunal tissue, which is a key enzyme for intestinal Arg synthesis. This and the known critical contribution of the intestine to whole body Arg synthesis in the neonate (75), and the limitation

of total parenteral nutrition (PN) to support Arg synthesis (90), would all seem to support that upregulation of Arg metabolism in the intestine of SBS piglets occurs. Thus, we may have identified a key metabolic adaptation that occurs in support of whole body Arg metabolism in the neonate given massive intestinal resection and while undergoing parenteral nutrition.

As SBS is a very heterogeneous disease with variable presentations and disease progression, research to generate new and better treatments is critical. To this end animal research can play an important role and the neonatal piglet is particularly useful. The piglet intestine has very similar anatomy and function to human neonates with minimal differences, such as a spiral colon. This model also shows signs of intestinal failure following resection and piglets thus become dependant on parenteral nutrition, similar to what is seen in human neonates with SBS (16).

The current research should be expanded to consider its further relevance for the common and serious problems observed in SBS infants. For example, central line infections are very common events with about 9 septic events per 1000 central catheter days (10). Sepsis can be considered likely to be the leading future cause of morbidity and mortality in this population, especially given the recent reduction in the severity of liver disease, the traditional main cause of death (22). To this end it is noteworthy that both Gln and Arg supplementation have been shown to improve clinical response to infections, including in cancer patients: fewer septic episodes, reduced length of hospital stay, reduction of radio-chemotherapy side effects on the intestine and reduction in abdominal sepsis (125). However, the effect of Arg supplementation in SBS septic infants is currently unknown. Would improvement of whole-body Arg metabolic status prevent infections in SBS infants? An equally important consideration is the negative impact of infection

on the probability of weaning from PN (10), again perhaps Arg or Gln supplementation, or a combination of both, would be beneficial for intestinal adaptation and weaning from PN.

Multiorgan failure can occur in SBS patients, most often in the presence of severe sepsis, but also independently, given both the liver and kidney are at risk of toxicity from prolonged PN (126). Thus, further investigation of the exact organ or organs responsible for the observed upregulation of whole body Arg synthesis in SBS is required. There are multiple ways of isolating the organs responsible for whole body Arg synthesis: one being stable isotope tracers with isolation of the organs by sampling the afferent and efferent blood supply (127). A follow up study to the current research should include assessment of multiple organs, such as liver, muscle, intestine and kidney, because it is important to identify the site of Arg upregulation. Another possible way to isolate the organ site(s) would be isotope tracer infusion and assessing tissue samples for quantification of presence of both intermediates and end products in the target organs in order to rank their specific synthesis contributions to whole body Arg (120).

The current study focused on an SBS model without ileum, since the ileum has been shown to have a higher adaptive potential and hence resection creates the most valid intestinal failure model (10). The ileum is also considered to be one of the sites of Arg absorption (128); however, it is not actually known if Arg synthesis itself varies from the jejunum to the ileum. This warrants further clarification and future studies should include different models of SBS, including our own anatomical variants that have either contain a remnant ileum or are devoid of it (JC as studied here) and models that vary in length of intestinal resection. The question remains would infants with resected ileum or with greater over all intestinal resection length require more Arg or supplementation than those who have a remnant ileum or less resection?

