Novel Treatments for Intestinal Failure Explored in Neonatal Piglets with Short Bowel Syndrome: Focusing on the Microbiome, Sepsis and Trophic Factors

by

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Abstract

Short bowel syndrome (SBS) is the leading cause of intestinal failure (IF) in infants, and occurs when there is a significant loss of intestine due to acquired or congenital reasons, leading to infants being unable to absorb sufficient nutrients for survival and growth. SBS traditionally had a mortality rate of 25-50%, with infants dying from complications of parenteral nutrition (PN). Over the last 25 years, this has drastically changed due to multidisciplinary IF teams, changes in PN, home parenteral nutrition (HPN) and antibacterial locks. In order for these infants to survive, the remnant bowel needs to undergo a compensatory process termed adaptation, with structural and functional changes of the intestine to improve nutrient absorption. For the risks associated with PN to be eliminated, children need to undergo adaptation and achieve enteral autonomy.

Glucagon-like peptide-2 (GLP-2) is produced by enteroendocrine L cells of the distal bowel and has been shown to stimulate intestinal adaptation. While GLP-2 analogues have helped SBS patients decrease PN volume, along with some patients achieve enteral autonomy, the mechanism by which GLP-2 exerts its intestinotrophic effects is still not fully understood. Insulin-like growth factor-1 (IGF-1) is one of the most abundant hormones in human milk and colostrum, and importantly the placenta secretes IGF-1 and levels increase in the infant at birth. IGF-1 is expressed primarily in the subepithelial myofibroblasts close to the GLP-2 receptors, and IGF-1 receptors are found in the epithelial crypt cells that are the drivers of villus lengthening and adaptation.

Every time a child with SBS becomes septic, this negatively impacts their enteral tolerance and therefore their gut adaptation. Sepsis in SBS children comes from central line

associated bloodstream infections (CLABSI), both from line contamination and potentially gut bacterial translocation. Many patients with SBS suffer from dysbiosis as a result of prematurity, prolonged hospitalization, recurrent antibiotics and altered intestinal anatomy. Probiotics have been suggested as a way to modify the microbiome in SBS but there have been few studies to date with mixed results. CLABSI risk is modulated with the use of antibacterial locks.

In these studies, we investigate novel treatment approaches to promote adaptation, and hence autonomy from PN, with probiotics and trophic factors, along with strategies to reduce CLABSI and maintaining catheter patency while on PN, including with a novel locking solution.

Studies were undertaken in a surgical animal model of SBS, with a 75% small bowel resection, with a jejunocolic anastomosis (JC). Piglets had a jugular venous catheter inserted for total PN (TPN) and a gastric tube for enteral nutrition (EN). Piglets were maintained in laboratory for 7-14 days. Structural adaptation was measured via bowel length, mucosal and intestinal weight and histopathology. Functional adaptation was measured via fat absorption and Üssing chamber. Trophic factor signalling was assessed using relative gene expression of tight junction proteins, trophic factors and their associated receptors. The microbiome structure was determined via PCR, while the microbiome function was determined via short chain fatty acid (SCFA) analysis by gas chromatography.

Our results demonstrated the benefits for catheter patency and reduced CLABSI with the use of a novel locking solution. We identified potential benefits of using probiotics in SBS, both for adaptation and modulating dysbiosis. We confirmed the benefits of GLP-2 analogues

for intestinal adaptation, but highlighted this is particularly when longer acting analogues are used and is not augmented by the use of IGF-1.

These studies of novel treatments in piglets demonstrate the importance of translational research in developing and testing new approaches for SBS. Moving forward, these studies will provide important preclinical data for strategies of modulating dysbiosis, CLABSI and adaptation for infants and children with SBS.

Preface

This thesis is an original work by Mirielle Lauren Pauline. The research project, of which this thesis is a part, obtained ethics approval from the University of Alberta Animal Care and Use Committee, study titles “Improving adaptation and outcomes in short bowel syndrome: studies in neonatal piglets”, study ID AUP00000155 and “Novel parenteral lipids for neonatal intestinal failure: studied in total parenterally fed piglets”, study ID AUP00003707.

Portions of the research conducted for this research form part of an institutional and international research collaboration, led by Dr. Justine M. Turner at the University of Alberta and Dr. Paul W. Wales at the University of Cincinnati. The literature review in chapters 1-4 represents my original work. Chapter 5 is materials and methods used broadly throughout the thesis.

Chapter 6 of this thesis has been submitted and peer-reviewed by the Journal of Parenteral and Enteral Nutrition (JPEN) and the requested revisions are being drafted for submission, Mirielle L. Pauline, Evan Labonne, Pamela R. Wizzard, Justine Turner, Paul W. Wales, “Association between 4%-tetrasodium EDTA and sepsis in neonatal piglets: A retrospective cohort study” This study was a retrospective review, designed by myself with assistance of J.M Turner and P.W. Wales. I was responsible for data review, analysis and manuscript composition. All authors contributed to the manuscript editing.

Chapter 7 of this thesis has been published by Mirielle Pauline, Janelle Fouhse, Tierah Hinchliffe, Pamela Wizzard, Patrick Nation, Hien Huynh, Paul Wales, Benjamin Willing and Justine Turner, “Probiotic treatment vs empiric oral antibiotics for managing dysbiosis in short bowel syndrome: Impact on the mucosal and stool microbiota, short-chain fatty acids, and

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Chapter 8 has been published by Mirielle Pauline, Patrick Nation, Pamela Wizzard, Tierah Hinchliffe, Tong Wu, Violetta Dimitriadou, Justine Turner and Paul Wales, "Comparing the intestinotrophic effects of 2 glucagon-like peptide-2 analogues in the treatment of short-bowel syndrome in neonatal piglets." *JPEN. Journal of parenteral and enteral nutrition*. First published online May 2020. Published in issue March 2021. Volume 45 issue 3. Pg 538-545. https://doi.org/10.1002/jpen.1853. J.M. Turner and P.W. Wales contributed to the conception and design of the research. I was responsible for data collection (assisted by P.R. Wizzard, T. Hinchliffe and T. Wu) and analysis and manuscript composition. V. Dimitriadou performed the pharmacokinetic study. Myself under supervision of P. Wizzard performed the fat absorption analysis. J.M. Turner, V. Dimitriadou and P.W. Wales contributed to the editing of the manuscript.

Chapter 9 has been completed and the manuscript is under revisions to be submitted to JPEN by Mirielle Pauline, Arjuna Srikrishnaraj, Pamela Wizzard, Caitlin Huynh, Rohan Persad, Evan Labonne, Kun Wang, Patrick Nation, Donna Vine, Patricia Brubaker, Paul Wales and Justine Turner, "Independent of its potential role in glucagon-like peptide-2 signaling, insulin-like growth factor-1 therapy has no additional benefit for intestinal adaptation in short bowel

piglets". This study was designed by myself and J.M Turner and P.W. Wales. I was responsible for data collection (assisted by P.R. Wizzard, C. Huynh, R. Persad and E. Labonne) and analysis and manuscript composition. The Üssing was completed by Kun Wang under supervision of D. F. Vine. A. Srikrishnaraj performed the gene expression analysis on intestinal growth factors and their receptors with supervision by P.L. Brubaker at the University of Toronto. P.N. Nation performed the analysis of histological specimens.

Dedication

I dedicate this thesis to my father:

Thank you for instilling my love of science into me. From chemistry sets, microscopes and just being my biggest supporter. You made me who I am.

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Thank you to Dr. Justine Turner and Dr. Paul Wales for providing me with the outstanding opportunity to purse a graduate degree in their lab, and for mentoring me on both my professional and personal growth and development. I feel unbelievably lucky to have ended up in this laboratory, and to have two extremely dedicated supervisors who helped instill this passion for research into me. Most importantly they both believed in me and my potential before I believed in myself, and I couldn't be more grateful for them pushing and encouraging me throughout the program. Thank you to Dr. Benjamin Willing for sharing his passion and understanding of the microbiome with me. Thank you to Dr. Hien Huynh for all his feedback throughout my program. Thank you to Dr. Sujata Persad for all your guidance throughout the years.

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Chapter 1. Neonatal Short Bowel Syndrome

1 Introduction:

Short bowel syndrome (SBS) is the leading cause of neonatal intestinal failure (IF) and traditionally had a mortality rate of 25-50%.[1] This has improved drastically due to the implementation of multidisciplinary teams, with an emphasis on strategies to ameliorate hepatic dysfunction.[2, 3] Parenteral nutrition (PN) is essential for survival and delivery of nutrition to these infants, yet comes with the risk of sepsis and cholestatic liver disease.[2] Sepsis negatively impacts enteral tolerance and gut adaptation in these infants with SBS, and adversely impacts time to achieving autonomy from PN, the ultimate goal of treatment.[4] While previously the goal of SBS treatment was to improve survival, now there needs to be an emphasis on promoting adaptation, preventing life-threatening sepsis and improving patient quality of life. This first chapter is a review of neonatal SBS, with a view to identifying areas to target for possible improvement with novel treatments, including etiology, nutrition, treatment, adaptation and quality of life parameters.

1.1 Definition

In order to review the literature on short bowel syndrome and intestinal failure, first an appropriate definition of SBS and IF is required. Unfortunately, a concise definition can be quite elusive. Some experts in the field have preferred “intestinal failure results from obstruction, dysmotility, surgical resection, congenital defect, or disease-associated loss of absorption and is characterized by the inability to maintain protein-energy, fluid, electrolyte, or micronutrient balance.”[5] Other definitions include “the impaired absorption of nutrients resulting in the failure to meet requirements for growth and development.”,[2] although this is also a definition

of IF. These matters of debate often come down to defining SBS and IF by either fecal energy loss, residual bowel length or the amount of parenteral nutrition required.[6] There are nutritional and anatomic criteria, but function has recently come up for discussion as a definition for IF and SBS.[6] Recently, pediatric IF has been defined as "the reduction of functional intestinal mass below that which can sustain life, resulting in dependence on supplemental parenteral support for a minimum of 60 days within a 74 consecutive day interval."[7] While SBS occurs secondary to IF, and is a subcategory of IF, due to loss of small bowel from congenital absence or surgical resection.[7]

With many different heterogenous aspects of SBS and IF and the previous lack of a concise definition, reviewing the literature can be confusing. Due to this, the current literature review will consider anatomy, etiology and nutrition in SBS as variably defined. This literature review and introduction aims to look at the different aspects of SBS and to evaluate based on the current literature the importance of each of these factors.

1.2 Anatomical Considerations

SBS within its name describes how this syndrome often begins with a massive small bowel resection, due to different reasons and often during the neonatal period.[4] The importance of length of the bowel as well as the site of remnant bowel has been of great debate. However, is more bowel length or bowel function more important? We will review literature in this regard, however first we need to understand the 3 anatomical subtypes of SBS (**Figure 1-1.**). Type 3, is with an intact colon with a small bowel anastomosis and typically some ileum is still present.[8] Type 2 is a small bowel resection with a partial colon resection with a

resulting jejunocolic anastomosis.[8] Type 1 is the most severe, with a small bowel resection and a high output jejunostomy.[8] The way type 1-3 is done throughout the literature can vary and the anatomy must be clarified to better understand the results. The area of SBS anatomy and definitions differ between pediatric versus adult SBS in terms of using length. For adults, length is used, while in neonates and infants this is challenging due to infant developmental changes and intestinal growth that varies with gestational age.[9] While values for length were often determined based on post-mortem data, the greatest predictor for length for infants based on prospective studies has been determined to be height at surgery, over age and weight.[9]

A single center retrospective cohort study by Belza et al. at SickKids in Toronto has examined the relationship between residual anatomy and independence from parenteral nutrition (enteral autonomy).[4] Here the authors analyzed 120 infants less than 12 months old with SBS between 2006 and 2013.[4] One of the authors goals was to see if enteral autonomy was achieved in these patients and what intestinal failure related factors, with a particular focus on anatomy, were predictors of achieving autonomy. The results show that 70% of the infants achieved enteral autonomy.[4] The authors findings suggest that "for every additional 1% of expected small bowel a child possesses, the odds of weaning from parenteral support increases by 3%."[4] The remnant colon must also be considered. The colon not only contributes to water and sodium absorption, but also the release of trophic factors like glucagon-like peptide-2 (GLP-2).[4] In this study the percentage of residual colon remaining was statistically significant in predicting the achievement of enteral autonomy.[4] Compared to the finding stated previously for small bowel percent, when infants had less than 50% of their small bowel, the colon then played a more important role in weaning rates from PN.[4] Patients with only 10% of

expected small intestine could achieve autonomy if the colon remnant was greater than 50%; but the median time to achieve enteral autonomy was higher, being over 6 years.[4]

In a single center retrospective review at Boston Children's hospital looking at neonates with SBS, intestinal length was found to be the primary predictor of weaning from PN.[10] In this study, patients who weaned had a mean small intestinal length of around 55cm, compared with 27cm in those who were still dependent on PN.[10] Interestingly for etiology, the frequency of gastroschisis was 3% in PN independent versus 27% in PN dependent patients.[10] Gastroschisis is a motility disorder so this brings into question the role of intestinal function in SBS.

In another retrospective review of pediatric patients with SBS, the authors looked into predictors of mortality within SBS patients.[11] In this study, cholestasis and age-adjusted small bowel length were the two major predictors of mortality in pediatric SBS.[11] The authors found that the percentage of small bowel length, rather than absolute length predicted survival of neonates with SBS. Patients having 10% or more of the expected small bowel length for gestational age were more likely to survive and wean from PN than those with less than 10%.[11] This paper also brings up the concept of the ileocecal valve (ICV). In this study the presence of the ICV was a primary predictor of weaning from PN.[11] Now, one could argue that the presence of the ICV means that there is potentially remaining colon that may contribute to increased absorptive function and adaptation.[11] Due to the strong correlation between reduced colonic length versus loss of the ICV due to the anatomy of the ICV to the colon, it was not possible to distinguish between the effects of these 2 covariates.[11]

The ileum may also be more likely present when ICV is intact and ileum has been shown to have greater potential for adaptation than the jejunum.[12] To understand this one has to look

at the structure of the intestine, with the length of the villi decreasing from proximal to distal small bowel, along with typically the proximal jejunum having a larger proportion of luminal nutrients than the distal ileum.[12, 13] In fact, in rat small intestine, the villi were nearly three times as tall in the upper duodenum comparted to the terminal ileum.[13] Interestingly, when ileal segments were inserted into the jejunum, the villi enlarged to the size of the jejunal villi in that area, and vice versa, jejunum into the ileum actually had decreased villi to the local environment.[13] This demonstrated that possibly the type of chyme present, either jejunal or ileal actually influenced the length of the villus present.[13] Similarly, the greater the amount of tissue resected, the greater the correlated adaptation.[12]

Finally, in a retrospective record review of neonates with SBS, the authors found that enteral feeding of breast milk and amino-acid based formula, as well with residual bowel length were associated with a shorter duration of PN.[14] Here, the authors found that presence of an intact ICV and frequency of catheter related infections were not significantly correlated with duration of PN.[14] When the authors performed multivariate analysis, only residual small bowel length was a significant independent predictor of duration of PN.[14] This study did not look into remnant large bowel and is a limitation of the study. As noted, the presence of the ICV may lead to better outcomes due to the presence of greater remnant colon length.