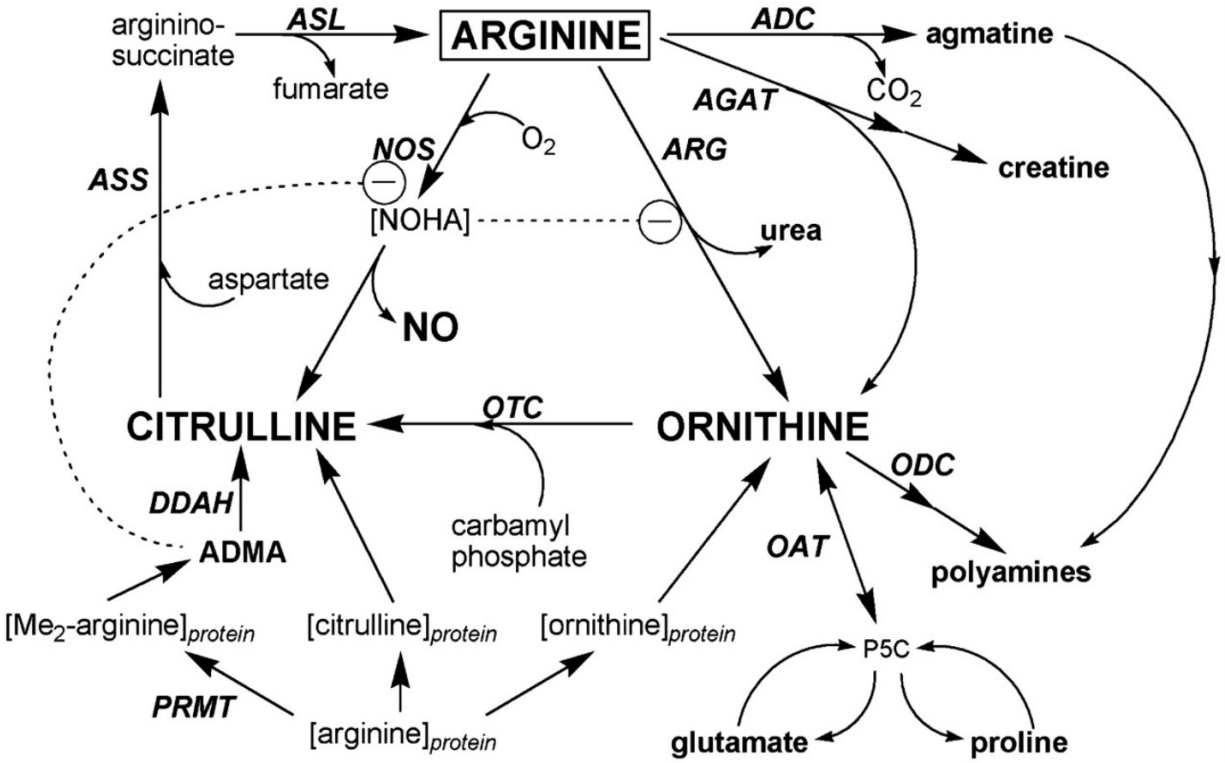
One of the main causes of SBS is necrotizing enterocolitis (NEC), which alone is responsible for 40-50% of SBS cases (6). As noted in this thesis, there is already preliminary information linking Arg status to the incidence of NEC (121). A better understanding of the role of Arg in nitric oxide (NO) synthesis, as a potent vasodilator, is required in preterm infants at risk of NEC. It is possible that low Arg and thus reduction in NO synthesis plays an important role in NEC pathophysiology. Studies have shown that patients with NEC have lower Arg levels and show improvement with Arg supplementation (117). Asymmetric dimethylarginine (ADMA) a bioproduct of Arg, inhibits the activity of nitric oxide synthase (NOS), a key enzyme for NO production (***Figure 3***). A study by Richir et al., assessed Arg and ADMA levels in infants with NEC in comparison to age matched healthy preterm infants (117). In infants with NEC, plasma concentrations of Arg, ADMA and ARG:ADMA ratio were all lower than in unaffected infants. While unexpected that ADMA would also decline, it seems that a balance between Arg and ADMA is of great importance, because within the NEC group, mortality was associated with a significantly lower plasma Arg and Arg:ADMA ratio than for surviving NEC infants. It is thus possible that decreased synthesis of NO from Arg is of critical importance in the pathogenesis of NEC, a major cause of severe SBS. This hypothesis is supported by the study by Lorenzo et al., examining the effect of Arg supplementation and L-NAME, an inhibitor of NOS, on NEC using isolated intestinal loops in neonatal piglets (120). The Arg supplemented group had a lower NEC histological grade, while L-NAME produced higher grade lesions and hemorrhagic congestion. Thus, additional research is warranted to assess which NOS inhibitor is important in NEC pathogenesis and the current paucity of whole body animal models for this purpose needs to be addressed. The piglet model is ideal for this research purpose and preterm piglet models of NEC are already well established (129-131). Would promoting a higher Arg:ADMA ratio or reduction

in NOS inhibitors, as in the in-vivo model, be a clinical preventative strategy to reduce NEC and hence, SBS? The dose of Arg supplementation that reverses NEC, presumably by adequately correcting the Arg:ADMA ratio, could be established readily in preterm piglet models, given the very high rates of NEC observed in such models. This would have important clinical translation to improve the health and outcomes of preterm infants given the high costs of this disease (132). Any strategy that could reduce the risk of NEC, could ultimately reduce SBS by up to 50%, which has a further economic benefit and more importantly further improves preterm infants health outcomes (132).

Lastly, this brings us to an important anomaly that should be taken into consideration: the so-called Arg paradox (114, 119). In essence, this states that cells prefer exogenous sources for NO production rather than innate endogenous synthesis. Hence, another limitation of our study was that we did not assess NOS activity, nor NO synthesis. An addition of a Cit tracer would have allowed the measurement of NO production in vivo in our model system. Since the increased synthesis viewed in our study, as we have argued, may be from an intestinal source, it is important to understand the utilization of precursors for Arg in intestinal NO production. I would hypothesise that intestinal NO production would be lower in SBS piglets as the intestine adapts to promote and prioritize Arg synthesis. This raises the possibility that supplementation of Cit or the dietary precursors may support Arg synthesis and at the same time improve NO production, which is likely to be beneficial in a severe intestinal illness, like SBS. Furthermore, the impact of parenteral vs enteral supplementation on intestinal NO production and intestinal NOS activity needs to be explored and has translational relevance to other critical illnesses, beyond SBS.

In conclusion, understanding the effect of intestinal resection on Arg synthesis in a valid neonatal animal model is more relevant to the care of these infants than translating knowledge obtained from adult animals or humans. This is especially the case, given the developmental differences in intestinal enzyme expression relevant to Arg synthesis. Our findings of poor utility of Cit are an excellent example of this premise, being contrary to some of the data supporting the use of Cit as a biomarker for adaptation in adults. Furthermore, we know that Arg is critically important for many organ functions, but especially in the intestine where it plays a role in NO synthesis, and research already shows supplementation improves the risk of a severe neonatal intestinal disease like NEC. Thus, ongoing research should be more neonate directed and should endeavour to increase our understanding of neonatal Arg metabolism under different disease models or conditions, as well as of the important role whole body Arg synthesis may have in neonatal disease pathogenesis and prevention.

Figure 3: Asymmetric dimethylarginine inhibition on nitric oxide synthase (NOS)



References

1. Kohler ES, Sankaranarayanan S, van Ginneken CJ, van Dijk P, Vermeulen JL, Ruijter JM, et al. The human neonatal small intestine has the potential for arginine synthesis; developmental changes in the expression of arginine-synthesizing and -catabolizing enzymes. BMC developmental biology. 2008;8:107.

2. Bines JE. Intestinal failure: A new era in clinical management. J Gastroenterol Hepatol. 2009;24 Suppl 3:S86-92.

3. Merritt RJ, Cohran V, Raphael BP, Sentongo T, Volpert D, Warner BW, et al. Intestinal Rehabilitation Programs in the Management of Pediatric Intestinal Failure and Short Bowel Syndrome. Journal of Pediatric Gastroenterology and Nutrition. 2017;65(5).

4. Wales PW, de Silva N, Kim J, Lecce L, To T, Moore A. Neonatal short bowel syndrome: population-based estimates of incidence and mortality rates. Journal of pediatric surgery. 2004;39(5):690-5.

5. Pironi L, Arends J, Baxter J, Bozzetti F, Pelaez RB, Cuerda C, et al. ESPEN endorsed recommendations. Definition and classification of intestinal failure in adults. Clin Nutr. 2015;34(2):171-80.

6. Thompson JS. Short Bowel Syndrome and Malabsorption - Causes and Prevention. Viszeralmedizin. 2014;30(3):174-8.

7. Amin SC, Pappas C, Iyengar H, Maheshwari A. Short bowel syndrome in the NICU. Clin Perinatol. 2013;40(1):53-68.

8. Wales PW, Christison-Lagay ER. Short bowel syndrome: epidemiology and etiology. Semin Pediatr Surg. 2010;19(1):3-9.

9. Buchman AL, Moukarzel AA, Bhuta S, Belle M, Ament ME, Eckhert CD, et al. Parenteral Nutrition Is Associated With Intestinal Morphologic and Functional Changes in Humans. JPEN Journal of Parenteral & Enteral Nutrition. 1995;19(6):453.

10. Belza C, Fitzgerald K, de Silva N, Avitzur Y, Steinberg K, Courtney-Martin G, et al. Predicting Intestinal Adaptation in Pediatric Intestinal Failure: A Retrospective Cohort Study. Ann Surg. 2017.

11. Tappenden KA. Intestinal adaptation following resection. JPEN J Parenter Enteral Nutr. 2014;38(1 Suppl):23S-31S.

12. Altmann GG. Influence of bile and pancreatic secretions on the size of the intestinal villi in the rat. American Journal of Anatomy. 1971;132(2):167.

13. Sukhotnik I, Shiloni E, Krausz MM, Yakirevich E, Sabo E, Mogilner J, et al. Low-fat diet impairs postresection intestinal adaptation in a rat model of short bowel syndrome. Journal of pediatric surgery. 2003;38(8):1182-7.

14. Williamson RCN. Intestinal Adaptation. N Engl J Med. 1978;298(25):1393-402.

15. Sangild PT, Ney DM, Sigalet DL, Vegge A, Burrin D. Animal models of gastrointestinal and liver diseases. Animal models of infant short bowel syndrome: translational relevance and challenges. American journal of physiology-gastrointestinal and liver physiology. 2014;307(12):G1147-G68.

16. Lim DW, Turner JM, Wales PW. Emerging Piglet Models of Neonatal Short Bowel Syndrome. JPEN J Parenter Enteral Nutr. 2015;39(6):636-43.

17. Soden JS. Clinical assessment of the child with intestinal failure. Semin Pediatr Surg. 2010;19(1):10-9.

18. Fallon EM, Nehra D, Potemkin AK, Gura KM, Simpser E, Compher C, et al. A.S.P.E.N. clinical guidelines: nutrition support of neonatal patients at risk for necrotizing enterocolitis. JPEN J Parenter Enteral Nutr. 2012;36(5):506-23.

19. Wong T, Gupte G. Complications of short bowel syndrome. Paediatrics and Child Health. 2015;25(9):418-21.

20. Fullerton BS, Hong CR, Jaksic T. Long-term outcomes of pediatric intestinal failure. Semin Pediatr Surg. 2017;26(5):328-35.

21. Chandra R, Kesavan A. Current treatment paradigms in pediatric short bowel syndrome. Clin J Gastroenterol. 2018;11(2):103-12.

22. Oliveira C, de Silva NT, Stanojevic S, Avitzur Y, Bayoumi AM, Ungar WJ, et al. Change of Outcomes in Pediatric Intestinal Failure: Use of Time-Series Analysis to Assess the Evolution of an Intestinal Rehabilitation Program. Journal Of The American College Of Surgeons. 2016;222(6):1180-8.e3.

23. Courtney CM, Warner BW. Pediatric intestinal failure-associated liver disease. Current Opinion in Pediatrics. 2017;29(3):363-70.

24. Nilesh MM, Heather ES, Sharon YI, Jorge AC-B, Sarah V, Elizabeth Anne F, et al. Guidelines for the Provision and Assessment of Nutrition Support Therapy in the Pediatric Critically Ill Patient: Society of Critical Care Medicine and American Society for Parenteral and Enteral Nutrition. Journal of Parenteral and Enteral Nutrition. 2017;41(5):706-42.

25. Ontario SHT. Guidelines for the Administration of Enteral and Parenteral_Nutrition in pediatrics. 2007.

26. Tappenden KA. Mechanisms of enteral nutrient-enhanced intestinal adaptation. Gastroenterology. 2006;130(2 Suppl 1):S93-9.

27. Dodge ME, Bertolo RF, Brunton JA. Enteral feeding induces early intestinal adaptation in a parenterally fed neonatal piglet model of short bowel syndrome. JPEN Journal Of Parenteral And Enteral Nutrition. 2012;36(2):205-12.

28. Carter BA, Cohran VC, Cole CR, Corkins MR, Dimmitt RA, Duggan C, et al. Original Articles: Outcomes from a 12-Week, Open-Label, Multicenter Clinical Trial of Teduglutide in Pediatric Short Bowel Syndrome. The Journal of Pediatrics. 2017;181:102-11.e5.

29. Drucker DJ, Yusta B. Physiology and Pharmacology of the Enteroendocrine Hormone Glucagon-Like Peptide-2. Annual Review of Physiology. 2014;76(1):561-83.