As eluded to previously, the role of the ICV has been controversial, with some studies showing increased time on PN without the ICV,[15, 16] but other studies finding no correlation.[11, 14] As mentioned previously, often there is a strong correlation between reduced colon length and loss of the ICV making it hard to distinguish if this increased time on PN is due to the importance of the valve or due to reduced colon length influencing this increased time on PN.[11]

It appears that when studied more vigorously, that the valve is simply a proxy for the colon length and the direct importance of the ICV is yet to be determined.[11, 14, 17]

1.3 The Process of Adaptation

Intestinal adaptation is an innate compensatory process, involving structural and functional changes to improve this reduced nutrient and fluid absorption of the remnant bowel due to resection.[12] Structural changes have been best demonstrated by animal studies,[2] including bowel lengthening, thickening, increased muscle calibre, along with the gold standard of increased villus height and crypt depth.[12] Functional changes involve increased nutrient transporter expression of the remnant bowel, increased crypt cell differentiation and slowed transit time.[12] The largest factor that stimulates adaptation, is the use of EN for luminal stimulation of the bowel.[12] Other factors are the amount of bowel resected and the anatomy left, along with the use of intestinotrophic factors.[12]

Following resection, the remnant small bowel with continue its self-renewal of the crypt cells in a process of crypt cell proliferation, migration and differentiation into the specialized mucosal cells of enterocytes, enteroendocrine cells, goblet cells and Paneth cells.[12] Adaptation mostly affects this rate of crypt cell proliferation, leading to the increased crypt depth and villus height that are our gold standard when measuring intestinal adaptation in animal models.[12] There is also an increase in angiogenesis to the remnant bowel, with increased oxygen and blood flow.[12] The bowel also dilates, leading to an increased surface area for contact of nutrients, a structural change, with slowed transit time for increased contact time, a functional

change.[12] For nutrient transporters and exchangers, there is an increase in sodium glucose cotransporters, Na^+/H^+ exchangers and Na^+/K^+ adenosine triphosphatases.[12]

1.3.1 Enteral Nutrition in Adaptation

Enteral nutrition (EN) is arguably the most important factor in gut adaptation. In adults given exclusively TPN for 14 days there was reduced total mucosal thickness, that could potentially cause increased intestinal permeability.[18] There was also a significant reduction in villus height.[19] EN was able to restore these changes[19] and is required to prevent this mucosal atrophy. These patients also had increased intestinal permeability with TPN, leading to the potential for bacterial translocation and luminal antigens to cross the gut barrier.[19] The decreased enzyme activity of the brush border, most substantially the disaccharidases has been demonstrated in adults on TPN as well.[20] After one week of TPN in rats compared to rats fed orally, there was a decrease in gut weight, mucosal weight, mucosal protein and DNA, along with a decrease in brush border enzymes including maltase, sucrase and lactase.[21]

In children on short-term 1 month use of TPN, this was unable to show much significant differences in brush border enzymes, while long term (greater than 9 months) led to villus atrophy, a decrease in brush border disaccharidase activity and a decrease in thymidine incorporation into DNA, a marker of DNA synthesis and cell proliferation.[22] While in animals this hypoplastic effect of TPN is evident very quickly, in children there is a longer period of TPN required for these significant affects to be seen.[22, 23]

This increased expression of nutrient processing proteins and brush border enzymes of the jejunum compared to ileum is not the only difference between these two sites for adaptation. As stated previously, there is this decrease in villi height from jejunum to ileum,

largely due to the change in nutrient density and composition as luminal contents move down the "healthy" small bowel.[24] This decrease in villi height and shape is even further seen with a decrease in the caliber of the lumen from the proximal to distal small bowel.[24] So therefore there is an importance of luminal nutrients, the complexity of these nutrients, along with gastrointestinal hormones.[24] The role of the intestinotrophic product of fermentation via the microbiota, short chain fatty acids (SCFAs), will be evaluated in a later chapter and not discussed here.

In rats given TPN, by 2 weeks there was a significant decrease in villi height, with increased villi with the use of elemental diet EN, even further increased with the addition of fibre to the EN.[25] This has also been seen in 4 week old piglets with a 75% proximal small bowel resection, where a complex diet of either pig chow or polymeric formula with the addition of fiber was able to have a significant increase in villus height compared to control animals, over that of a less complex diet polymeric formula without fibre or elemental formula.[26]

This brings up the importance of the nutrient composition and complexity, specifically of EN. Since breast-milk is considered the most optimal nutrition for infants, what about the composition of the colostrum on adaptation. In 4 week old piglets with a 75% bowel resection, they were either given pig chow, polymeric infant formula or polymeric infant formula supplemented with colostrum protein concentrate.[27] The piglets that received the colostrum supplemented polymeric formula, had increased villus length and crypt depth in both jejunum and ileum.[27] Colostrum has immunoglobulins, hormones, growth factors, specific proteins and amino acids making colostrum promote adaptation in this piglet model of SBS.[27]

1.4 Clinical features

1.4.1 Etiology:

Knowing the etiology of SBS is important as with each different etiology comes different treatments, complications and potential surgical management. The main causes of SBS in neonates and infants, the focus of this thesis, are congenital and perinatal diseases, including intestinal atresia, gastroschisis, malrotation/volvulus and long-segment Hirschsprung's disease.[8] At least 30% of most reported cases are due to necrotizing enterocolitis, making it the most common cause of SBS.[8] SBS also develops outside of the neonatal period, accounting for approximately 20% of cases.[8]

1.4.2 Presentation, Malabsorption and Malnutrition

To understand how malabsorption and malnutrition occur in SBS it is important to understand the process of digestion and absorption of nutrients under normal physiological state and the site-specific implications of resection. The intestine has to be able to sense the nutrient load and complexity at the different sites along the intestinal tract and lead to neural, hormonal and other responses to aid in the optimal absorption and digestion.[24] This is all largely based on in the normal physiological state there is this increased nutrient load at the proximal bowel, and therefore increased absorptive capacity.[28] There is a release of hormones from the endocrine cells of both the stomach and intestine, with fat being the most effective at stimulating these endocrine cells in the distal duodenum and jejunum.[29] The pyloric sphincter controls the movement of stomach contents into the duodenum, whereas discussed above there is mixing with pancreatic and biliary secretions.[28] Macronutrient digestion and absorption primarily occurs here, due to these bile and pancreatic secretions along with longer villi

containing digestive enzymes and nutrient transporters.[28] Carbohydrates are primarily absorbed by the proximal small bowel, with amylase released by the pancreas for digestion and specific disaccharide and monosaccharide transporters.[28] Now, with more complex carbohydrates these get broken down along the intestine, leading to the production of SCFAs in the colon that will be reviewed later on.[28] Cholecystokinin (CKK) is released from enteroendocrine cells of the duodenum, jejunum and proximal ileum to inhibit gastric emptying and promote satiety, while peptide YY (PYY) is released in response to nutrient stimulation to slow intestinal motility.[28] The distal ileal and colonic L cells produce PYY.[28]

Moving towards the ileum, there are high amounts of glucagon-like peptide-1 (GLP-1) secreting L cells and neurotensin-secreting N cells, to act as an ileal brake, and slow gastric emptying if they sense luminal fats in the ileum, specifically long-chain fatty acids producing the most potent response.[29] The tight junctions of the ileum are less permeable than the jejunum, leading to less water entering the ileum compared to the jejunum when hyperosmotic meals are eaten and therefore helps maintain proper hydration.[28]

Now, the pH of the gut typically increases as we move from proximal to distal bowel, due to the parietal cells of the fundus of the stomach producing hydrochloric acid to denature dietary proteins.[28] In the duodenum, the chyme is mixed with these pancreatic enzymes that increase the pH, including bicarbonate, trypsinogen and chymotrypsinogen.[28] A cascade occurs, where larger more complex oligopeptides are broken down by the brush border enzymes of the jejunum and ileum.[28] Pancreatic lipases mix with emulsified fat in the duodenum where the majority of lipid digestion occurs.[28]

Site specific digestion and absorption is important to understand for why malnutrition and malabsorption occurs with different SBS anatomies. Iron is reduced into its ferrous form and up-taken by transporters found in both the duodenum and proximal jejunum.[28] Lactase is synthesized in the jejunum and proximal ileum, with bile salt resorption in the ileum.[28] The distal ileum is vital for absorption of vitamin B_{12}, while the colon has transporters for folate uptake.[28] Magnesium is absorbed in the distal small bowel and colon.[28]

Therefore, patients with a jejunoileal anastomosis (JI), with the presence of both jejunum, ileum and colon can have transient gastric acid hypersecretion and impaired digestion (**Figure 1-2.**). This is due to a decrease in CCK and secretin feedback inhibition, leading to an increased pH of the proximal small bowel, altering the pancreatic enzymes and impairing digestion.[28] These patients have the presence of ileum, that as discussed has greater innate adaptive capacity than the jejunum, along with the colon that aids in fluid and electrolyte absorption.[28]

In patients with a jejunocolic anastomosis (JC), with loss of ileum that has greater adaptive capacity, it makes sense that these patients have more variable probability of PN dependence.[28] With loss of the ileum, there is increased risk of osmotic diarrhea after hyperosmotic meals due to changes in the tight junction proteins between jejunum and ileum.[28] There is loss of the bile salt resorption and vitamin B_{12} absorption of the ileum as well.[28] If there is partial loss of the colon, diarrhea is further exacerbated, along with the loss of ileum for water absorbing capacity.[28] This altered bile salt resorption can lead to fat malabsorption and choleretic diarrhea as well.[28] With these changes with fat malabsorption, there can be decreased circulating calcium and magnesium leading to decreased oxalate salts,

therefore free oxalate is absorbed and can result in hyperoxaluria and nephrolithiasis.[30] Bile salts help maintain solubility in the gallbladder, so with depleted bile salts this can lead to the precipitation of cholesterol, with cholesterol crystallization leading to gallstone formation.[30]

Patients with an end jejunostomy, lack both the ileum and colon, so they differ from jejunocolic patients largely due to the lack of capacity for the colon for fluid absorption, and therefore have increased stomal output and significant fluid and nutrient malabsorption.[28] Like jejunocolic patients they also have vitamin B_{12} deficiencies and impaired bile salt reabsorption.[28] With loss of distal small bowel and colon they also have a magnesium deficiency.[28]

1.4.3 Complications:

Some definitions of SBS are based on the duration of PN dependency. The issue with this is that a definition based only on length of PN has a selection bias for survivors because patients who die early after massive small bowel resection are excluded.[8] Long term dependency on PN delivered through an intravenous catheter has significant complications, in particular intestinal failure-associated liver disease (IFALD) and sepsis. IFALD was reported previously to develop in 40-60% of infants who require long term TPN.[31]

1.4.3.1 Intestinal Failure Associated Liver Disease:

Since the advent of parenteral nutrition this therapy has been plagued by associated liver disease. The clinical spectrum of IFALD included hepatic steatosis, cholestasis, cholelithiasis and hepatic fibrosis.[31] Hepatic steatosis is also known as fatty liver, it is when the liver weight is composed of more than 5% lipid (mainly triglyceride).[32] Cholestasis is an interruption in bile flow, in the case of intestinal failure often due to an excretory failure of the

hepatocytes with an accumulation of bile constituents in the blood.[33] Cholelithiasis is the formation of gallstones. Liver fibrosis is a scarring process that is non-physiological with excessive extracellular matrix deposition occurring, leading to irreversible tissue damage and disruption of liver function and even liver failure.[34] All of this can progress to cirrhosis and portal hypertension, and then liver failure, with this occurring more commonly in infants and neonates than adults.[35] Portal hypertension is an elevated portal pressure, that is caused by the disruption of hepatic sinusoids that leads to increased resistance in the portal venous system.[36] In 24 neonates with a clinical history of receiving TPN and who had died, autopsies and a medical record review was performed by reviewers blinded to knowledge of liver pathology.[37] The research confirmed the significant relationship between the duration of TPN and liver injury.[37] The severity of fibrosis correlated with the duration of TPN infusion.[37] With more severe cholestasis there was also more severe bile duct proliferation.[37] The authors reported no significant differences for birth weight, gestational age and occurrence of necrotizing enterocolitis or sepsis between groups.[37] This is contrary to many other studies, but, the authors reported patients small for gestational age were found to have more severe liver changes.[37] Infants with an extremely short bowel have the greatest risk for development of IFALD.[38]

In a retrospective study looking at ten children with cholestasis included in a home PN (HPN) program, the study analyzed the influence of lipid emulsions on cholestasis onset.[39] A change in the lipid delivery preceded more than half of the episodes of cholestasis.[39] A temporary decrease in lipid administration lead to normalization of bilirubin in 74% of the

cholestasis episodes.[39] The authors state that in the future the long term use of lipid emulsions containing a lower proportion of polyunsaturated fatty acids need to be investigated.[39]

The lipid composition of PN has had a large impact on SBS patient outcomes. Long chain polyunsaturated fatty acids (LC-PUFAs) are incorporated into cell phospholipid membranes where they are involved in cell signaling, inflammation, blood vessel tone, platelet aggregation and the immune system.[40] The main LC-PUFAs are the down chain product of alpha-linolenic and linoleic acid, omega-3 and omega-6 fatty acids.[40] These LC-PUFAs interact with each other with shared metabolic pathways and a strong negative feedback of the end products.[40] Arachidonic acid, one of the main omega-6 fatty acids, leads to the production of eicosanoids that are pro-inflammatory.[40] In the past, soy-based lipid emulsions were used that had a omega-6 to omega-3 fatty acid ratio of 5.5:1.[40]

1.4.3.2 Central Line Associated Bloodstream Infections

While the previous sections have emphasized the importance of PN for survival for patients with SBS, it comes with the associated risks of sepsis and cholestasis. Specifically, a major source of sepsis arises from central line associated bloodstream infections (CLABSI) from line contamination. When a patient develops CLABSI, often the first step is to remove that central venous catheter (CVC), and as the literature has shown these patients rely on TPN and that CVC to survive.[41] Inside the CVC a biofilm can form, a mixture of polysaccharides, proteins and DNA that have many different bacterial species throughout this community.[41] A biofilm is a complex microbial community, with intra and inter species variation, where the cells are attached to in this case the catheter or other bacteria and embedded in extracellular polymeric substances (EPS) or glycocalyx.[41]

With the improvements in morbidity and mortality for patients with SBS and IF it is no longer sufficient to aim solely for survival of the patient, but we must aim to improve quality of life. Sepsis is not only life threatening, but also decreases quality of life for these patients.[4, 42, 43] Infants are most prone, over other age groups, to recurrent catheter infections.[1] Recurrent hospitalization is one reason for decreased quality of life and conversely provision of HPN is important to improve quality of life and decrease health care costs.[1] The incidence of septic episode for HPN is 2.1 per 1000 central venous catheter days (CVCD), the highest infection rates are observed in children with the poorest outcomes.[1] A major impact on the incidence of sepsis and on final outcomes is decreased motility of the remnant bowel.[1] A significantly slowed motility causes bacterial overgrowth, translocation of bacteria and recurrent sepsis.[1] Catheter related sepsis is the most important factor that influences the cost of HPN leading to hospitalization.[1] The risk of sepsis is higher at the hospital than during HPN due to nosocomial flora and other factors.[1]

When Terra et al. looked at catheter related sepsis on HPN, the authors found that the length of the remnant small bowel was related to the frequency of catheter sepsis.[44] Patients with remnant small bowel <50cm have a higher frequency of catheter-related sepsis.[44] Those with remnant small bowel <50cm were often septic due to enteric microorganisms, suggesting the potential for bacterial translocation when patients have a short remnant small bowel.[44]

In a meta-analysis of pediatric patients with IF with long-term clinical outcomes, sepsis was determined to be the primary factor associated with mortality and liver failure, along with sepsis rates being predictive of IFALD.[45] The Pediatric Intestinal Failure Consortium when looking retrospectively at infants (less than 12 months old) with IF on PN for greater than 60

continuous days) found the incidence of septic episodes were 8.9 per 1000 CVCD, with the cohort experiencing a 27% mortality rate.[46] More recently, Raghu et al. evaluated pediatric intestinal transplant outcomes for patients transplanted form 1985-2017, and found that sepsis remained the largest contributor to patient death.[47]

Now, this data makes it very clear that novel ways to reduce sepsis are vital for children with SBS and IF, locking solutions are a strategy that has emerged for preventing sepsis along with maintaining venous access and will be discussed further in 1.5.2.2.

1.4.3.3 Small Bowel Bacterial Overgrowth

Small bowel bacterial overgrowth (SBBO) is essentially what its name implies, it occurs when there is a large increase in the density of the bacteria in the small bowel.[48] The density of the bacteria present in the proximal small bowel normally is quite sparse, and it increases as you move distally down the small bowel towards the colon.[48] There are physiological mechanisms that work together to suppress excessive bacterial colonization of the small intestine.[48] These mechanisms include the low pH in the stomach, IgA, defensins, and very importantly, the bowel motility.[48] As well, it is believed that the ICV prevents the high density flora of the colon from retrograde movement into the distal small bowel where the bacteria density is lower.[48] The symptoms of SBBO include chronic diarrhea, steatorrhea, macrocytic anemia, weight loss and less commonly protein losing enteropathy.[49]

SBBO occurs in many conditions where the normal mechanisms that keep bacterial colonization in a "healthy" state are disrupted by anatomical abnormalities, changes in gastric acid secretion, changes in intestinal motility, SBS, chronic disorders and immunodeficiences.[50] Specific cut off values for what number of microbiota present is diagnosed as SBBO is vague,

recently a more solidified definition was released of an excess of bacteria >10^5 CFU/mL in duodenojejunal aspiriate.[7] SBBO often occurs due to this overgrowth of colonic bacteria into the small bowel, and due to this there is growing support for the definition to include an excess of bacteria >10^3 CFU/mL, if the species identified are normally present in the colon.[7]

1.4.3.4 Ways to Diagnose Small Bowel Bacterial Overgrowth

The gold standard for diagnosis of SBBO is a jejunal aspirate, but in a real patient setting, this is often not feasible; especially within a pediatric population. It has been reported that the gold standard method has a specificity of 100%, while methods like hydrogen breath tests have a specificity of 44%.[51] Instead of a jejunal aspirate, often diagnosis is made based on symptoms like chronic diarrhea, bloating and gas.[7, 49] The recent definition has come to be the CFU counts listed above, or a glucose or lactose hydrogen breath test with either double peak or a peak of >20 parts per million hydrogen above basal within 90 min of lactulose ingestion.[7]

For the gold standard jejunal aspirate to diagnose SBBO, there are some limitations. First, an endoscopy is not only expensive but invasive, often involving sedation in children.[50] As well, many of the microbes of the gastrointestinal tract (GIT) do not grow on routine media for bacterial cultures, leading to the potential of an underestimate of the true number of bacteria present.[50]

For the hydrogen breath test, this test is detecting H_2 gas in the expired breath.[50] H_2 gas is detected as it is a by-product of carbohydrate fermentation by mainly anaerobic luminal bacteria of the small bowel.[50] This H_2 gas is thought to get from the luminal bacteria to the lungs to be expired. The H_2 gas is being absorbed through the intestinal mucosa into the bloodstream where it is then transported to the lungs.[50] This assumption is because humans do

not expire any H_2 when fasting.[50] For SBBO, the glucose breath hydrogen test and the lactulose breath hydrogen test are the most commonly used for SBBO detection.[50] The sensitivity of the glucose breath hydrogen test is 44% while the specificity is 80%, while the sensitivity is 31% and specificity is 86% for the lactulose breath hydrogen test.[50] A problem with the hydrogen breath test is that false negatives can occur due to some patients producing little to no H_2 while some producing large amounts of methane.[50] Some bacteria, like *Methanobrevibacter smithii* use H_2 to produce methane and this can lead to a false negative, as well, SBBO can be due to bacteria that are not large H_2 producers.[50]

1.5 Management:

1.5.1 Multiple Disciplinary Care and Recent Advances to Care

Multi-disciplinary care centres have emerged as the gold standard in management of SBS.[52] Multidisciplinary care centres emerged with the goal of improving clinical care for SBS, more specifically, for infants and children with SBS.[53] These multidisciplinary teams include specialists from surgery, neonatology, gastroenterology, transplantation, nursing, nutrition, pharmacy, social work and palliative care.[53] From the GIFT program (Group for the Improvement of Intestinal Function and Treatment) in Canada at the Hospital for Sick Children, within the first 2 years saw earlier assessment for transplant service with increased rates of transplantation and decreased mortality from IFALD.[53] Although at the time of the retrospective study by Diamond et al., the overall mortality rate did not decrease, the mortality from liver disease was decreased significantly.[53] In a retrospective study by Modi et al. at the

Center for Advanced Intestinal Rehabilitation there was significantly increased survival with a multi-disciplinary approach.[53]

The GIFT program looked into the outcomes in a retrospective cohort study of children before the commencement of the intestinal rehabilitation program, during the first years, and during the later years of the program.[54] An interesting comparison to the previous paper from the GIFT program occurred, while at the commencement of the GIFT program transplant numbers went up, later there had been an increase in patients who were not listed for transplant, and in fact, patients have been removed from the transplant waitlist due to clinical improvement.[54] Now, there is improved survival for patients waiting for intestinal transplantation.[54] The case of death has moved from IFALD and sepsis before the GIFT program and to non-intestinal failure related comorbidities as the GIFT program continues.[54] With multidisciplinary teams, along with care advancements and lipid composition changes quickly evolving the outcomes, morbidity and mortality associated with pediatric SBS, the focus for care advancement has shifted towards the microbiome, sepsis and trophic factors.

1.5.2 Medical Management

1.5.2.1 Management of Diarrhea and Malabsorption Beyond TPN

Patients with gastric acid hypersecretion, like in those with a jejunoileal anastomosis and altered CKK and secretin feedback, can be given a proton pump inhibitor or H_2 antagonist.[28] For vitamin B_{12} and magnesium deficiency these can be supplemented.[28] Many patients will experience fat absorption impairment, and if so this can lead to fat soluble vitamins like A, D, E and K being depleted, and therefore need to be supplemented.[30]

As stated previously, these patients often get a large volume of diarrhea, due to the loss of the ileal brake due to loss of ileum and proximal colon and therefore loss of these hormones like GLP-1, GLP-2, neurotensin and PYY and lack of an inhibitory feedback loop.[30] Therefore decreasing diarrhea and slowing intestinal motility can be treated with anti-diarrheal agents like codeine phosphate and loperamide.[55] Of course, these treatments are not without side effects and in many SBS patients accurate dosing can be a problem.[55] Somatostatin/octreotide is also used as it reduced salivary, gastric and pancreatobiliary secretions, slows small bowel transit and potentially gastric emptying.[55] This can reduce intestinal fluid and sodium output.[55]

1.5.2.2 Management of CLABSI

1.5.2.3 Heparin

The previous standard of care was often the use of heparin to prevent clotting in the central line. For HPN, low molecular weight heparin was used as a prophylactic to prevent catheter related thrombosis.[56] The cumulative thrombosis-free survival after 5 years was 93% with the use of low weight heparin or vitamin K antagonists, while 48% without prophylaxis.[56] Catheter occlusion occurred in 44% of the no prophylaxis group, and 6% in the prophylaxis group.[56] There was a trend towards, but not significant, for CVCD of 4.6 without prophylaxis and 2.1 for prophylaxis ($p=0.06$).[56] No bleeding complications occurred with the use of heparin in this study.[56] Unfortunately, there is no evidence of heparin preventing infections throughout the literature.[57]

Other studies have brought up the concern that heparin flushes work only due to the nature of the flush itself, the pulsatile flush removing thrombotic materials from the catheter rather than the heparin itself.[58, 59] The efficacy of heparin locks is questioned throughout the

literature, with some studies reporting no statistical difference in catheter occlusions or use of thrombolytic agents when comparing heparin to a saline flush.[58] There were no adverse effects in that study.[58] Heparin also only has a short half-life of 60-90 minutes.[58]

Another concern is heparin-induced thrombocytopenia (HIT), an autoimmune reaction to the heparin occurring typically 5-10 days after treatment.[60] The risk is much lower for low molecular weight heparin then higher doses, but brings about the concern especially with it occurring more often in surgery patients.[60] A common bacteria that causes CLABSI is *Staphylococcus aureus,* and there is growing concern that heparin can increase biofilm formation, specifically of *S. aureus*.[61]

1.5.2.4 Alcohol Locks

In a meta-analysis by Rahhal et al, the authors looked at the safety and effectiveness of ethanol locks compared to heparin locks in IF.[62] Ethanol locks compared to heparin led to a decrease of CLABSI by 6.27 per 1000 CVCD and a 63% reduction in CLABSI overall.[62] For catheter outcomes, there was a decrease in catheter replacement by 4.56 per 1000 CVCD, but there was an increase in catheter repair rates by 1.67 per 1000 CVCD.[62] An important topic that comes up from this study is that the primary endpoint was the rate of CLABSI, with a secondary outcome of catheter replacement and repair.[62] Whether CLABSI or occlusions are the primary outcome is different between studies and brings up the question of which is more important clinically. Another topic that comes up is this increase in catheter repair with ethanol, and the effect of ethanol on catheter integrity, particularly the concern for precipitation and the use of polyurethane catheters with ethanol.[62]

In a systematic review looking at ethanol use in pediatric IF compared to heparin locks, the authors similarly found reduced CLABSI per 1000 CVCDs by 7.67 events and decreased CLABSI rate by 81%.[63] For catheter outcomes, catheter replacements were decreased by 5.07 events per 1000 CVCD, with adverse events of thrombotic events.[63]

Unfortunately, in the United States orphan drug designation was recently given to dehydrated alcohol, leading to the cost of 70% alcohol skyrocketing, from $10/day to $1000/day.[64] This study on the cost-effectiveness of ethanol locking solution since this cost increase like the previous studies showed a 40% reduction in CLABSI frequency with the use of ethanol compared to without ethanol.[64] Unfortunately, the $1000/day ethanol would have to decrease to $68/day to be cost-effective, and $63/day to be cost-saving.[64]

Now due to this increased cost of ethanol locks, along with concerns of precipitate and catheter patency, with heparin not decreasing CLABSI and possibly promoting biofilm formation and HIT, it is apparent how an alternative locking solution is needed for pediatric IF and SBS.

1.5.2.5 Antibiotic Lock Therapies

Antibiotic lock therapy has had some interest, but comes with additional clinical concerns. In a review Justo et al. emphasized the lack of consensus on antibiotic concentrations, additives, stability, compatibility, biofilms, antibiotic resistance and dwell times.[41] Antibiotic lock therapies are recommended for patients with a history of CLABSI.[41, 57] Antibiotic lock therapy has been used for catheter salvage, where patients have CLABSI and the CVC was not removed.[41, 65] CLABSI often develops due to biofilm formation, and antibiotic susceptibility when a biofilm develops decreases by 10-1000 fold, with different antibiotic susceptibilities throughout this biofilm.[41] It is very difficult to find an antibiotic that all bacteria

in the community are susceptible to, along with getting through the EPS, that is the most abundant at the deepest layer of the biofilm protecting the bacteria.[41]

1.5.2.6 Taurolidine Citrate

Taurolidine citrate is also used, Lambe et al. has a positive study with this locking solution used prophylactically in children with IF.[66] With taurolidine citrate CLABSI rates per 1000 CVCD went from 4.16 to 0.25 in patients given taurolidine due to having previous CLABSI incidents.[66] Now, taurolidine citrate has been shown throughout the literature to be effective at decreasing CLABSI from gram-negative organisms, with less effect on gram positive organisms, but is not effective at improving catheter patency/venous access and can have thrombolytic events with it.[67]

1.5.2.7 Sodium Bicarbonate

Sodium bicarbonate has mixed results throughout the literature on its efficacy for CLABSI and catheter patency. In adults on hemodialysis, sodium bicarbonate compared to normal saline has had decreased catheter loss rates due to CLASBI and catheter related thrombosis.[68] For pediatric IF, preliminary results shown a decrease in CLABSI per 1000 CVCDs of 2.77 with ethanol lock to 0 with sodium bicarbonate, although not a statistically significant result due to small sample size.[69]

1.5.2.8 Current State of line Locks

The previous studies have shown there are many locking solutions, but with ranging costs, catheter patency concerns, antibiotic susceptibilities and concern over antibiotic resistance. Most importantly, the locking therapies we study often only target catheter patency concerns or CLABSI. Biofilms are not only a source of bacteria in the catheter, but also a site for

thrombus and fibrin sheath formation. Therefore, CLABSI and catheter thromboses are not mutually exclusive and potentially both could be targeted. For patients with a CVC, and especially for pediatric IF and SBS patients, a locking solution that prevents CLABSI and helps maintain catheter patency would be the ideal locking solution. A locking solution that decreases CLABSI, will also decrease line loss and replacements. With sepsis being the largest risk factor for mortality in SBS, septic episodes decreasing enteral tolerance and increasing hospitalizations that decrease quality of life, we need ways to prevent sepsis in this at risk population that are not only available but also cost effective.

1.5.3 Management of SBBO

When treating conditions like SBBO, broad spectrum antibiotic therapy is often used not with the goal to completely deplete the GIT microbiome, but to reduce the number of pathogenic bacteria present.[70] Metronidazole is one common antibiotic used, with the course usually lasting 7-14 days with the antibiotics targeting both anaerobic and aerobic bacteria.[70] In a study by Piper et al. the authors found a decrease in the abundance of Clostridia (a beneficial bacteria) and an increase in Proteobacteria (a marker of dysbiosis and pathogenic bacteria) after 1 week of antibiotic treatment.[70, 71]

1.5.4 Surgical Management

1.5.4.1 Colon in continuity

As discussed above, PYY and GLP-1 both inhibit gastric emptying, gastric acid secretion and small bowel motility.[28] PYY and GLP-1 are produced by the distal ileum and colon, therefore in dogs with a small bowel resection, Landor et al. looked at gastric hypersecretion of proximal

one third of the small bowel, mid one third of the small bowel or distal one third.[72] The dogs had the highest gastric acid hypersecretion with loss of jejunum over loss of ileum with colon in continuity.[72] With colon in continuity, there is not this decreased PYY and GLP-1, and therefore these hormones are able to inhibit gastric emptying, gastric secretion and small bowel motility, and therefore improve gastric acid hypersecretion.[28] This is why in patients with a jejunostomy, that lack colon in continuity, they lack these benefits of PYY and GLP-1 and have gastric acid hypersecretion, increased gastric emptying and increased transit time.[28]

In a 25 year retrospective analysis of children with SBS who required PN for greater than 3 months, the study showed that small bowel length, an intact ICV, intestinal continuity and preservation of the colon are all important factors for survival and adaptation.[17] Here, patients with remnant small bowel length greater than 38cm were more likely to survive and wean from PN and the ileocecal valve becomes important when patients have less than 15cm of small bowel remaining.[17] The authors stated similarly to other studies,[11] that evaluation of the impact of the ICV is difficult because usually due to anatomy with loss of the ICV parts of the colon and terminal ileum are also resected.[17] These results are interesting in comparison to the study by Belza et al. where the colon became important for weaning from PN when there was loss of 50% or more of the small bowel.[4] Similarly, that patients with only 10% of the small intestine can achieve autonomy if they have over 50% of their colon.[4] The present study found that patients with an intact colon or with greater than 50% of colon were more likely to adapt.[17] Similarly to other papers,[4, 10] the authors reported that 77% of the survivors had intestinal adaptation.[17]

As eluded to in Belza et al., the colon is important when less than 10% of the small bowel is remaining, the importance of the colon is further summarized by Goulet et al.[73] Here, the review states the importance of the colon for fluid and electrolytes absorption, absorption of medium-chain triglycerides (MCTs) and the production of SCFAs for colonic energy salvage. The colon is colonized by some of the most abundant bacteria for SCFA production that we will review further. While the presence of colon is important in predicting outcomes for SBS, especially when only a very small remnant of small bowel is remaining, mucosal adaptive changes have not been well supported by the literature.[74] In adults with SBS, Joly et al. found that in patients with a JC anastomosis that adaptation within the large intestine included a significant increase in crypt depth and the number of cells per crypt.[74] Importantly, the authors state how in animal models an increase in mucosal mass of the colon has been well documented, but "in animal models with SBS, the adaptive response is temporally regulated that confirms the importance of studying time."[74] This study was performed at least 2 years after the initial resection, and the authors suggest that an adaptive hyperproliferative response occurs early on after resection, leading to more cells produced and deeper crypts, and this is compensated with time.[74] Surprisingly, this study did not look at villus height.[74]

1.5.4.2 Lengthening Procedures

This brings us to the concept of lengthening of the bowel. If residual bowel is a predictor of survival and the ability to achieve autonomy, logically, if using different treatments, if one could increase the length of the bowel this would be beneficial. One method is a serial transverse enteroplasty (STEP). Javid et al. performed a single center retrospective review and

found that the majority of children with SBS were weaned off of PN following a STEP procedure.[75] Of the STEP patients, 80% had improved enteral tolerance and 60% achieved enteral autonomy after the STEP procedure.[75] With that, 40% had no improvements in EN with 83% of these patients having SBS due to gastroschisis.[75] The authors and the data suggests that STEP procedures for patients with SBS due to gastroschisis may produce less optimal outcomes.[75] This is hypothesized due to inherent issues with intestinal dysmotility, like with gastroschisis.[75] Due to the small sample size, it is hard to determine whether patients with intestinal dysmotility have already poorer outcomes before the STEP procedure, or that the STEP procedure produces less optimal outcomes for these patients.[75]

1.5.4.3 Transplantation

The last resort typically is intestinal transplant when patients have failed all other attempts at intestinal rehabilitation.[76] The four conditions to be listed for transplant is the presence of IFALD, loss of central venous access (3-6 losses in children), recurrent catheter related sepsis or a single episode of fungal sepsis, and recurrent bouts of severe dehydration or metabolic abnormalities.[76] Whether a patient receives a combined liver-intestine transplant largely depends on if there is advanced liver disease versus moderate biochemical and histologic liver disease.[76] Greater than 50% of patients listed for intestinal transplant are simultaneously listed for liver transplant.[76] Of course, patients with progressive cholestasis have an additional risk of death, therefore those patients listed for combined liver intestine transplant have the highest mortality rates on the waitlist, with infants in this group having the worst outcomes.[38] While we move into a new era with decreased IFALD, due to multidisciplinary teams and a change in lipid composition, of course there are decreasing rates

of transplant required.[38] In a cross sectional study of the long term outcomes in children with neonatal SBS, the survival rate for children on long term PN was 100%, compared to a survival rate of 60% after intestinal transplant, supporting that intestinal transplant while life-saving should be a used after all other attempts at intestinal rehabilitation have failed.[77] The rate of CLABSI is a risk factor for intestinal transplant, and therefore needs to be prevented and managed.[77] Gastroschisis is also a risk factor for nutritional failure leading to intestinal transplant.[77] Similarly in a different international study, the overall survival at 1 year for intestinal transplant patients was 73% and at 5 years was 57%.[47] One year survival was strongly associated with being liver inclusive grafts. Patient survival was strongly associated with elective transplantation versus hospitalized status.[47] Here, sepsis remains the largest contributor to patient death.[47]

1.6 Conclusions

This introduction has highlighted just how far care for infants and children with SBS has come, however there are clearly also some areas requiring improvement. Arguably one of the major current clinical challenges for infants with SBS is sepsis, given that mortality and morbidity from IFALD has recently been so dramatically reduced. We are in an excellent position to address this concern with advances in our understanding of the gut microbiome. As we will discuss in Chapter 2, changes in the gut microbiome have been associated with increased gut permeability and decreased barrier function specifically in SBS and this must be considered a risk factor for sepsis that can be targeted for treatment.