30. Kim ES, Keam SJ. Teduglutide: A Review in Short Bowel Syndrome. Drugs. 2017;77(3):345-52.

31. Diamond IR, de Silva N, Pencharz PB, Kim JH, Wales PW, Group for the Improvement of Intestinal F, et al. Neonatal short bowel syndrome outcomes after the establishment of the first Canadian multidisciplinary intestinal rehabilitation program: preliminary experience. J Pediatr Surg. 2007;42(5):806-11.

32. Stanger JD, Oliveira C, Blackmore C, Avitzur Y, Wales PW. The impact of multi-disciplinary intestinal rehabilitation programs on the outcome of pediatric patients with intestinal failure: a systematic review and meta-analysis. J Pediatr Surg. 2013;48(5):983-92.

33. Ramos Boluda E. Pediatric small bowel transplantation. Current opinion in organ transplantation. 2015;20(5):550-6.

34. Dowling RH, Booth CC. Structural and functional changes following small intestinal resection in the rat. Clinical science. 1967;32(1):139-49.

35. Pereira PM, Bines JE. New growth factor therapies aimed at improving intestinal adaptation in short bowel syndrome. Journal of gastroenterology and hepatology. 2006;21(6):932-40.

36. Cisler JJ, Buchman AL. Intestinal adaptation in short bowel syndrome. Journal of Investigative Medicine. 2005;53(8):402-13.

37. McDuffie LA, Bucher BT, Erwin CR, Wakeman D, White FV, Warner BW. Intestinal adaptation after small bowel resection in human infants. J Pediatr Surg. 2011;46(6):1045-51.

38. Helmrath MA, Erwin CR, Shin CE, Warner BW. Enterocyte apoptosis is increased following small bowel resection. Journal of Gastrointestinal Surgery. 1998;2(1):44-9.

39. Martin CA, Perrone EE, Longshore SW, Toste P, Bitter K, Nair R, et al. Intestinal resection induces angiogenesis within adapting intestinal villi. Journal of pediatric surgery. 2009;44(6):1077-83.

40. Christopher J. Sonnenday MM. Disorders of the Exocrine Pancreas. In: Hammer GD, McPhee SJ, editors. Pathophysiology of Disease: An Introduction to Clinical Medicine, 7e. New York, NY: McGraw-Hill Education; 2013.

41. Barrett KE, Barman SM, Boitano S, Brooks HL. Digestion, Absorption, & Nutritional Principles. Ganongâ€™s Review of Medical Physiology, 25e. New York, NY: McGraw-Hill Education; 2016.

42. Mescher AL. Chapter 16. Organs Associated with the Digestive Tract. Basic Histology, 13e. New York, NY: The McGraw-Hill Companies; 2013.

43. Taqi E, Wallace LE, de Heuvel E, Chelikani PK, Zheng H, Berthoud H-R, et al. CAPS Paper: The influence of nutrients, biliary-pancreatic secretions, and systemic trophic hormones on intestinal adaptation in a Roux-en-Y bypass model. Journal of Pediatric Surgery. 2010;45:987-95.

44. Fenyö G. Morphological changes of the adapting small intestine deprived of gastric, duodenal, biliary and pancreatic secretions in the rat. European Surgical Research Europäische Chirurgische Forschung Recherches Chirurgicales Européennes. 1977;9(2):122-30.

45. Williamson RC, Bauer FL, Ross JS, Malt RA. Proximal enterectomy stimulates distal hyperplasia more than bypass or pancreaticobiliary diversion. Gastroenterology. 1978;74(1):16-23.

46. Weser E, Heller R, Tawil T. Stimulation of mucosal growth in the rat ileum by bile and pancreatic secretions after jejunal resection. Gastroenterology. 1977;73(3):524-9.

47. Musch MW, Bookstein C, Rocha F, Lucioni A, Ren H, Daniel J, et al. Region-specific adaptation of apical Na/H exchangers after extensive proximal small bowel resection. American Journal of Physiology - Gastrointestinal and Liver Physiology. 2002;283(4):G975-G85.

48. Sacks AI, Acra SA, Dykes W, Polk DB, Barnard JA, Pietsch J, et al. Intestinal Na+/H+ exchanger activity is up-regulated by bowel resection in the weanling rat. Pediatric research. 1993;33(3):215-20.

49. Ziegler TR, Fernández-Estívariz C, Gu LH, Bazargan N, Umeakunne K, Wallace TM, et al. Distribution of the H+/peptide transporter PepT1 in human intestine: up-regulated expression in the colonic mucosa of patients with short-bowel syndrome. American Journal of Clinical Nutrition. 2002;75(5):922-30 9p.

50. Algin O, Evrimler S, Arslan H. Advances in radiologic evaluation of small bowel diseases. Journal of computer assisted tomography. 2013;37(6):862-71.

51. Herfarth H, Palmer L. Risk of Radiation and Choice of Imaging. Digestive Diseases. 2009;27(3):278.

52. Kaufman AB, Rubin DT. Risks of Radiation Injury in IBD Patients. Inflammatory Bowel Disease Monitor. 2010;10(4):126.

53. Nicoll D, Lu CM, Pignone M, McPhee SJ. Decision Making. Pocket Guide to Diagnostic Tests, 6e. New York, NY: The McGraw-Hill Companies; 2012.

54. Gaca AM, Jaffe TA, Delaney S, Yoshizumi T, Toncheva G, Nguyen G, et al. Radiation doses from small-bowel follow-through and abdomen/pelvis MDCT in pediatric Crohn disease. Pediatr Radiol. 2008;38(3):285-91.