We emphasized the importance of the hormones involved in normal gut function and in adaptation, also the effects when these are perturbed given certain anatomies. The trophic factor that has received the most attention to date as a potential therapy is GLP-2. It is secreted via the enteroendocrine L cells of the ileum of the small bowel.[78] Intestinotrophic GLP-2 analogues like teduglutide and apraglutide have previously been studied in both animal and human studies looking at their effects on adaptation in the management of SBS.[78, 79] However, as we will discuss in Chapter 3, there remain gaps in our understanding of the actions of these hormones and key clinical goals that may be better optimized with this treatment. The new era of research into clinical advancement of care for children with SBS will include a focus on the microbiome, along with how to reduce sepsis and how to utilize trophic factors as a therapy for infants and children.

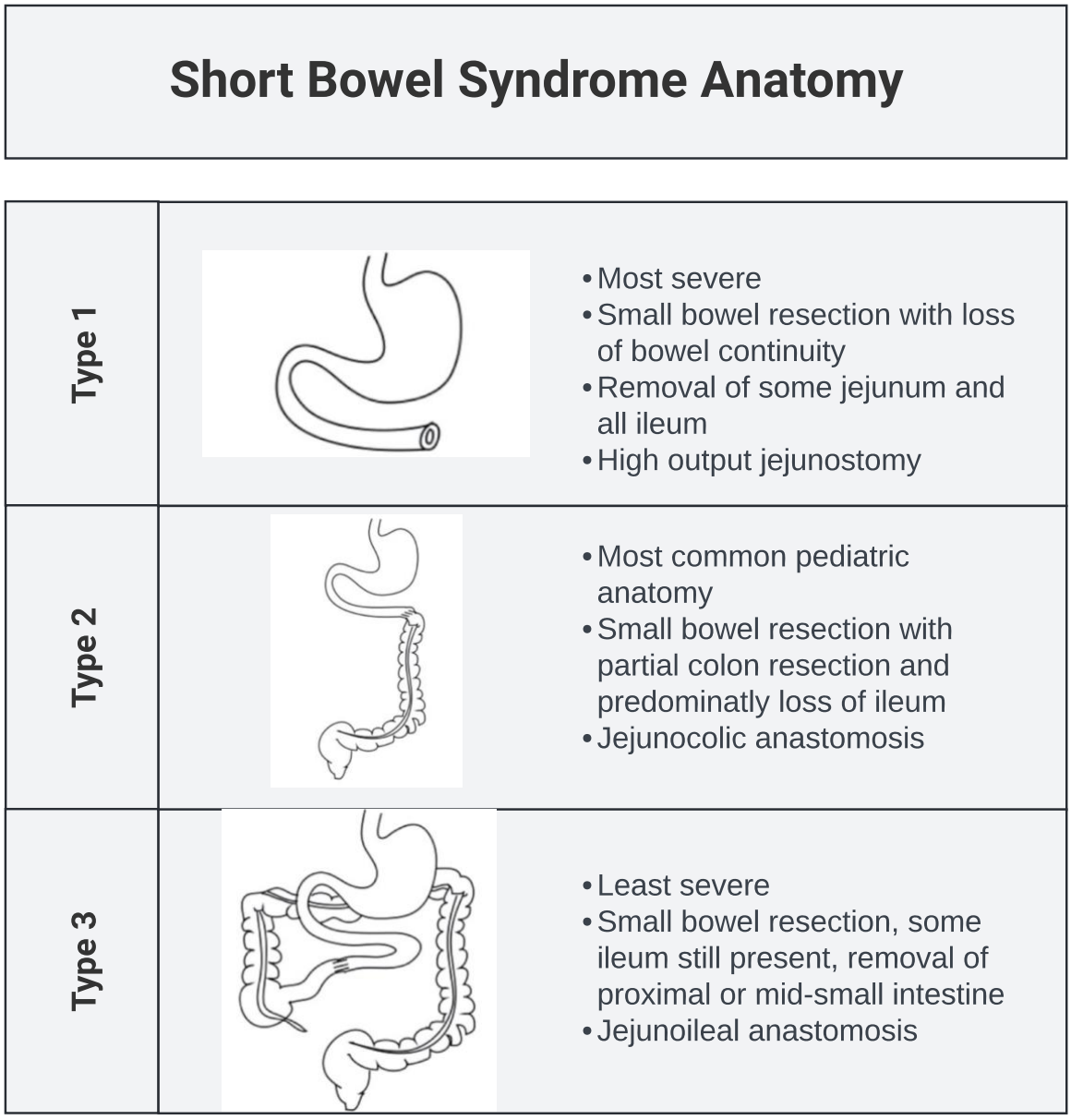


Figure 1-1. Short Bowel Syndrome Anatomy.

Adapted from Suri, Lim, Tappenden. [28, 80, 81]

Short Bowel Syndrome Anatomical Consequences

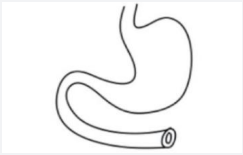
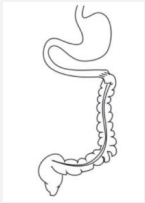
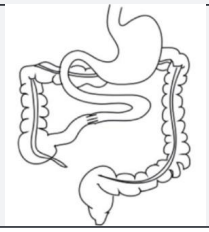
Type 1		• Most severe • Small bowel resection with loss of bowel continuity • Removal of some jejunum and all ileum • High output jejunostomy	• Lack of colon for fluid absorption • High stomal output • Fluid and nutrient malabsorption • Vitamin B12 deficiency • Loss of bile salt resorption • Magnesium deficiency
Type 2		• Most common pediatric anatomy • Small bowel resection with partial colon resection and predominatly loss of ileum • Jejunocolic anastomosis	• Osmotic diarrhea • Loss of bile salt resorption • Vitamin B12 deficiency • Fat malabsorption • Choleretic diarrhea • Fat soluble vitamin deficiency • Gallstones
Type 3		• Least severe • Small bowel resection, some ileum still present, removal of proximal or mid-small intestine • Jejunoileal anastomosis	• Decreased CCK and secretin feedback inhibition • Increased pH of proximal small bowel • Altered pancreatic enzymes • Gastric acid hypersecretion • Impaired digestion

Figure 1-2. Consequences of Short Bowel Syndrome Anatomies.

Adapted from Suri, Lim, Tappenden.[28, 80, 81]

Chapter 2. The Microbiome in Short Bowel Syndrome

2.1 Pursuing microbiome based solutions for SBS

While we have covered the importance of preventing CLABSI and sepsis in neonatal SBS, an emerging field is the role of the microbiome in SBS. Specifically, how the microbiome is altered due to anatomy, diet, antibiotics and other treatment factors, and how this may predispose to CLABSI and sepsis. With these changes to the microbiome, there are multiple different pathologies that can occur, from dysbiosis to SBBO, to D-lactic acidosis. The microbiome is changing drastically in infants while their GIT is developing, coincidental with these first years of SBS treatment. While the microbiome is more stable in adulthood, the infant GIT microbiome is more transient during this time and able to be modulated, in both a positive and negative manner. First, we need to understand the microbiome structure and function in SBS, but then we will cover how clinicians use tools to modulate the microbiome, the emerging research in this area and its implications for SBS.

2.2 Microbiome Introduction:

The neonatal gut is quickly colonized by bacteria after birth, termed the GIT microbiome.[82] A "healthy" microbiome that is balanced and has high stability and diversity will interact with the host's immune system and can suppress the expansion and colonization by "unhealthy" bacteria.[82] The GIT microbiome has been described to contain more than 50 different phylum of bacteria, however, only 4 major phyla predominate including: Firmicutes, Bacteroidetes, Actinobacteria and Proteobacteria.[83] A marker of an unhealthy or unstable gut has often been attributed to an abundance of Proteobacteria.[82]

To understand the gut microbiome we will review the literature first in relation to the different taxonomic bacteria that compose the microbiome. Next, we will look at the phylum that colonize the GIT of infants after birth. Then, host factors that influence the microbiome including diet, genetics, environment and antibiotics. Along with the products of bacterial fermentation. Lastly, we will look at the relationship between the gut microbiome and gut barrier function, intestinal inflammation and SBBO.

Before we can review the literature, it is important to review bacterial taxonomy: characterization, classification and nomenclature of bacteria.[84] Classification is the placement of bacteria into groups or taxa based on similarities, but recently, there have been many changes and arguments to change how bacteria are classified.[84] Traditionally, taxonomy has defined species by genetic and expressed characteristics so that members are monophyletic and have a large amount of genomic and phenotypic coherence.[84] Monophyletic means that the strains of bacteria that make up the species all share a recent common ancestor.[85] Now, many are arguing that for bacteria, species definitions should include a genome sequence.[84] The history of how bacteria have been classified has evolved, with the first definitions being based on phenotypic traits, that lead to blurry classifications.[84] Following this, due to new methods, comparisons were able to be made via the DNA-DNA hybridization.[84] The degree of DHH between the genomes of two bacteria is a measure of how genomically similar they are.[84, 85] Luckily, now 16s ribosomal ribonucleic acid (rRNA) has become an available method, allowing genealogical reconstruction based on ribosomal RNA genes.[84, 85] The concept primarily used to describe bacteria is called the phylogenetic species concept. The phylogenetic species concept

defines “a microbial species as a group of strains that share certain diagnostic traits and that are genetically cohesive and share a unique recent common ancestor.”[84, 85]

2.2.1 Genome Fingerprinting:

To evaluate specific strains of bacteria based on polymorphisms between strains, a rapid approach that is often used is called genome fingerprinting.[85] These fingerprints are fragments of DNA that are generated from individual genes or whole genomes of bacteria.[85] This gene sequencing done via PCR amplification of the gene fragments, the common way to characterize these gene sequences is termed ribotyping.[85] This involves the localization of small subunit rRNA (SSU rRNA) genes on genome fragments.[85] First, the sample is digested by a restriction enzyme allowing these fragments to be separated by gel electrophoresis that is transferred to a nylon membrane and labelled with an SSU rRNA gene probe.[85] Here the term operon becomes important, an operon is a functioning unit of DNA containing a cluster of genes under the control of a single promoter. Species of bacteria can have 1 to 15 rRNA operons, and the number of operons that are present is conserved for each species of bacteria.[85] Differences in the genome sequence of bacteria sequences leads to the endonuclease enzyme cutting it in different locations, leading to restriction fragments of different lengths.[85] The different sizes and number of bands that are produced by different bacteria allow this pattern to be created, that is a genome fingerprint of the bacteria that is termed ribotyping.[85]

The 16S rRNA has different degrees of variability in different regions.[86] Conserved regions, almost all bacteria have, are the same sequence and this won’t help distinguish between bacteria.[86] The variable regions typically a genera or larger group share the same sequence, while the highly variable have shared sequences for many species.[86] Therefore a PCR

primer is able to bind this conserved region present on almost all bacteria. This allows the amplification of the DNA, and then can use the variable and highly variable regions to identify the bacteria potentially down to the species level.[86]

2.2.2 Alpha Diversity: Shannon and Simpson Diversity Measures

Alpha diversity is the diversity of a species within a particular ecosystem, in the case of the microbiome, it is the diversity of a species in the microbiome.[87] It is also termed species richness, as it is the number of species present within that ecosystem. To measure this, there are three commonly used alpha diversity measures; species observed, Shannon index, and Simpson index.[88] To measure diversity, the methods are comprised of differing weights of richness and evenness.[88] Richness is the number of different taxa observed in the community without taking into account the frequency of these taxa.[88] Evenness is how evenly distributed these taxa frequencies are in the community.[88] Species observed is synonymous with richness and only measures richness, not evenness.[88] Shannon index puts equal weight on richness and evenness, while Simpson index puts more weight on evenness while still measuring richness.[88]

2.2.2.1 Issues with the Alpha Diversity Measures

There are four main issues why the application of alpha diversity measures can be cumbersome. First, there are a number of different diversity measures. For example, the species observed, Shannon index and Simpson index.[88] Secondly, the nomenclatures to describe diversity is complex.[88] Diversity being divided into richness and evenness varies in how much weight is assigned to each based on different methods.[88] There is differences in how well certain bacteria can be identified down to the species level, and this leads to incomplete

sampling.[88] Along with this, it is possible to have no change in alpha diversity while having a change in beta diversity.[88]

2.2.3 Beta Diversity: Bray Curtis and Binary Jaccard

As previously described, alpha diversity is of a single sample, it is a local sample, conversely, the regional component, the diversity of a collection of samples is termed gamma diversity.[88] The relationship between alpha and gamma diversity is termed beta diversity.[88] The importance of beta diversity measures become clear when looking at the composition of a community.[88] For example, alpha diversity is useful when looking at changes over time of community structure, but a community could have a shift in the taxa that comprise the community, but could still have a similar number and abundance of taxa, leading to no change in alpha diversity while the taxa have changed.[88]

To determine beta diversity, much like alpha diversity, there are multiple different measures that can be used. The three common measures are Jaccard, Bray-Curtis and Sørensen.[88] Important terms for determining beta diversity are species replacement, richness difference and nestedness.[89] Species replacement is also termed turnover, it is the increases and decreases in species as they replace each other over time, impacted by environmental filtering, competition and more.[89] It also involves the ecological tolerance and niches of species.[89] Richness difference is the differences in how many species are in one community compared to another.[89] It allows the diversity of niches that occur throughout the GIT to be analyzed.[89] Lastly, there is nestedness, which is a type of richness difference pattern, nestedness occurs when a species at a site, being a subset of the species, are richer at this specific site.[89] The Jaccard measure is the proportion of unshared species out of the total

number of species recorded from the two sites.[90] The Sørensen measure is similar to the Jaccard measure, but, the Sørensen measure gives double the weight to shared species.[90] The Bray-Curtis measure is a modified version of the Sørensen index, where the values range from 0-1 and data is often reduced to presence or absence.[90]

2.3 Omics, Metagenomics, Metabolomics, Metaproteomics, Metatranscriptomics

While these previous measures are used to measure and detect the bacteria that are present, an important new area of study looks into the function of the bacteria that are present.[91] The first component, metagenomics is a way to bridge between the taxonomic composition of the GIT microbiome and the functional potential of the GIT microbiome.[91] However, to get more information about the function of the GIT microbiome, more specific omics measures can be used. Omics is a group of methodologies that are used to characterize a class of biomolecules, including metagenomics, metabolomics, metaproteomics and metatranscriptomics.[91] Metabolomics, measures the intracellular and/or extracellular metabolites in and around a microbial community.[91] Metaproteomics characterizes the function of bacteria by applying the analysis of the complete set of proteins expressed by a bacteria (proteomes) but applied to mixed species of the GIT microbiome.[91] Metatranscriptomics is the analysis of the RNA of microbial communities, but often used to infer the function/activity of these bacteria.[91] Metagenomics is the functional and sequence-based analysis of the microbial genomes of a sample, from complete genome arrangements to isolated genes.[92, 93] These methods allow a functional assessment of the GIT microbiome.