55. Tappenden KA. Pathophysiology of short bowel syndrome: considerations of resected and residual anatomy. JPEN J Parenter Enteral Nutr. 2014;38(1 Suppl):14S-22S.

56. Hill PG. Faecal fat: time to give it up. Annals of Clinical Biochemistry. 2001;38(3):164.

57. Raman M, Fenton T, Crotty P, Ghosh S, Rioux K, Hundal R. A novel method to identify fat malabsorption: The Serum Retinyl Palmitate Test. Clinica Chimica Acta. 2015;438:103-6.

58. Suri M, Turner JM, Sigalet DL, Wizzard PR, Nation PN, Ball RO, et al. Exogenous glucagon-like peptide-2 improves outcomes of intestinal adaptation in a distal-intestinal resection neonatal piglet model of short bowel syndrome. Pediatr Res. 2014;76(4):370-7.

59. Y Gavrieli YS, Ben-Sasson SA. - Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation doi:10.1083/jcb.119.3.493. - The Rockefeller University Press; 1992. p. - 493.

60. O'Brien DP, Nelson LA, Stern LE, Williams JL, Kemp CJ, Wang Q, et al. Epithelial permeability is not increased in rats following small bowel resection. J Surg Res. 2001;97(1):65-70.

61. Falcone Jr RA, Shin CE, Stern LE, Wang Z, Erwin CR, Soleimani M, et al. Differential Expression of Ileal Na+/H+ Exchanger Isoforms after Enterectomy. Journal of Surgical Research. 1999;86(2):192-7.

62. Chaves M, Smith MW, Williamson RC. Increased activity of digestive enzymes in ileal enterocytes adapting to proximal small bowel resection. Gut. 1987;28(8):981.

63. Schulzke JD, Fromm M, Bentzel CJ, Zeitz M, Menge H, Riecken EO. Ion transport in the experimental short bowel syndrome of the rat. Gastroenterology. 1992;102(2):497-504.

64. Urban E, Zingery AA, Michel AM, Whitney SC. Permeability of polyethylene glycol in remnant small bowel after massive intestinal resection. Proceedings Of The Society For Experimental Biology And MedicineSociety For Experimental Biology And Medicine (New York, NY). 1983;172(2):207-13.

65. Sigalet DL, Martin GR, Poole A. Differential sugar absorption as a marker for adaptation in short bowel syndrome. Journal of pediatric surgery. 2000;35(5):661-4.

66. Salles Teixeira TF, Boroni Moreira AP, Silva Souza NC, Frias R, Gouveia Peluzio MdC. Intestinal permeability measurements: general aspects and possible pitfalls. Nutricion Hospitalaria. 2014;29(3):269-81 13p.

67. Chiba M, Sanada Y, Toki A. Markers of enteral adaptation in pediatric short bowel syndrome. Pediatr Int. 2017;59(8):911-6.

68. Bourdon A, Rougé C, Legrand A, Robert CD, Piloquet H, Vodovar M, et al. Urinary citrulline in very low birth weight preterm infants receiving intravenous nutrition. British Journal of Nutrition. 2012;108(7):1150-4 5p.

69. Fitzgibbons S, Ching YA, Valim C, Zhou J, Iglesias J, Duggan C, et al. Relationship between serum citrulline levels and progression to parenteral nutrition independence in children with short bowel syndrome. J Pediatr Surg. 2009;44(5):928-32.

70. Luo M, Fernández-Estívariz C, Manatunga AK, Bazargan N, Gu LH, Jones DP, et al. Are plasma citrulline and glutamine biomarkers of intestinal absorptive function in patients with short bowel syndrome? JPEN Journal of Parenteral & Enteral Nutrition. 2007;31(1):1-7 p.

71. Peters JH, Wierdsma NJ, Teerlink T, van Leeuwen PA, Mulder CJ, van Bodegraven AA. Poor diagnostic accuracy of a single fasting plasma citrulline concentration to assess intestinal energy absorption capacity. Am J Gastroenterol. 2007;102(12):2814-9.

72. Peters JHC, Mulder CJJ, van Bodegraven AA. Fasting plasma citrulline concentrations do not reflect intestinal absorption capacity. JPENJournal Of Parenteral And Enteral Nutrition. 2008;32(3):288; author reply 9.

73. Poole A, Deane A, Summers M, Fletcher J, Chapman M. The relationship between fasting plasma citrulline concentration and small intestinal function in the critically ill. Crit Care. 2015;19:16.

74. Urschel KL, Rafii M, Pencharz PB, Ball RO. A multitracer stable isotope quantification of the effects of arginine intake on whole body arginine metabolism in neonatal piglets. Am J Physiol Endocrinol Metab. 2007;293(3):E811-8.

75. Wilkinson DL, Bertolo RF, Brunton JA, Shoveller AK, Pencharz PB, Ball RO. Arginine synthesis is regulated by dietary arginine intake in the enterally fed neonatal piglet. Am J Physiol Endocrinol Metab. 2004;287(3):E454-62.

76. Ball RO, Urschel KL, Pencharz PB. Nutritional consequences of interspecies differences in arginine and lysine metabolism. J Nutr. 2007;137(6 Suppl 2):1626S-41S.

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

78. Brunton JA, Bertolo RF, Pencharz PB, Ball RO. Proline ameliorates arginine deficiency during enteral but not parenteral feeding in neonatal piglets. Am J Physiol. 1999;277(2 Pt 1):E223-31.

79. Urschel KL, Shoveller AK, Uwiera RR, Pencharz PB, Ball RO. Citrulline is an effective arginine precursor in enterally fed neonatal piglets. J Nutr. 2006;136(7):1806-13.