2.4 Taxonomic Composition of the GIT Microbiome

Proteobacteria, as mentioned earlier, are often considered "unhealthy" bacteria, but there is much more to the story. Proteobacteria are facultative anaerobic bacteria, meaning that they can grow in the presence or absence of oxygen.[94] Proteobacteria have been implicated in many diseases, from metabolic disorders to inflammatory bowel disease.[83] Most diseases wherein a Proteobacteria signature has been implicated have an underlying theme of inflammation.[83] Proteobacteria can be divided into six classes: Alphaproteobacteria, Betaproteobacteria, Gammaproteobacteria, Deltaproteobacteria, Epsilonproteobacteria and Aetaproteobacteria.[83] Importantly, bacteria in the family E*nterobacteriaceae* that are part of the class Gammaproteobacteria are often implicated in diseases, like prediabetes and type 2 diabetes.[83] Proteobacteria are gram negative bacteria and thus produce lipopolysaccharides (LPS).[83] Low grade inflammation that is sustained by LPS and the development of metabolic disorders has been well established.[83] In fact, LPS endotoxin is the only known bacteria product that when injected subcutaneously into mice can induce obesity and insulin resistance via an inflammation-mediated pathway.[95]

In a study performed by Fei et al. analyzed the relative abundance of bacteria in a morbidly obese human as they lost weight.[95] They found that *Enterobacter* relative abundance decreased from 35% to undetectable as the volunteer lost weight.[95] While the volunteer lost weight they had a decreased abundance of endotoxin biosynthetic genes in their GIT that was correlated with a decreased circulating endotoxin load.[95] When the authors isolated *Enterobacter cloacae B29* from the gut of the volunteer and put this strain into the gut of germfree mice fed a high-fat diet the mice not only developed obesity and insulin resistance,

but surprisingly the germfree mice not exposed to the bacteria, but fed a high fat diet, did not exhibit the same disease phenotypes.[95]

The next phylums we will look at are the gram positive Firmicutes and gram negative Bacteroidetes. Bacteroidetes and Firmicutes can be anaerobic or aerobic. The reason these need to be investigated together is that often when one of these phylum increases, the other phylum will decrease.[96] Members of the genus *Bacteroides* that are a part of the phylum Bacteroidetes have been shown to synthesize linoleic acid and convert primary bile acids to secondary bile acids.[97] Members of the genus *Bacteroides* are the predominant organisms that participate in carbohydrate metabolism.[97]

In the literature Let et al. looked at the relative proportion of Bacteroidetes and Firmicutes in obese people compared to lean people.[98] They studied 12 obese people who were either assigned to a fat restricted or to a carbohydrate restricted low calorie diet.[98] The gut microbiota was monitored via 16S rRNA sequencing for 1 year.[98] Before the diet therapy obese people had fewer Bacteroidetes and more Firmicutes.[98] Over time, on either of the diets the relative abundance of Bacteroidetes increased and the abundance of Firmicutes decreased.[98]

Another study of importance by Ismail et al. looked at the fecal microbial composition of children and adults, obese versus normal weight.[99] Fecal samples were collected from all subjects and total DNA was extracted via a conventional PCR.[99] The proportions of the phyla Firmicutes and Bacteroidetes were significantly increased for the obese group compared to the normal weight group.[99]

The last phylum to look at is the phylum Actinobacteria. A genus of Actinobacteria, called *Bifidobacteria* have become the focus of many probiotics.[100] Actinobacteria are gram

positive and anaerobic bacteria.[100] The literature is well established that infants born via vaginal delivery have a higher diversity in the phylum Actinobacteria.[100] *Bifidobacteria* in particular is important in infants and will be covered in more detail next.

2.5 The Developing Microbiome

The microbiome has coevolved with its host, allowing the microbiome to not only shape the phenotypes of our ancestors, but also within-species transmission of microbiota across generations of humans.[101] Labor and birth are the first major exposure to microbiota and is believed to be where this intergenerational microbiota transfer from mother to infant occurs.[101] Immediately after birth, there is a decrease in gut alpha diversity, most likely due to the selective pressures of milk substrates on the taxonomic composition of the GIT microbiome.[101] By the time the infant is 1 week old, the GIT microbiome is very similar to that of a 1 month old infant.[101] During the first 6 months of life, an infant that is able to breast feed will have a GIT microbiome that is largely influenced by the selective pressures of the components of breast milk.[101] After breast feeding ends, the GIT microbiome will now start to be influenced by solid feeds that will have selective pressures for bacterial populations with metabolic activities for these food components and the microbial diversity of the intestine steadily increases until around 3 years old.[101] This increase in diversity is due to increased diversity of foods, gut maturation and immune system maturation.[101]

A higher abundance of *Bifidobacterium* have been shown in breast fed infants.[100] In fact, Bezirtzoglou et al. found that when looking at fecal bacteria of breast fed versus formula fed

newborns, breast fed infants had more than 2 times increased numbers of *Bifidobacterium* present in their feces compared to formula fed infants.[102]

There are different theories on how the initial bacterial niches are established. The first argument is that facultative anaerobic Proteobacteria make the initial niche, using up the oxygen that is present within the gut.[82] This decrease in oxygen favors colonization by obligate anaerobes, the Proteobacteria are then replaced by obligate anaerobic Firmicutes and Bacteroidetes.[82] The other argument is that these "pioneer" facultative anaerobes first colonize, and this initial colonization that is acquired at birth is heavily influenced by the mode of delivery.[103] Then the two obligates anaerobes are *Bacteroides* and *Bifidobacterium*.[103] The authors here argue that this process is much more complicated than just a low-oxygen environment allowing the obligate anaerobes to become dominant.[103]

The first argument for why this is a more complex relationship is shown in a study by Jost et al.[104] The authors looked at maternal feces, breast milk and corresponding neonatal feces.[104] Multiple obligate gut associated anaerobic genera of bacteria are shared between maternal feces, breast milk and neonatal feces; specifically, a viable strain of *Bifidobacterium breve*.[104] The authors argue that this suggests that maternal gut bacteria reach breast milk via an entero-mammary pathway that is in turn able to influence the developing neonatal GIT microbiome.[104]

The second argument is that both *Bacteroides* and *Bifidobacterium* are consumers of human milk oligosaccharides (HMOs).[103] Marcobal et al. showed that infants lack the enzymes for milk glycan digestion, yet members of the infant GIT microbiome allow this group of important carbohydrates to be digested.[105] Interestingly, the authors state that *Bifidobacterium*

breve are unable to use diverse HMOs, but can grow using short chain oligosacchairdes.[105] Breastfed infants distal GIT typically have *Bifidobacterium longum, Bifidobacterium breve* and *Bifidobacterium bifidum*.[106] Arguably, I believe that the two different perspectives are saying the same thing. These initial "pioneer bacteria", i.e. the Proteobacteria make the first niche, that depletes oxygen and helps create the environment for these obligate anaerobes, that have their niche furthered by the presence of HMOs.

More recent research has come out in regards to the microbiome and NEC. Of note, one case-control prospective study looked at infants who developed NEC and unaffected controls prior to disease onset.[107] The authors found that the microbiome undergoes dynamic development during the first 2 months of life, with degree of prematurity being a major factor contributing to colonization.[107] As well, for infants with early versus late onset NEC, the pattern of microbial progression was different.[107] While in early onset, the abundances of *Clostridium sensu stricto* were significantly higher, for late onset, *Escherichia/Shigella* from Gammaproteobacteria were increased prior to disease onset and were significantly higher in cases than controls 6 days before NEC onset.[107]

2.6 Fecal Microbiota, Resident Microbiome and Transient Microbiome

Often, in the studies evaluated so far, the fecal microbiota is used to generalize changes to the entire gut microbiome. However bacteria are present not only along the entire lumen of the intestine, there are also bacteria that are adherent to the mucosa.[108] In healthy adults, the microbiota of the feces most resembles the microbiota of the distal lumen of the colon.[108] In adults it has often been found that microbiota samples from one individual at multiple different

sites of the human colon are more similar to each other than to matched samples from other subjects.[108, 109] With differences between individuals being significantly greater than intrasubject differences, there is one exception; the variation between stool and adherent mucosal microbiota.[109]

The epithelial cells that line the intestine turn over rapidly, at a rate of up to one to three billion cells per hour in the small intestine, and about 1/10th that rate in the colon.[110] The bacteria present in the gut need to be able to withstand this epithelial cell turnover and peristalsis.[110] Bacteria that are able to establish themselves and become commensal residents of the GIT microbiome are called autochthonous components, while bacterial species that are more sporadic and don't become a resident are termed allochthonous components.[110] In a paper by Gordon et al. the authors propose that these resident bacteria that are attached to the mucosa are part of a biofilm like community.[110] For this to be considered a biofilm, the authors propose it must satisfy these characteristics: "it must have a polymer-based matrix (such as polysaccharide); it must be capable of recognizing components evolved by microbes to mediate their attachment and thus oppose their washout; and it must facilitate nutrient harvest and exchange."[110] The intestinal mucus layer is a dense matrix of polysaccharides, a polymer-based matrix, so it fits this criteria.[110] The mucus layer also opposes washout of microbes as the mucus turnover is more rapid than the underlying epithelium, but is slower than the transit time of food.[110] For the last criterion, for nutrient harvest exchange, some bacteria can actually attach and graze on mucus-associated glycan, while other bacteria can use SCFAs from microbial fermentation to get energy.[110]

2.7 Diet

Diet has been recognized as a large contributor influencing the microbiome, and this extends past breast milk versus formula fed infants.[94] In a study by De Filippo et al. the authors looked at the microbiome of children eating a modern western diet versus a rural diet, using 16S rRNA sequencing of children's feces.[111] Children eating a rural diet high in fibre had a significant increase in Bacteroidetes and a decrease in Firmicutes.[111] They also found the rural diet associated with significantly more fecal short chain fatty acids (SCFAs) compared to the western diet.[111] As well, *Enterobacteriaceae Shigella/Escherichia,* which are from the phylum Proteobacteria and class Gammaproteobacteria, were underrepresented in the rural compared to the western children.[111]

Within nutrition, it has been shown that long term high uptake of animal proteins, amino acids and fats increases the relative amounts of *Bacteroides.*[112] While, low protein and increased carbohydrates increased the amount of *Prevotella*.[112] A high protein diet also increases the levels of SCFAs produced along with some potential toxins like sulfide and ammonia.[112]

2.8 Short Chain Fatty Acids (SCFAs)

Recently, the focus of research into the microbiome has shifted away from looking solely into what bacteria are present, but examining more specifically what the bacteria are doing. Functions of the gut bacteria include the fermentation of indigestible components of food into metabolites that can then be absorbed, the synthesis of vitamins, the removal of toxic compounds, outcompeting pathogenic bacteria, aiding in the intestinal barrier and stimulation

and regulation of the immune response.[91] As described previously, often omics can be used to analyze the function of the GIT microbiome.[91] SCFAs are the products of the microbial metabolism within the GIT and it has been established that they play a role in many different parts of the body, therefore having huge biological significance.[113] The three main SCFAs are butyrate, acetate and propionate. These SCFAs are often produced when a primary polysaccharide is degraded by bacteria, often in the colon, releasing small polysaccharides, oligosaccharides and SCFAs.[113] Up until recently, SCFAs were considered to be the end products of fermentation; however, bacterial groups have been identified that are able to use one SCFA to produce another.[113] Acetate and propionate are predominantly produced by bacteria belonging to Bacteroidetes, while butyrate is primarily produced by bacteria from Firmicutes.[114] SCFAs are able to bind to G-protein coupled receptors (GPCRs) and induce modification of transcription factors.[113]

In a study by Lu et al. the authors looked at dietary supplementation of acetate, propionate and butyrate on mice fed a high fat diet.[115] The authors not only found that the SCFA supplementation significantly inhibited the body weight gain induced by high-fat diet feeding, but also caused significant changes in the expression levels of G-protein coupled receptor 43 (GPCR43) and GPCR41.[115] As well, the SCFA supplementation influenced the bacterial community structure in the feces, with a reduction in the proportion of Firmicutes and an increase in the proportion of Bacteroidetes.[115] GPCR43/41 are both considered free fatty acid receptors 2/3 respectively (FFAR2/3), and are widely expressed in the small intestine and colon as well as other tissues and organs throughout the body.[115] Some other sites with these

FFA receptors include the pancreas, adipocytes, sympathetic nervous system and immune cells.[116]

High fiber diets increase fecal bulking, SCFA production and increase transit rates along the large intestine.[117] Slow transit rates actually encourage growth of slower growing bacteria in the GIT, having increased contact time with fiber etc., often including hydrogen-utilizing methanogens.[118] Most absorbed butyrate (95%) is actually metabolized by the colonic epithelium enterocytes, and therefore there are low concentrations of butyrate in portal blood.[117]

2.9 Dysbiosis

Dysbiosis is an imbalance in the taxonomic composition of the gut microbiota that is believed to have negative functional significance for the host.[82] In metabolic disorders, dysbiosis often includes an increased prevalence of Proteobacteria as discussed previously.[82] Stress conditions can lead to decreased microbial diversity and promote the expansion of specific taxa of bacteria, leading to dysbiosis.[112] The gut microbiota is beginning to be thought of as an organ. Like other organs, the function of the microbiome relies on a stable cellular composition, which was previously discussed involving the phyla Bacteroidetes, Firmicutes, Actinobacteria and a small ratio of Proteobacteria.[112] Large intraindividual shifts in the ratios of these different phyla, or even the expansion of a new bacterial group leads to this “unhealthy” and often disease promoting imbalance termed dysbiosis.[112] Dysbiosis is being linked to many diseases, either contributing to the disease development or the severity of the disease, including inflammatory bowel diseases, metabolic disorders, autoimmune diseases and

neurological disorders.[112] Dysbiosis can even trigger disease in the first few weeks of life as the microbiome is establishing and developing, this has been documented in NEC.[112] But, in dysbiosis it has become clear that "good" and "bad" bacteria can become blurred as symbiotic bacteria can become pathogenic when present in abnormally large numbers in the microbiome, or taking over another bacteria's niche.[112] These bacteria are termed pathobionts, as they are symbiotic when present in normal amounts but become pathogenic when they have large expansion.[112]

The microbiome is very resilient to many exogenous and endogenous factors, yet most of the time a single factor alone is not going to induce dysbiosis.[112] This resilience is due to the capacity of the GIT microbiome to adapt to variability in nutrient availability and changing environment conditions.[112] Often, to lead to dysbiosis there will be a combination of several factors leading to large changes in microbial groups and eventually a shift that is pathological.[112] This is quite dependent on the bacterial groups that are changed due to these factors, with changes in the phyla Bacteroidetes and Firmicutes often not leading to a pathological state, while increased numbers of *Enterobacteriaceae* rapidly expanding often leading to a greater likelihood of inflammation and/or dysbiosis.[112]

2.9.1 Dysbiosis and the Low Oxygen Hypothesis

Observations for dysbiosis in inflammatory bowel syndrome have shown a decrease in obligate anaerobes of the phylum Firmicutes and an increase in facultative anaerobes from the family *Enterobacteriaceae*.[119] The low oxygen hypothesis stems from this shift in bacterial communities from obligate to facultative anaerobes, suggesting a disruption in anaerobic fermentation, and suggests a role for oxygen in intestinal dysbiosis.[119] Therefore the hypothesis

states that an increase in oxygen in the gut is the cause of dysbiosis in inflammatory bowel disease.[119] With dysbiosis, there is often a decrease in diversity and a decrease in these dominant obligate anaerobes, along with an increase of the subdominant facultative anaerobes, sometimes with an increase in detection of certain aerobes.[119] The microbiome is composed like any ecosystem; of niches, different environments, intra and inter species competition and more. Therefore, this increase in oxygen disrupts anaerobic fermentation, allowing a selective advantage for these facultative anaerobes and some obligate aerobes to now outcompete and take over the niches of obligate anaerobes.[119] The obligate anaerobes are unable to compete for their niche anymore, being at a disadvantage with this oxygen present, that the facultative anaerobes use to their advantage.[119]

In a study by Hartmen et al. the authors were in a unique position following 17 small bowel transplants to study the microbial ecology of the human small bowel with an ileostomy, allowing access to ileal effluent samples and mucosal biopsies.[120] Following transplant, the small bowel was dominated by facultative anaerobes like *Lactobacilli* and *Enterobacter.*[120] This has also been noted in other patients with an ileostomy but not a bowel transplant, leading to authors to suspect that this change in the flora of the GIT is due to oxygen entering via the ileostomy.[120] After surgical closure of the ileostomy, the bacterial communities of the GIT reverted to the normal bacterial communities that are dominated by obligate anaerobes like Bacteroides and Clostridia.[120]

NEC is an inflammatory intestinal disorder affecting preterm infants.[121] Wang et al. looked at 10 NEC preterm infants and 10 matched control preterm infants at a single center and looked at fecal samples via PCR and 16S rRNA sequencing.[121] The authors not only found that

the NEC infants had less diversity with an increase in Gammaproteobacteria but also had a high mean number of previous days of antibiotics.[121] The authors state that their results may suggest that the severe lack of microbiota diversity with single dominant microorganisms might be favoured by the use of empiric and widespread antibiotics, commonly given to mothers before preterm birth.[121] It could be argued that the limited microbiota diversity of NEC patients that are often in hospital can lead to microbiome changes via clinical care and environmental conditions. The authors show how previous research found that providing breast milk and probiotics have decreased the incidence of NEC and changed the GIT microbiome.[121]

2.9.2 Prevention of Dysbiosis

2.9.2.1 Prebiotics

NEC as a consequence of dysbiosis in preterm infants is a disease model that allows us to compare different treatments for neonatal onset dysbiosis. NEC is typically due to reduced bacterial diversity, decreased obligate anaerobes and increased relative abundance of a few facultative anaerobes like *Enterobacteriaceae*.[122] Prebiotics are non-digestible food ingredients that are metabolized by bacteria of the GIT, ideally by beneficial bacteria like *Bifidobacterium* and *Lactobacillus*.[123] When these non-digestible food ingredients reach the colon they are metabolized by beneficial bacteria, and this in turn will increase the numbers or activity/function of these bacteria.[123] Diet is one of the main factors controlling the GIT microbiome, so it makes sense that using fibre, protein, resistant starch, oligosaccharides, amino acids etc. that are selectively utilized by certain bacteria could drastically impact the microbiome.[123] As discussed above, prebiotics can not only impact numbers of bacteria, but also the production of metabolites like SCFAs. The prebiotics must not be hydrolyzed or

absorbed in the stomach or small intestine, must be selective for beneficial commensal bacteria in the colon and their fermentation should induce beneficial luminal/systemic effects within the host.[123] The benefit here is that bacteria do not need to be added, unlike with the methods discussed in 2.8.2.2 and 2.8.2.3.