80. Urschel KL, Shoveller AK, Pencharz PB, Ball RO. Arginine synthesis does not occur during first-pass hepatic metabolism in the neonatal piglet. Am J Physiol Endocrinol Metab. 2005;288(6):E1244-51.

81. Bertolo RF, Brunton JA, Pencharz PB, Ball RO. Arginine, ornithine, and proline interconversion is dependent on small intestinal metabolism in neonatal pigs. Am J Physiol Endocrinol Metab. 2003;284(5):E915-22.

82. Wakabayashi Y, Yamada E, Yoshida T, Takahashi H. Arginine becomes an essential amino acid after massive resection of rat small intestine. J Biol Chem. 1994;269(51):32667-71.

83. Dejong CH, Welters CF, Deutz NE, Heineman E, Soeters PB. Renal arginine metabolism in fasted rats with subacute short bowel syndrome. Clin Sci (Lond). 1998;95(4):409-18.

84. Sangild PT. Gut Responses to Enteral Nutrition in Preterm Infants and Animals. Experimental biology and medicine -maywood nj-. 2006;231(11):1695-711.

85. Turner JM, Wales PW, Nation PN, Wizzard P, Pendlebury C, Sergi C, et al. Novel neonatal piglet models of surgical short bowel syndrome with intestinal failure. Journal of Pediatric Gastroenterology & Nutrition. 2011;52(1):9-16 8p.

86. Moughan PJ, Birtles MJ, Cranwell PD, Smith WC, Pedraza M. The piglet as a model animal for studying aspects of digestion and absorption in milk-fed human infants. World review of nutrition and dietetics. 1992;67:40-113.

87. Jiang PP, Sangild PT. Intestinal proteomics in pig models of necrotising enterocolitis, short bowel syndrome and intrauterine growth restriction. Proteomics clinical applications. 2014;8(9-10):700-14.

88. Wykes LJ, Ball RO, Pencharz PB. Development and validation of a total parenteral nutrition model in the neonatal piglet. Journal of nutrition. 1993(7).

89. Duro D, Kamin D, Duggan C. Overview of pediatric short bowel syndrome. J Pediatr Gastroenterol Nutr. 2008;47 Suppl 1:S33-6.

90. Urschel KL, Evans AR, Wilkinson CW, Pencharz PB, Ball RO. Parenterally fed neonatal piglets have a low rate of endogenous arginine synthesis from circulating proline. J Nutr. 2007;137(3):601-6.

91. Ligthart-Melis GC, van de Poll MC, Dejong CH, Boelens PG, Deutz NE, van Leeuwen PA. The route of administration (enteral or parenteral) affects the conversion of isotopically labeled L-[2-15N]glutamine into citrulline and arginine in humans. JPEN J Parenter Enteral Nutr. 2007;31(5):343-48; discussion 9-50.

92. Lim DW, Levesque CL, Vine DF, Muto M, Koepke JR, Nation PN, et al. Synergy of glucagon-like peptide-2 and epidermal growth factor coadministration on intestinal adaptation in neonatal piglets with short bowel syndrome. Am J Physiol Gastrointest Liver Physiol. 2017;312(4):G390-G404.

93. O'Keefe SJ, Buchman AL, Fishbein TM, Jeejeebhoy KN, Jeppesen PB, Shaffer J. Short bowel syndrome and intestinal failure: consensus definitions and overview. Clin Gastroenterol Hepatol. 2006;4(1):6-10.

94. Sukhotnik I, Siplovich L, Shiloni E, Mor-Vaknin N, Harmon CM, Coran AG. Intestinal adaptation in short-bowel syndrome in infants and children: a collective review. Pediatr Surg Int. 2002;18(4):258-63.

95. Reinshagen K, Adams R, Trunk M, Wessel LM. The chronic liver disease in patients with short bowel syndrome: etiology and treatment. Minerva Pediatr. 2009;61(3):273-81.

96. Crenn P, Coudray-Lucas C, Cynober L, Messing B. Post-absorptive plasma citrulline concentration: a marker of intestinal failure in humans. Transplant Proc. 1998;30(6):2528.

97. Crenn P, Messing B, Cynober L. Citrulline as a biomarker of intestinal failure due to enterocyte mass reduction. Clin Nutr. 2008;27(3):328-39.

98. Josephson J, Turner JM, Field CJ, Wizzard PR, Nation PN, Sergi C, et al. Parenteral Soy Oil and Fish Oil Emulsions: Impact of Dose Restriction on Bile Flow and Brain Size of Parenteral Nutrition-Fed Neonatal Piglets. JPEN J Parenter Enteral Nutr. 2015;39(6):677-87.

99. Crenn P, Vahedi K, Lavergne-Slove A, Cynober L, Matuchansky C, Messing B. Plasma citrulline: A marker of enterocyte mass in villous atrophy-associated small bowel disease. Gastroenterology. 2003;124(5):1210-9.

100. Crenn P, Coudray–Lucas C, Thuillier F, Cynober L, Messing B. Postabsorptive plasma citrulline concentration is a marker of absorptive enterocyte mass and intestinal failure in humans. Gastroenterology. 2000;119(6):1496-505.

101. Curis E, Crenn P, Cynober L. Citrulline and the gut. Curr Opin Clin Nutr Metab Care. 2007;10(5):620-6.

102. Fragkos KC, Forbes A. Citrulline as a marker of intestinal function and absorption in clinical settings: A systematic review and meta-analysis. United European Gastroenterol J. 2018;6(2):181-91.

103. Gutierrez IM, Fisher JG, Ben-Ishay O, Jones BA, Kang KH, Hull MA, et al. Citrulline levels following proximal versus distal small bowel resection. J Pediatr Surg. 2014;49(5):741-4.