Prebiotics have been postulated as prevention for NEC, but in meta-analysis the use of prebiotics in pre-term infants decreased sepsis, length of hospital stay and time to full EN, but there was no difference in morbidity due to NEC.[124]

2.9.2.2 Probiotics

Growing evidence is supporting that neonates might be one of the most vulnerable populations to disturbances of the GIT microbiome.[125] Probiotic efficacy in low birth weight neonates and premature infants is beginning to show promising effects for infants that are at an increased risk of developing NEC.[115]

In a systematic review by Deshpande et al. the authors looked at the effects of probiotic supplementation in preterm and very low birth weight neonates.[126] To be included the studies had to have started probiotic supplementation within the first 10 days and continued for 7 or more days.[126] The systematic review found that there was a 30% reduction in the incidence of NEC with probiotic therapy, and the risk of death was also significantly lower.[126] The risk of sepsis was not significantly different.[126]

There is data to suggest that for NEC specifically, a mixture of different bacteria could be more beneficial than a single species.[127] In a prospective cohort study, the authors treated infants in the NICU with a probiotic mixture called FloraBABY.[127] All infants were less than 32 weeks gestational age, being preterm infants.[127] The comparison group was the final 17 months

prior to the commencement of the probiotic, the treatment group was the 17 months after the commencement of the probiotic.[127] There were no cases of sepsis identified throughout the study that were due to the organisms present in the probiotic.[127] There was a significant decrease in the incidence of NEC after the introduction of probiotics, with a nonsignificant decrease in death and a significant decrease in the combined outcome of death or NEC.[127]

2.9.2.3 Fecal Microbial Transplant

Fecal microbial transplant (FMT), has become increasing popular due to its curative ability for *Clostridium difficile* infections.[128, 129] It is using donor stool in a capsule, frozen, freeze dried or other methods that is administered via enema, orally or via colonoscopy.[129] While probiotics were always the gold standard for this, probiotics contain a less diverse assortment of bacteria than all of the microbes of the colonic microbiome in a healthy individual.[129] FMT like probiotics lacks consensus on who it should be administered to along with dosing, frequency of dosing, the optimal source of donor feces, how it is processed, along with how long after antibiotic use it can be given.[129] The argument for the use of FMT, the more diverse microbial sample to be delivered, is also the same argument against it. Controlling the donor source, matching for age or for specific disease states is difficult. Importantly, FMT delivers not only 'good' bacteria, but also 'bad' bacteria, and it is difficult to know how these 'bad' more opportunistic bacteria in a disease state will act. Conversely, prebiotics allow the existing bacteria to be fed, while probiotics introduce 'good' bacteria that ideally are known down to the strain of bacteria. While prebiotics are arguably the safest, it is difficult to target which bacteria are metabolizing the prebiotic. While probiotics are arguably the most targeted, they need to be regulated and dosed properly, along with being able to have one or a few strains of

bacteria outcompete established niches of a microbiome. FMT involves the most diverse microbiome that can be administered but includes both 'good' and 'bad' bacteria, along with needing regulated donor stool, dosing, administration and frequency.

While there is limited studies on FMT for preventing NEC, there is a new concept emerging called fecal filtrate transplantation (FFT), where using micropore filtering bacteria are removed from the donor feces, with bacteriophages still present in the sample.[122] In pre-term cesarian-delivered piglets given rectal FMT, or FFT rectally or oro-gastrically, FFT prevented NEC in this model, while FMT did not.[122]

2.10 The Microbiome in Short Bowel Syndrome

2.10.1 Introduction

From the previous literature review, it has become clear that many factors play into determining an individual's structure of the microbiome, including genetics, diet and environment. Given the dramatic anatomical, functional and nutritional impact of short bowel syndrome, as well as the diverse etiologies it is logical to assume dramatic changes in the intestinal microbiome will occur.[130] Gut microbial dysbiosis is often presumed to result in this syndrome from frequent use of broad-spectrum antibiotic use, lack of anatomical safeguards like the ICV and changes in intestinal motility. The resulting dysbiosis itself may delay enteral diet advancement, increase the risk of bacterial translocation, worsen liver disease and more.[130]

2.10.2 The Microbiome in Adults with Short Bowel Syndrome

While the purpose of this literature review is in the field of pediatrics, as there have been so few studies to date it is reasonable to review the adult literature. In a study by Huang

et al. the authors looked at fecal sample microbial composition from 5 adults with type 2 SBS, and 5 adults with type 3 SBS, as well as healthy adult controls.[131] The authors found that bacterial alpha diversity decreases in SBS patients and was positively correlated to the remaining small bowel length.[131] There was no differences in bacterial alpha diversity between the two types of SBS patients.[131] Patients with SBS type 2 had increased Proteobacteria and were deficient in Firmicutes and Bacteroidetes.[131] In patients with type 3 SBS, their microbiomes were dominated by *Lactobacillus* and *Prevotella* while commensal bacteria from *Lachnospiraceae, Ruminococcaceae* and *Bacteroidaceae* declined.[131] An important aspect of this finding is that patients with SBS type 2 lack the ICV while with type 3 the valve is present.[131] The duration that the SBS patients were on PN was positively related to the proportion of *Enterobacteriaceae* but negatively related to the proportion of *Lactobacillus*.[131] For metabolic functions, type 2 SBS patients, the functional pathways of citrate cycle and branched-chain aromatic amino acid biosynthesis were increased.[131] For type 3, the functional profiles of pyrimidine and purine metabolism were dominant.[131] This study excluded patients that were receiving antibiotics, which helps reduce that possible confounder but also leads to strict inclusion criteria that leads to a small sample size as the majority of SBS patients do receive antibiotics.[131]

In a pilot study by Boccia et al. the authors looked at fecal microbiota samples from 12 SBS patients on HPN compared to 16 controls (which were healthy relatives and housemates, living in the same place and eating a similar diet).[132] The authors used both culture-dependent methods and qRT-PCR to analyze the microbial profiles.[132] The culture-dependent method showed statistically lower concentrations of Bacteroidetes and Firmicutes in SBS patients

compared to controls.[132] The qRT-PCR method showed that *Methanobrevibacter smithii* was also significantly lower in SBS patients compared to controls.[132] As well, the total bacterial count was significantly higher in the controls compared to the SBS patients.[132]

2.10.3 The Microbiome in Children with SBS

We will start with a study by Davidovics et al. where the authors looked at the fecal microbiota of 9 children with SBS and 8 healthy children ages 4 months to 8 years.[130] The authors not only found that the fecal microbiota was different in SBS children compared to healthy children, but also that the stool had significantly greater abundance of Gammaproteobacteria.[130] SBS patients who had increased stool frequency tended to have increased abundance of *Lactobacillus*.[130] Interestingly, previously discussed increased Proteobacteria could be a marker of dysbiosis,[94] Proteobacteria comprised 22% of the relative abundance of SBS samples, conversely, Proteobacteria comprised <1% of the relative abundance of the healthy controls samples.[130] More specifically, SBS patients had increased relative abundances of OTUs of *Escherichia/Shigella* and *Streptococcus.*[130] In addition, 7 out of the 9 SBS children had received metronidazole within 6 months prior to sample collection, with all 9 receiving some type of antibiotic in the past 6 months.[130] The authors state that a possible limitation of the study is that children with SBS were younger than the healthy children.[130] As well discussed previously, the microbiome is temporally changing and developing in the first years of life, so this could be a major limitation of the paper. As well, the authors do not look at possible different anatomical aspects of SBS (like the presence or absence of the ICV) and how this could affect the microbiome.

Next, in a paper by Lilja et al. the authors looked at fecal samples from children diagnosed with SBS during the neonatal period compared to their healthy siblings as controls.[133] The study looked at 11 children, ages 1.5-7 years compared to 7 healthy siblings.[133] At the time of the study, 5 of the 11 SBS children had not been weaned from PN.[133] The Shannon diversity index is significantly reduced in children with SBS not weaned from PN compared to children that were weaned, and none of these children had the ICV remaining.[133] Even in the children that were weaned from PN, only 1 child reached Shannon diversity indexes at the same level as controls.[133] The authors correctly identified the major limitations of their study being age, small sample size, intestinal length and antibiotic treatment.[133] This altered microbiome in the SBS patients shows the potential for conditions like dysbiosis, SBBO and bowel inflammation to delay or prevent weaning from PN due to a relation to the GIT microbiome.

Now these results are not universal, in fact in germ free rats compared to normal colonized rats with a 75% small bowel resection, the germ free rats had greater markers of adaptation.[134] This potentially shows that in the short term of 7 days the microbiome may not be a major player in adaptation after small bowel resection, but the later issues of dysbiosis and its consequences along with colonic energy salvage are more important.

2.10.4 Negative Impact of Dysbiosis in Short Bowel Syndrome

2.10.4.1 Growth

Piper et al. hypothesized that children with SBS and poor growth would exhibit more severe gut microbiota dysbiosis compared to SBS children that were growing adequately, despite having similar intestinal anatomy.[71] The authors looked at fecal samples from 8 children

with SBS and 3 healthy controls over 3 months.[71] The results showed that SBS children had a significant reduction of Firmicutes order Clostridiales compared to healthy children.[71] Children with SBS and poor growth were deficient in bacteria known to produce SCFAs and had an expansion of *Enterobacteriaceae* compared to SBS children with adequate growth.[71] The authors also looked at key genes for metabolic function, SBS children that were poor growers were deficient in genes needed for gluconeogenesis but enriched in branched and aromatic amino acid synthesis and citrate cycle pathway genes.[71] Five of the SBS children were undergoing treatment for presumed SBBO.[71] Similarly to one of the previous articles,[130] the study found that all healthy controls had <5% Proteobacteria *Enterobacteriaceae* compared with an average of 23% in children with SBS.[71] Interestingly, while 5 SBS patients were receiving PN and 3 were not, there was no significant difference in *Enterobacteriaceae* between these groups.[71] There was no significant difference between SBS patients and controls in overall bacterial diversity or richness,[71] in contrast to the previous studies.[130, 132] All 5 patients with poor growth were treated with empiric antibiotics for presumed SBBO, in comparison to none of the adequate growers with SBS being treated for SBBO.[71] Due to this, the poor growers had a significantly increased antibiotic exposure compared to the adequate growers.[71] Some major limitations were the use of fecal samples, that represent the distal gut microbiome, so they were unable to make inferences about the proximal small bowel.[71]

In an almost follow up study to the previous study by Piper et al. the authors of this study by Engelstad et al. looked at fecal samples and remnant small bowel length and the correlation with intestinal dysbiosis and linear growth.[135] In this age matched cohort study when they analyzed the fecal samples via 16S rRNA, they not only found that compared to

healthy controls, the SBS patients had an altered microbiome and an increase in the relative abundance of Proteobacteria and Fusobacteria, but also a decrease in the bacteria phyla considered beneficial like Bacteroidetes.[135] Patients were then separated into those with SBS with remnant small bowel ≥35cm, and those with <35cm small bowel. Those SBS patients with <35cm of remnant bowel had a microbiome that was dominated with Proteobacteria compared to the ≥35cm group.[135] Interestingly, further changes in the Proteobacteria phylum were seen when the authors broke the data down into the different members of the phylum.[135] The healthy matched controls had a more diverse Protobacteria phylum, while the <35cm group had a less diverse Proteobacteria phylum with the genus *Escherichia/Shigella* composing a majority of the phylums.[135] In contrast, *Bifidobacteria* and *Veillonella* that are often early colonizers of the healthy infant GIT microbiome only make up <0.01% of the GIT microbiome of the <35cm remnant small bowel group, with *Bifidobacteria* and *Veillonella* making up on average 16% each of the ≥35cm remnant small bowel group.[135] The authors also found that the percent EN was inversely related to the relative abundance of Proteobacteria.[135] In this study, although the weight Z scores were not different between the two SBS groups, the linear height growth was, with linear height stunting occurring with the <35cm remnant small bowel group.[135]

As discussed under the role of EN on adaptation, the different SBS anatomy will impact not only the probability of adaptation, but not covered is the impact on the microbiome and SCFAs. Luminal nutrients will affect not only the microbiome of the GIT, but also the products of fermentation of these luminal nutrients, SCFAs. Especially, the possible trophic roles of SCFAs on intestinal adaptation that will be covered below.[24]

In weaning piglets fed fructooligosaccharides without SBS, a fermentable fibre that produces SCFAs along with lactate, for 7 days while weaning, there was significantly increased crypt depth as compared to controls.[136] There was also significantly increased SCFAs, logically due to the fermentation of the fructooligosaccharides, along with the butyrate measured in the luminal samples being positively correlated with crypt depth and the number of epithelial, mitotic and mucin-containing cells.[136]

Now, in rats after an 80% small bowel resection given metronidazole with the goal to reduce fermentation, these rats had decreased cecal SCFAs, along with reduced intestinal adaption measured via mucosal dry weight, protein and DNA compared to controls.[137] As discussed, EN is vital to adaptation, and patients on 100% TPN undergo mucosal atrophy. When SCFAs were added to TPN or as an intercaecal infusion for 7 days in rats, there was significantly reduced mucosal atrophy compared to 100% TPN rats without SCFAs.[138] Further to this, in rats with an 80% small bowel resection given TPN with SCFAs, this reduced the mucosal atrophy associated with being on 100% TPN.[139] These changes have been postulated to be due to SCFAs upregulating messenger RNA (mRNA) levels of proglucagon, the parent hormone for many hormones including GLP-2 and GLP-1, along with basolateral intestinal nutrient transport based on studies in resected rats.[140, 141] In piglets, SCFAs in TPN have shown increased glucose, amino acid and dipeptide transport are enhanced.[142, 143]

SCFAs not only have receptors throughout the GI tract and human body, but also are absorbed by the colonic epithelium and play a role in colonocyte metabolism. Bartholome et al. added SCFA supplementation to TPN of surgical piglets with SBS.[144] Butyrate was determined to be the SCFA responsible for inducing structural aspects of intestinal adaptation including

increased villus height in the duodenum, jejunum and ileum.[144] All these results show the importance of SCFAs as a trophic factor in the lumen of the intestine in response to nutrition or given exogenously.

2.10.4.2 Sepsis from Bacterial Translocation

Bacterial translocation occurs when viable (i.e. alive) bacteria pass from the gastrointestinal tract to other sites, including the mesenteric lymph node complex, liver, spleen and bloodstream.[145] There are three ways in which bacterial translocation is promoted from the GIT; SBBO, compromised host immune defences and increased permeability/damage of the intestinal mucosa.[145] The mesenteric lymph node is the first organ where bacteria will often be found following bacterial translocation due to SBBO.[145] The degree of translocation of bacteria to the mesenteric lymph node is different for different phylum of bacteria, with the degree of translocation of some species of *Enterobacteriaceae* to the mesenteric lymph node being directly related to the levels of these bacteria in the small intestine and cecum.[145] The differences in bacterial translocation are hypothesized to be due to how sensitive the different species of bacteria are to oxygen, with the least sensitive obligate anaerobes translocating at greater efficiencies than more sensitive obligate anerobes.[145]

It is known that bacterial translocation occurs in the clinical setting and has led to increased infections and complications in patients that undergo abdominal surgery.[146] Schimpl et al. looked at rats with different models of SBS, including jejunal resection, ileal resection or sham.[147] After 2 weeks, samples were taken from portal vein, vena cava, mesenteric lymph nodes, liver and spleen.[147] All SBS rats were reported to have bacterial overgrowth.[147] Bacterial translocation was determined via having bacteria in any of these organs or systemically and was

seen in 12% of the sham animals, 70% of the jejunal resection, 58% in the ileal resection and 35% in the ileal resection with ICV remaining.[147] Although the findings in this study show levels of bacterial translocation and SBBO that is much higher than what is seen in piglet models of SBS and in the pediatric population, the relationship between the increased bacterial translocation with SBBO and decreased bacterial translocation with ICV present are important first steps in research into this field.[147]

As described in a previously reviewed study by Lilja et al., when looking at fecal samples from SBS children, the authors found that in children that were still on PN, 4 out of 5 had been examined for multiple episodes of suspected SBBO.[133] Interestingly, in these children with SBBO symptoms treated with oral broad spectrum antibiotics *Enterobacteriaceae* was the most relative abundant taxonomic family.[133]

2.10.4.3 Liver Disease

With hepatic cholestasis occurring in many SBS children during intestinal adaptation and septic episodes contributing to this inflammatory state and liver cholestasis, the role of dysbiosis in liver disease has come into question.[70] In a study by Korpela et al. the authors looked at the possible link between the GIT microbiome and IFALD.[148] An increase in Proteobacteria and Actinobacteria was seen in patients with IF, with Proteobacteria being associated with liver steatosis, fibrosis, liver inflammation and intestinal inflammation.[148]

The previous study also saw an increase in *Lactobacilli,*[148] there is a relationship with this genus of bacteria and D-lactic acidosis that will be expanded on later, but this increase also has effects on liver steatosis.[148] Briefly, following bile acid deconjugation, the GIT microbiome can use 7-α-dehydroxylation to further transform the bile acids.[148] This modification via the GIT

microbiome can alter the downstream bile acids that activate FXR and TGR5.[148] Therefore, an increase in *Lactobacillus* due to dysbiosis could promote liver steatosis with this increase in bile acid deconjugation via the bacteria, and this leads to changes in TGR5 and FXR regulation.[148]

In a 4 week old piglet model with a 75% small bowel resection, the authors looked at the microbiome and histological evidence of IFALD and FXR signaling.[149] The results found that microbial dysbiosis occurred, with a decrease in *Clostridium* and *Bacteroides* following the small bowel resection and a significant change in bile acid metabolism leading to steatorrhea, diarrhea and liver damage.[149] There was also a blunted intestinal FXR activation response, that the authors suggested could be due to the changes in bile acid metabolism.[149] In the sham piglets, the *Clostridium* and *Bacteroides* species were the largest bile acid transforming bacteria present.[149] For the SBS piglets, the proportion of *Bacteroides* species was decreased.[149] The proportion of primary bile acids was increased for the SBS animals compared to the sham animals, with the proportion of secondary and tertiary bile acids decreased in the SBS animals.[149] As discussed previously, bacteria like *Bacteroides* and *Clostridium* help create secondary bile acids, so this relationship between the SBS animals dysbiosis and changes in bile acids makes sense.