104. Stultz JS, Tillman EM, Helms RA. Plasma citrulline concentration as a biomarker for bowel loss and adaptation in hospitalized pediatric patients requiring parenteral nutrition. Nutr Clin Pract. 2011;26(6):681-7.

105. Diamanti A, Panetta F, Gandullia P, Morini F, Noto C, Torre G, et al. Plasma citrulline as marker of bowel adaptation in children with short bowel syndrome. Langenbeck's Archives of Surgery. 2011(7):1041.

106. Bailly-Botuha C, Colomb V, Thioulouse E, Berthe MC, Garcette K, Dubern B, et al. Plasma citrulline concentration reflects enterocyte mass in children with short bowel syndrome. Pediatr Res. 2009;65(5):559-63.

107. Rabier D, Kamoun P. Metabolism of citrulline in man. Amino Acids. 1995;9(4):299-316.

108. Fjermestad H, Hvistendahl M, Jeppesen PB. Fasting and Postprandial Plasma Citrulline and the Correlation to Intestinal Function Evaluated by 72-Hour Metabolic Balance Studies in Short Bowel Jejunostomy Patients With Intestinal Failure. JPEN J Parenter Enteral Nutr. 2017:148607116687497.

109. Lauverjat M, Hadj Aissa A, Vanhems P, Boulétreau P, Fouque D, Chambrier C. Chronic dehydration may impair renal function in patients with chronic intestinal failure on long-term parenteral nutrition. Clinical Nutrition. 2006;25(1):75-81.

110. Melis GC, Boelens PG, van der Sijp JRM, Popovici T, De Bandt J-P, Cynober L, et al. The feeding route (enteral or parenteral) affects the plasma response of the dipetide Ala-Gln and the amino acids

glutamine, citrulline and arginine, with the administration of Ala-Gln in preoperative patients. British Journal of Nutrition. 2007;94(1):19-26.

111. Lim DW, Wales PW, Mi S, Yap JY, Curtis JM, Mager DR, et al. Glucagon-Like Peptide-2 Alters Bile Acid Metabolism in Parenteral Nutrition--Associated Liver Disease. JPEN J Parenter Enteral Nutr. 2016;40(1):22-35.

112. Amin SC, Pappas C, Iyengar H, Maheshwari A. Short Bowel Syndrome in the Nicu. Clinics in perinatology. 2013;40(1):10.1016/j.clp.2012.12.003.

113. Wang WW, Qiao SY, Li DF. Amino acids and gut function. Amino Acids. 2009;37(1):105-10.

114. Morris JSM. Arginine: beyond protein. The American Journal of Clinical Nutrition. 2006;83(2):508S-12S.

115. Wu G. Amino acids: metabolism, functions, and nutrition. Amino Acids. 2009;37(1):1-17.

116. Polycarpou E, Zachaki S, Tsolia M, Papaevangelou V, Polycarpou N, Briana Despina D, et al. Enteral L-Arginine Supplementation for Prevention of Necrotizing Enterocolitis in Very Low Birth Weight Neonates. Journal of Parenteral and Enteral Nutrition. 2013;37(5):617-22.

117. Richir MC, Siroen MP, van Elburg RM, Fetter WP, Quik F, Nijveldt RJ, et al. Low plasma concentrations of arginine and asymmetric dimethylarginine in premature infants with necrotizing enterocolitis. Br J Nutr. 2007;97(5):906-11.

118. Oliveira C, de Silva NT, Stanojevic S, Avitzur Y, Bayoumi AM, Ungar WJ, et al. Change of Outcomes in Pediatric Intestinal Failure: Use of Time-Series Analysis to Assess the Evolution of an Intestinal Rehabilitation Program. J Am Coll Surg. 2016;222(6):1180-8 e3.

119. Wu G, Morris SM, Jr. Arginine metabolism: nitric oxide and beyond. Biochem J. 1998;336 (Pt 1):1-17.

120. Di Lorenzo M, Bass J, Krantis A. Use of l-arginine in the treatment of experimental necrotizing enterocolitis. Journal of Pediatric Surgery. 1995;30(2):235-41.

121. Mitchell K, Lyttle A, Amin H, Shaireen H, Robertson HL, Lodha AK. Arginine supplementation in prevention of necrotizing enterocolitis in the premature infant: an updated systematic review. BMC Pediatrics. 2014;14:226.

122. Burrin DG, Stoll B, Jiang R, Chang X, Hartmann B, Holst JJ, et al. Minimal enteral nutrient requirements for intestinal growth in neonatal piglets: how much is enough? Am J Clin Nutr. 2000;71(6):1603-10.

123. Bollhalder L, Pfeil AM, Tomonaga Y, Schwenkglenks M. Meta-analyses: A systematic literature review and meta-analysis of randomized clinical trials of parenteral glutamine supplementation. Clinical Nutrition. 2013;32:213-23.

124. García-de-Lorenzo A, Zarazaga A, García-Luna PP, Gonzalez-Huix F, López-Martínez J, Miján A, et al. Clinical evidence for enteral nutritional support with glutamine:: A systematic review. Nutrition. 2003;19(9):805-11.

125. Field CJ, Johnson IAN, Pratt VC. Glutamine and arginine: immunonutrients for improved health. Medicine & Science in Sports & Exercise. 2000;32(7).

126. Carroll RE, Benedetti E, Schowalter JP, Buchman AL. Management and Complications of Short Bowel Syndrome: an Updated Review. Curr Gastroenterol Rep. 2016;18(7):40.

127. Young VR, Yu Y-M, Krempf M. 2 - Protein and Amino Acid Turnover Using the Stable Isotopes 15N, 13C, and 2H as Probes. New Techniques in Nutritional Research: Academic Press; 1991. p. 17-72.