It has become evident that the relationship between dysbiosis to IFALD is multifactorial, with many different factors playing into its development. The first, is less secondary bile acid producers present with SBS, leading to an increased primary/secondary bile acid ratio, with decreased hepatic and intestinal FXR signaling, and an increase in bile synthesis with a decrease in bile acid transporters, an increase in cholestasis and development of IFALD.[150] Another side, is the loss of SCFA producers with dysbiosis, leading to decreased enterocyte feeding,

decreased epithelial barrier function, increased endotoxins, bacterial translocation and development of IFALD.[150] Conversely, decreased SCFAs can also led to a decrease in B-cell maturation and immunoglobulin secretion, leading to pro-inflammatory TLR signalling, decreased epithelial barrier function, increased bacterial translocation and IFALD.[150] The change in the GIT microbiome has a wide range of effects, with as seen here multiple different effects on the systems that help prevent liver disease.

2.10.4.4 Small Bowel Bacterial Overgrowth and Short Bowel Syndrome

Some of the major concerns with SBBO and SBS is malabsorption, bacterial translocation and D-lactic acidosis (**Figure 2-1.**).[70] In a pilot study by Cole et al. the authors looked at PN dependent infants with SBS and evaluated the impact of feeding route and intestinal permeability on bloodstream infections, SBBO and systemic immune responses over a 4 month period.[3] The authors used fecal calprotectin as a biomarker for SBBO, but used a hydrogen breath test to diagnose SBBO.[3] Of the 10 infants studied, blood stream infection incidence was high, at 80% and SBBO occurred in 50% of the infants.[3] SBBO increased the odds of a blood stream infection more than 7 fold.[3] A SBBO diagnosis was not related to bowel length or the degree of enteral tolerance in these children, but the authors only had a sample size of 5.[3] Fecal calprotectin levels were significantly higher in children with SBS compared to healthy age-matched controls.[3] As well, children with SBS diagnosed with SBBO (via breath hydrogen test) had significantly higher fecal calprotectin levels compared to children with SBS without SBBO.[3] For intestinal permeability, there was no correlation between the index of intestinal permeability and the proportion of enteral feeding tolerated or fecal calprotectin.[3] Then the authors looked at systemic proinflammatory cytokines and found that children with SBS had

significantly higher serum concentrations of TNF-α at baseline compared with the age-matched healthy children.[3] These TNF-α levels decreased over time.[3] Children with SBS that were able to tolerate higher amounts of enteral nutrition exhibited lower serum levels of proinflammatory cytokines.[3]

As previously discussed, SBBO occurs in children with SBS, but there is a further complication of SBBO, termed D-lactic acidosis.[50] This is due to the chronic carbohydrate malabsorption that can occur in SBS patients, leading to an overgrowth of bacteria that produce D-lactate.[50] While as stated in the previous chapter, there are limitations to many of the methods used to diagnose SBBO, it has been suggested that in patients with SBS D-lactate serum concentrations can be used to diagnose SBBO.[50] This would be a non-invasive method, but it is rarely used due to limitations.[50] Serum levels of D-lactate are not indicative of whether the D-lactic acid producing bacteria are present in the small bowel or colon.[50]

2.11 Management of dysbiosis in Short Bowel Syndrome beyond antibiotics:

2.11.1 Introduction

Probiotics, much like SBS, have had elusive definitions over the years with many companies selling products they term "probiotics" but actually don't meet the criteria.[151] The WHO definition is "a probiotic is live microorganisms which confer a health benefit on the host."[151] Arguments around a stricter definition from the International Scientific Association for Probiotics and Prebiotics include: should the definition include the well-defined beneficial commensal microbes that have been studied, should the definition include traditional fermented food containing live microbes, and what is an appropriate level of evidence for

determining health benefits for probiotics.[151] An argument against the word "live" has recently been brought up, as other microbial based nonviable products that don't meet the "live" requirement become available.[151] For the purposes of this review, we will use the definition of a probiotic provided by each study we review. It is suggested that the "ideal" probiotic should remain viable at the level of the intestine and should adhere to the intestinal epithelium and be able to confer these significant health benefits to the host.[152] Here, for these bacteria to remain viable they must be alive.

What about the term "paraprobiotic" or "ghost probiotics"? These ghost probiotics are often defined as "nonviable microbial cells (intact or broken) or crude cell extracts (with complex chemical composition), which, when administered (orally or topically) in adequate amounts, confer a benefit on the human or animal consumer.[153] This definition does miss SCFAs and should be included. Inactivated probiotics are known to be able to mediate the Th1 to Th2 switch of the immune response.[153] A benefit of the use of paraprobiotics is that these "dead" bacteria are much safer in terms of the risk of infection.[153]

2.11.2 Probiotics and Gut Barrier Function

Some of the major factors that have been reported to increase the risk of bacterial translocation in SBS patients are intestinal permeability, compromised host immune defences and overgrowth of pathogenic intestinal bacteria.[154] Interestingly, intestinal permeability decreases as infants age and is greatest during infancy.[154] Intestinal permeability decreases faster in breast fed infants than those fed with adapted formulas.[154]

SBBO has been associated with injury to the intestinal barrier in SBS.[154, 155] D'Antiga et al. looked at intestinal permeability of 6 children with SBS using sugar absorption tests, which

are a non-invasive way to assess intestinal permeability.[156] They found that increased intestinal permeability occurred in 3 of the 6 patients with SBS, and was associated with a recent episode of sepsis and severe liver disease.[156] Bines et al. found that when looking at 4 children with SBS who required long-term parenteral nutrition who started on an amino acid based complete infant formula, intestinal permeability to lactulose decreased with the amino acid based formula.[157] The authors also found a reduction in hospitalizations, episodes of proven and suspected bacterial sepsis and central line insertions.[157]

Now, probiotics have been suggested to stabilize the intestinal barrier function and decrease gastrointestinal symptoms in children with atopic dermatitis.[158] Along with decreasing the increased intestinal permeability characteristic of children with food allergies.[158] In a study by Sentongo et al. the authors looked at the effects of probiotic *Lactobacillus rhamnosus* on intestinal permeability in children with SBS.[154] Permeability was measured via lactulose-to-mannitol ratio in children with SBS.[154] The results found no significant effect of the probiotic on intestinal permeability, but, they also found that fecal colonization with *Lactobacillus* species did not differ between the probiotic and placebo group.[154] It is possible that the dosage, length of administration, probiotic chosen or use of a single species probiotic wasn't adequate to cause any significant differences.

2.11.3 Probiotics in Animal Models of Short Bowel Syndrome

First, let's begin with a study by Eizaguirre et al. where the authors looked at probiotic supplementation in 126 adult Wistar rats with an 80% gut resection.[159] After 10 days, the animals were euthanized and mesenteric lymph nodes were recovered along with peripheral and portal blood.[159] A positive identification of bacterial translocation was determined if

bacteria was present both in the mesenteric lymph node and the blood.[159] The authors definition of a probiotic was "probiotics are live organisms that survive passage through the gastrointestinal tract and have beneficial effects on the host."[159] The probiotic used here was *Bifidobacterium lactis.*[159] The incidence of bacterial translocation was 6% in the control rats, while 87% of the untreated resection rats had bacterial translocation and 50% of the resection rats treated with the probiotic had bacterial translocation.[159] While these results do show a significant difference in bacteria cultured from both the mesenteric lymph node and blood, a major limitation of the study is that they do not look at the microbiome or permeability.[159] This would help further support whether the bacteria present in the mesenteric lymph nodes and blood was due to bacterial translocation, but, the reason it was not sequenced is when published many of the current microbiome analytical tools were not available or were cost prohibitive.[159]

More recently, in 2011 a study by Tolga Muftuoglu et al. looked at Wistar-Albino rats with and without a resection treated with probiotics.[160] The authors found a statistically significant difference in villus length, crypt depth, goblet cell count in the villus and crypt, mitosis and immunohistochemical evaluation in the jejunum of the SBS group untreated compared to the SBS group treated with probiotics.[160] The definition of a probiotic here was "probiotic bacteria are microorganisms that are of human origin, non-pathogenic and resistant to acid and bile. Probiotic bacteria are colonized in the intestines after oral administration and have beneficial effects on the host by inhibiting localization of pathogen bacteria in the intestines and stimulating the immune system."[160] Interestingly, the authors did not look at the effect of the probiotic on the microbiome of the rats, which seems to be a major limitation of

the paper. The probiotic used for this study had multiple species, being *Lactobacillus acidophilus, Bifidobacteria* and *Streptococcus thermophilus.*[160]

2.11.4 Probiotics in Pediatric Short Bowel Syndrome

We will begin with a study by Kanamori et al. looking at symbiotic therapy for over 1 year for 7 SBS patients with refractory enterocolitis.[161] Here, synbiotics are defined as "the combined used of probiotics and prebiotics and is expected to have a stronger effect on intestinal diseases than probiotic or prebiotics alone."[161] The symbiotic cocktail had *Bifidobacterium breve, Lactobacillus casei* and galactooligosaccharides.[161] In 6 out of the 7 patients, body weight gain was accelerated in the patients after starting symbiotic therapy.[161] In the feces, after starting symbiotic therapy there was high levels of *Bifidobacterium breve* and *Lactobacillus casei* detected.[161] In 6 out of the 7 patients, there was a significant increase of SCFA content in the feces.[161] From this, in a case study the authors looked at 1 girl with SBS from this group. The patients intestinal absorptive function and motility were improved.[161, 162] While the levels of *E. coli* and *Candida* were high prior to the commencement of therapy, with treatment the levels decreased in the feces with treatment.[162] As well, prior to treatment the ratio of facultative anerobic bacteria to total bacteria was very high, but this ratio was reduced after treatment started.[162] Weight gain was dramatically accelerated with treatment.[162]

Uchida et al. looked at symbiotic therapy in 4 pediatric SBS patients.[163] The symbiotic much like the previous study had *Bifidobacterium breve, Lactobacillus casei* and galactooligosaccharides.[163] The authors define probiotics as "live microorganisms, which beneficially affect the host animal by suppressing proliferation of pathogenic bacteria and improving intestinal microbial balance."[163] Prebiotics were defined as "nondigestive food

ingredients that stimulate growth and activity of a limited number of bacteria in the host's colon, thus improving host health.[163] The fecal microbiome in the SBS patients was abnormal, with low anaerobic bacteria, including Bacteroidaceae and *Bifidobacteria* compared to healthy controls.[163] After 3 months of treatment, the fecal microbiome had increased in total bacteria, including *Bifidobacterium,* and *Lactobacillus* in all patients.[163] Prior to treatment, the levels of SCFAs in the feces were very low compared to healthy controls.[163] The levels of SCFAs in the feces increased in 3 of the 4 SBS patients after 3 months of symbiotic therapy, and in 1 of these patients the levels of SCFAs was not only significantly increased but the levels were similar to the healthy controls.[163]

A male infant with SBS was administered probiotics *Lactobacillus plantarum* and *Lactobacillus GG* due to an abnormal small bowel flora that was concerning for potential impact on the function of his residual jejunum.[164] Stool samples prior to supplementation were lacking any detectable *Lactobacilli,* 3 days after supplementation this species was detectable in stool.[164] The patients had improved sodium balance and decreased stool frequency following probiotic therapy.[164]

2.11.5 Probiotics to Treat Small Bowel Bacterial Overgrowth and D-Lactic Acidosis in Short Bowel Syndrome

The standard treatment for D-lactic acidosis is the restriction of oral carbohydrates and administration of broad spectrum antibiotics.[165] In a case study by Uchida et al. the authors looked at a 22 year old patient with SBS with D-lactic acidosis that was treated with probiotics and an oral antibiotic.[165] The probiotic contained *Bifidobacterium breve* and *Lactobacillus casei* and the antibiotic used was Kanamycin.[165] In 8 months of follow up, there were no major attacks of encephalopathy or metabolic acidosis.[165] The authors did not look at the changes in

the microbiome of fecal samples, so it is hard to determine whether the probiotic or the antibiotic were responsible for the resolution.[165]

2.11.6 Safety of Probiotics

Lactobacillus and *Bifidobacterium* are extremely rare causes of infection in humans.[166] Now, strains of probiotics should be chosen from the commensal flora of humans, as well, these strains should not carry known or intrinsic resistance to antibiotics.[166] The importance for no known resistances is in the rare case of a probiotic derived infection.[166] In the case of SBS especially, strains added should be restricted to L-lactic acid producers, and not D-lactic acid producers, in order to prevent D-lactic acidosis.[166] This stems from an overgrowth of commensal *Lactobacillus* that as discussed can be an issue for SBS patients, and is frequently associated with D-lactic acidosis.[166]

While probiotics have a growing body of evidence in regards to potential benefits and safety, they should be used with caution, and with evidence based medicine, especially in premature infants, patients with a central line and/or patients with immune deficiencies.[166] As well, the properties of different probiotic species can vary and be strain specific so one should not make generalizations about one probiotic strain without confirmation in separate studies.[166] As well, different ages involve different levels of development of the GIT microbiome, and lead to different possible targets and effects of different species.[101]

Another possible risk is that probiotics are often regulated as dietary supplements rather than as pharmaceuticals or biological products, and therefore there is usually no requirement to demonstrate safety, purity or potency before marketing the probiotic.[152] Furthermore, a goal among many probiotics is to have the probiotic remain viable throughout

the gastrointestinal tract and to be able to have good adherence to the intestinal mucosa.[152] Of course, with increased adherence, there is increased risk of bacterial translocation.[152] Therefore is it hypothesized that the most potent probiotics may have increased risk of pathogenicity.[152]

Formula feeds are starting to be supplemented with probiotic. In infants fed a formula containing *Lactobacillus johnsonii,* a known D-lactate producing bacteria, the authors sought out to determine if urinary D-lactate excretions would increase.[167] After 4 weeks of probiotic supplemented formula, versus formula fed or breastfed infants, the D-lactate excretion did not differ between the two formula groups, but, was high in the formula groups compared to the breastfed infants.[167] This study showed no evidence that supplementation with this specific probiotic strain increased the risk of D-lactic acidosis.[167]

2.11.7 Complications with Probiotic Treatment for Short Bowel Syndrome

Although there are studies emerging on the potential benefits of probiotics for SBS, there is concern for complications. The probiotic *Lactobacillus,* is generally not considered a pathogen and is a commensal bacteria of the human microbiome, especially in the infant microbiome, but, in compromised hosts it can cause disease.[168] In two case studies by Kunz et al. the authors looked into possible complications with the use of *Lactobacillus GG* for pediatric SBS.[168]

In a male infant with SBS that was TPN dependent and had developed cholestasis *Lactobacillus GG* was started, and after 23 days of treatment the patient experienced fever and diarrhea, with other symptoms consistent with sepsis.[168] There was a positive blood culture for *Lactobacillus,* and after 10 days of ampicillin treatment the blood cultures came back negative.[168] An endoscopy was performed, and the infant's intestine was inflamed and

friable.[168] The authors suspect that bacteria translocation occurred across the fragile intestine, although, fingerprinting of the isolate along with mesenteric lymph node cultures was not performed so whether bacteria translocation occurred or catheter related sepsis it is hard to determine the exact origin.[168]

In the second case study, a male infant with SBS and TPN dependent with cholestatic liver disease was started on *Lactobacillus GG* regimen.[168] After 169 days of treatment, the patient developed an elevated temperature, tachycardia and increased apneic events.[168] Blood cultures were performed and antibiotics ceftriaxone and ampicillin were administered, with a positive blood culture for *Lactobacillus.*[168] In this case study, the authors were able to confirm that the sepsis was due to the probiotic strain of bacteria due to DNA fingerprinting.[168] The authors suspected that the patient's intestinal inflammation led to translocation of the probiotic across the intestinal lumen.[168] Some questions that arise from this, is potentially not probiotics in general, but *Lactobacillus GG* as a single strain probiotic is not safe in SBS. As well, potentially probiotics are not viable in patients with severe liver disease.