128. Silk DB, Perrett D, Clark ML. Jejunal and ileal absorption of dibasic amino acids and an arginine-containing dipeptide in cystinuria. Gastroenterology. 1975;68(6):1426-32.

129. Sangild PT, Thymann T, Schmidt M, Stoll B, Burrin DG, Buddington RK. Invited review: the preterm pig as a model in pediatric gastroenterology. J Anim Sci. 2013;91(10):4713-29.

130. Sodhi C, Richardson W, Gribar S, Hackam DJ. The development of animal models for the study of necrotizing enterocolitis. Disease Models & Mechanisms. 2008;1(2-3):94-8.

131. Sulistyo A, Rahman A, Biouss G, Antounians L, Zani A. Animal models of necrotizing enterocolitis: review of the literature and state of the art. Innovative Surgical Sciences2018. p. 87.

132. Kosar C, Steinberg K, de Silva N, Avitzur Y, Wales PW. Cost of ambulatory care for the pediatric intestinal failure patient: One-year follow-up after primary discharge. J Pediatr Surg. 2016;51(5):798-803.

Appendix

Table 8: qPCR enzymes primer and probes sequences

Arginase II	Forward	CAAGCCTTGTATCTCTTCCCC
	Probe	ACTAAGAGACGTGGACCCTCCTGAA
	Reverse	GTCTGTTCCATGACCTTCTGG
Ornithine decarboxylase	Forward	GGGCGAGGGATCTTGATATTG
	Probe	ACGTGGGAAGTGGCTGTACTGAC
	Reverse	AGATACATGCTGAAACCGACC
Ornithine aminotransferase	Forward	CCCACAACTACCATCCTTTACC
	Probe	ACATCCCAAACGTAAACACTTTCCCTCT
	Revere	GAAGTCGAAGTATCTCCTGCC
Arginosuccinase synthase	Forward	ACGCTCATTTAGACATCGAGG
	Probe	TGTACACCAGCTCGGCGAATTTCA
	Reverse	TGGCGGACAAATTCACACTC
Pyrroline-5-carboxylate reductase	Forward	CCCAGCCCATCAAGAAGA
	Probe	CTTCCGGTCCTTGCTCATC
	Reverse	AGCTTCACCTTGTCCAGAAC
Gapdh	Forward	CACTCTTCCACTTTTGATGCTG
	Probe	ACCACTTCGTCAAGCTCATTTCCTGT
	Reverse	CCTGTTGCTGTAGCCAAATTC

Table 9: Day 0 Serum amino acid data in piglets fed arginine deficient diet (μM)

	20% EN Fed		40% EN Fed		Total Std. error	P-Value	Sow Fed control	
	Sham	JC	Sham	JC				
	Mean	Mean	Mean	Mean			Mean	Std. Error
Methionine	-39.2[a]	46.6[b]	-32.0[a]	-4.4 [ab]	11.1	0.008	13.6	15.9
Alanine	-485.9	-252.9	-662.0	-459.2	71.7	0.19	-143.7	135.1
Asparagine	-120.6	-50.8	-135.4	-66.4	15.9	0.14	-14.1	19.3
Glycine	-296.8	82.0	103.0	-54.4	96.1	0.42	361.7	164.9
Leucine	0.5	60.3	80.8	106.0	15.0	0.10	88.3	25.8
Lysine	64.9	152.0	215.1	203.1	27.3	0.20	71.0	29.5
Phenylalanine	9.7	16.8	25.8	45.0	4.5	0.08	38.5	9.8
Serine	9.0	164.9	31.6	36.0	27.5	0.13	45.8	26.2
Tyrosine	-114.2 [ab]	-29.3[b]	-144.7 [a]	-74.0 [ab]	16.6	0.04	39.5	17.2
Valine	-140.0	-27.3	-82.2	-13.0	25.7	0.32	28.3	24.3

Jejunocolic- JC
Superscripts represent differences between treatment groups

Table 10: Day 7 Serum amino acids in piglets fed arginine deficient diet (μM)

	20% EN fed		40% EN fed		Total Std. Error	P-Value	Sow fed control	
	Sham	JC	Sham	JC				
	Mean	Mean	Mean	Mean			Mean	Std. Error
Methionine	28.1[a]	101.4[b]	70.2[ab]	84.5[ab]	8.1	0.003	133.3	9.1
Alanine	350.9[a]	467.6[ac]	726.0[b]	669.0[bc]	37.4	0.0001	694.5	58.5
Asparagine	22.1[a]	41.3 [b]	36.3[ab]	49.1[b]	2.7	0.005	134.1	15.1
Glycine	704.1[a]	787.9 [a]	1858.0[b]	1005.6[ab]	148.5	0.008	1506.7	209.4
Leucine	110.3[a]	129.7 [a]	258.4 [b]	244.8 [b]	16.2	0.0001	378.5	30.5
Lysine	241.4[a]	262.9 [a]	495.9 [b]	324.8 [ab]	27.1	0.0001	282.8	50.3
Phenylalanine	32.8 [a]	32.8 [a]	71.0 [b]	84.0 [b]	5.1	0.0001	115.2	7.5
Serine	269.5[a]	332.1 [a]	492.4 [b]	415.6 [ab]	23.3	0.001	382.2	10.9
Tyrosine	40.1 [a]	66.5 [a]	62.7 [a]	111.8 [b]	5.3	0.000	306.2	26.5
Valine	104.3[a]	132.1[ab]	177.4 [b]	176.6[ab]	9.7	0.013	270.0	17.5

Jejunocolic- JC

Superscripts represent differences between treatment groups