A 5 year old girl with SBS was on the probiotics *Lactobacillus acidophilus, Bifidobacterium longum* with fructose oligosaccharide due to watery diarrhea. 2 weeks after the probiotic administration began the onset of neurologic symptoms consistent with D-lactic acidosis started.[169] D-lactic acidosis was confirmed with plasma D-lactate levels of 5.537 mmol/L and attributed to the probiotic.[169]

In an 11 month old male with SBS, he presented with fever and hypoxia following receiving *Lactobacillus rhamnosus GG* twice daily for 5 weeks via a gastrostomy tube for treatment of rotavirus-related diarrhea.[170] Blood cultures drawn were positive for *Lactobacillus*

sp. and *Candida albicans.*[170] The catheter was removed and the patient was treated with ampicillin and gentamicin for 7 days and with amphotericin for 21 days.[170] The authors were able to confirm that the probiotic strain was the bacteria isolated in the blood.[170]

2.11.8 Probiotic Conclusions in Short Bowel Syndrome

While an overwhelming amount of the animal and human data for probiotics in SBS is positive, there are concerns that require further investigation for safe probiotic use in SBS. As well, what the best probiotic species would be for energy salvage, potency, duration etc. For risks, *Lactobacillus rhamnosus GG* given alone in multiple case studies has had negative effects.[168-170] Interestingly, considering the factors we mention in choosing the best probiotic, especially for SBS, *Lactobacillus rhamnosus* is among one of the most adhesive strains of probiotic studied.[171]

This review shows the importance of studying probiotics in animal models prior to human use, allowing us to study the effects on the GIT microbiome, energy salvage, adaptation and intestinal permeability.

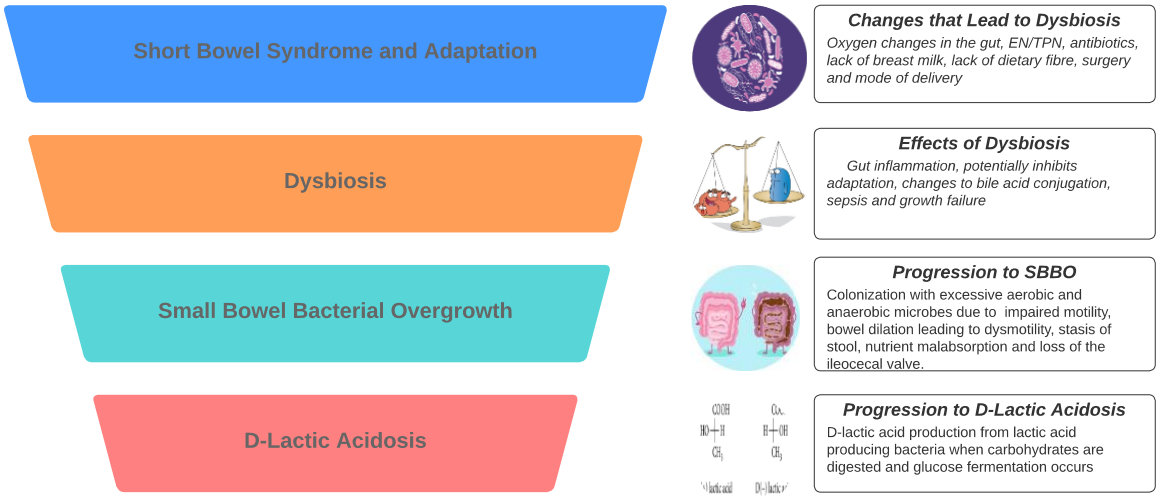


Figure 2-1. Pathogenesis of Short Bowel Syndrome on the Microbiome.

Chapter 3. Novel Medical Solutions for Enteral Autonomy: Trophic Factors

3 Introduction

In order to improve quality of life, weaning from PN, reducing days on PN, or achieving enteral autonomy, should be the goal of every IF treatment program. In SBS as discussed previously, this is achieved through gut adaptation. Recently, GLP-2 analogues have gained interest as trophic therapies aiming to improve adaptation in the management of SBS.[79] The relationship between the many different trophic factors, focusing primarily on GLP-2, insulin-like growth factor-1 (IGF-1) and epidermal growth factor (EGF) in gut development and adaptation is a novel field. Animal models along with the emerging human data gives us a glimpse how these trophic factors work individually and together. Along with the potential to give them exogenously to improve adaptation.

3.1 Glucagon-like Peptide-2 (GLP-2)

GLP-2 first gained interest in 1969, when a patient with a glucagon-secreting renal tumor presented with slow gastrointestinal transit and constipation from excessive glucagon produced via the tumor.[172, 173] The patient had marked changes in small bowel structure with elongated villi, motility and absorptive function that reversed when the tumour was removed.[173] This was then recreated in mice, where nude mice with proglucagon-producing tumors had significant proliferation of the small intestinal epithelium.[174] GLP-2 was determined to be the cause, stimulating crypt cell proliferation, increased bowel weight and villus growth.[174] The authors went on to determine that GLP-2 not only enhanced nutrient absorption in healthy mice,[175] but also in rats with experimental SBS on PN.[176]

Now, we know that GLP-2 is an intestinotrophic hormone produced by the enteroendocrine L cells of the distal small bowel and right colon.[172] It is a 33 amino acid peptide at the carboxyterminal end of proglucagon.[172] It has a short half-life, of about 7 minutes,[172] due to rapid degradation by the enzyme dipeptidyl peptidase-IV (DPP-IV).[177] GLP-2s role under normal physiological circumstances when stimulated by luminal nutrients in the distal intestine is to promote nutrient and fluid absorption.[28] GLP-2 is produced by the enteroendocrine cells of the distal small bowel, while its receptors are present throughout the GIT, being most abundant in the jejunum.[178]

Previously we reviewed how anatomy effects SBS symptoms, what wasn't discussed and is highly relevant to anatomical differences in adaptation is GLP-2 (**Figure 3-1.**). Individuals or animals with a jejunoileal resection still have the enteroendocrine L cells of the distal small bowel that produce GLP-2, along with the GLP-2 receptors in the proximal small bowel. Conversely, those with a jejunocolic anastomosis or jejunostomy have decreased endogenous GLP-2 due to a loss of the GLP-2 producing L cells. However, given a JC anastomosis with colon in continuity some studies show upregulated plasma levels of GLP-2, presumably through nutrient stimulation of residual colonic L cells.[28]

3.1.1 Glucagon-like Peptide 2 Development in Humans

As stated previously, GLP-2 is involved in the ileal break, due to this GLP-2 has been postulated when given exogenously to be able to help this ileal break in patients who have lost it, typically those without a terminal ileum and colon and that have severe malabsorption.[179] In adult patients, given 400 μg subcutaneously twice daily of GLP-2 without terminal ileum and

colon, treatment with GLP-2 improved intestinal absorption, with time to 50% gastric emptying increasing significantly, with small bowel transit time not changing.[179]

On that note, it is known that those with SBS with preserved colon have improved adaptation, and it has been demonstrated that patients with SBS with preserved colon have elevated fasting plasma concentrations of both GLP-1 and GLP-2, and significantly larger area under the curve of GLP-1 and GLP-2 after meals compared to age and sex matched controls.[180] In adults with SBS given 400 μg subcutaneously of GLP-2 three times daily (TID) with PN kept constant, over 2 years GLP-2 was able to decrease fecal weight and allowed SBS patients to be maintained with lower oral intakes.[181] While this was in a small sample size, this study was an important first step to demonstrating GLP-2s potential role in functional adaptation.

3.1.2 Glucagon-like Peptide 2 in Animal Studies

Since GLP-2 is produced primarily by the enteroendocrine L cells of the distal small bowel, it was hypothesized that with loss of that distal ileum, piglets would have decreased endogenous GLP-2 production, and therefore decreased capacity for adaptation.[182] Hua et al. looked at the ability to wean from TPN and promote EN with piglets with a 75% distal or mid resection, i.e. with or without ileum where these GLP-2 producing L cells are predominantly found.[182] The SBS piglets without ileum had limited innate capacity for adaptation and weaning from TPN, while the piglets with ileum had greater adaptation along with increased plasma GLP-2 levels.[182]

In another study, pre-term piglets with a 50% resection and a jejunostomy were given TPN with or without GLP-2 treatment and compared to unresected piglets.[183] After 5 days piglets were given EN to allow for fecal fat measures.[183] These resected piglets similar to the

previous study in older neonatal piglets, did not have an increase in endogenous GLP-2 when enteral nutrients were introduced, but the non-resected piglets did have an increase in endogenous GLP-2.[183] GLP-2 treatment led to increased relative absorption of energy and macronutrients from the stoma, along with an increase in the relative small bowel weight and percentage mucosa.[183]

Next, in a similar two week study in piglets with 75% mid JI or distal JC small bowel resection compared to sham controls, piglets were given GLP-2 or saline intravenously and structural and functional adaptation was assessed.[184] While JI piglets on GLP-2 compared to on saline had greater EN given, the JC piglets had more significant results with GLP-2 with fewer days on PN, greater EN tolerance, along with decreased diarrhea.[184] Both JC and JI piglets given GLP-2 had increased small bowel length compared to their respective saline groups.[184] While structural adaptation was improved for both JI and JC piglets, the piglets with decreased innate ability to produce GLP-2, the JC piglets without ileum, had the greatest clinical/functional results with GLP-2 therapy.

3.2 Development of Teduglutide first in its class GLP-2 Analogue

As stated previously, the short half-life of GLP-2 is due to the ability of DPP-IV to degrade it, this is largely due to the alanine residue at position 2 where GLP-2 is cleaved.[177, 185] Therefore, this was a likely target for drug development where amino acid substitution could reduce the affinity for DPP-IV and create a GLP-2 analogue with a longer half-life. The first such therapy, teduglutide, was created with the valine for alanine substitution in position 2, leading

to a half-life of around 2 hours.[172, 185] Teduglutide has been approved in children 1 year of age or older in Canada and the US, along with in adults at the same dosing regimen.[186]

In one of the first published teduglutide studies in adults, teduglutide was given subcutaneously for 21 days, once or twice a day at 0.05, 0.10 or 0.15 mg/kg/day.[187] Teduglutide at all doses increased absolute and relative absorption, urine weight and urine sodium excretion.[187] The investigators were able to perform intestinal mucosa biopsies, allowing them to evaluate villus height and crypt depth. In those with end jejunostomy, patients had significantly increase villus height, crypt depth and mitotic index, this was not seen in SBS patients with colon in continuity.[187]

In adults receiving teduglutide versus placebo those with a jejunostomy/ileostomy had the most significant results on teduglutide for absolute PN volume, compared to not only placebo but also to the other anatomies.[188] Teduglutide had an intermediate effect on those with <50% colon-in-continuity, a colostomy or both.[188] These results suggest that results in adults can be highly variable on teduglutide depending on different factors. Typically, those with higher baseline PN volume requirements had the most significant decrease in PN volume on teduglutide, especially those with jejunotomies or ileostomies.[188]

3.2.1 Teduglutide Pharmacology

Like all drugs, GLP-2 analogue pharmacology should be considered in terms of pharmacokinetics and pharmacodynamics, the principles of which will be briefly reviewed.

Pharmacokinetics (PK) focuses on how the body interacts with the drug, while pharmacodynamics (PD) focuses on how the drug effects the body.[189] There are four main areas to understand for PK studies, absorption, distribution, metabolism and excretion.[189] Absorption,

as the name implies, is how that drug is brought into systemic circulation.[189] This is important for the speed and concentration of the drug that will get to the targeted location, so in the case of GLP-2 analogues, how fast and what concentration GLP-2 will get to systemically and then to its receptors.[189] This brings up first-pass metabolism. When a drug is given orally, it will undergo first-pass metabolism, as it is processed by the liver, gut, digestive enzymes etc., this will decrease the amount of that medication that is delivered systemically in the case of oral medications/feeds.[189] For the case of subcutaneously injected GLP-2 analogues, the absorption of these medications into the plasma will create the area under the curve (AUC) for the plasma concentration.[189]

Next, is distribution of the drug throughout the body.[189] Distribution is important for getting the effective drug concentration, i.e., the amount of drug required for the drug to reach its targeted area (the volume of distribution) and is influenced by the properties of the drug along with the physiology of the patient taking that treatment.[189]

Metabolism is the processing of the drug by the body.[189] In the case of pediatric SBS for GLP-2 analogues, this is influenced by the ability of DPP-IV to cleave the analogue, along with the typically higher metabolism seen in pediatrics.

Excretion is how that drug is eliminated by the body.[189] GLP-2 analogues are eliminated by the kidneys, so kidney function can impact how quickly these analogues are eliminated.[190] An important term that comes up from PK studies is the half-life of a drug, so the amount of time for the concentrations in the blood to reach 50%.[189] It is calculated by the volume of distribution over clearance, so how a drug is distributed along with how long for the body to metabolise and excrete that drug will effect this half-life.[189]

Pharmacodynamics, is the physiologic effects or actions of that drug.[191] How a drug exerts its effects is through receptor binding, post-receptor effects and chemical interactions.[191] Emax is the maximum effect of a drug on the clinically decided target, in the case of GLP-2 analogues this would be on adaptation.[191] EC50, like the half-life, is the drug concentration to get half of the maximum effect of that drug.[191] The last term to understand for pharmacodynamics is the Hill coefficient, which is the slope of the relationship between drug concentration and effect, i.e. when the concentration of the drug is changed how significant of a change in effect is there.[191]

Some important questions for GLP-2 analogues include Kd, the relationship between the drug binding the receptor and the concentration of the drug already at the receptor site.[191] The smaller the Kd value, the greater the affinity of the drug for the target (i.e. GLP-2 receptor).[191] Receptor occupancy, the more receptors that are occupied by a drug, typically the greater effect of that drug, but all receptors don't have to be occupied for many drugs to get their Emax.[191] With long-term exposure to a drug, can get downregulation of the receptor, i.e. a decreased number of GLP-2 receptors with chronic GLP-2 analogue use.[191] There are a few exceptions to this rule where can get an upregulation of receptors with long-term exposure.[191]

One of the first dosing studies for teduglutide involved daily subcutaneous administration in healthy volunteers over 8 days, helping determine safety, pharmacokinetics and safety of the drug.[192] When assessing teduglutide that is largely excreted by the kidneys, those with less developed kidneys or renal impairment need to be considered. In 3 patients with renal impairment versus three with healthy subjects with normal renal function given 10 mg/kg of teduglutide as a single dose a PK study was performed.[193] AUC and C_{max} were raised

for those in end stage renal disease versus healthy subjects.[193] These results suggested that the dose should be reduced by around 50% in patients with moderate and severe renal impairment.[193] This study was performed in adults without SBS. Questions when relating this study to SBS patients, especially in pediatrics, is that while these patients have less developed kidneys, they also have a higher metabolism than adults.

3.2.2 Clinical Trials in Adults

Since the work on teduglutide began in adults it is important to look at the studies in adult SBS first. Much of the work has been discussed under teduglutide development. At a single centre, teduglutide in adults with SBS was able to have 61% of patients achieving complete EN independence at a median of 10 months, and PN requirements were reduced.[194] Of the patients that achieved EN independence, 91% had colon.

Plasma citrulline has been used throughout the literature as a rough marker of enterocyte mass, as it is produced by enterocytes, and therefore higher citrulline levels would indicate greater adaptation.[195] At 24 weeks, teduglutide at both doses of 0.05 and 0.10 mg/kg/day had significantly increased plasma citrulline, along with reductions in PN volume requirements.[195]

Teduglutide has not been shown to have significant effects on gastric emptying in both healthy volunteers or patients with SBS.[196, 197] While there was no effect on gastric emptying, there was a trend ($p=0.075$) towards increased gut transit time for teduglutide over placebo in 8 adults with SBS on HPN given teduglutide at 0.05 mg/kg/day for 7 days.[197] There was no effect on mucosal permeability, measured via urine mannitol, lactulose or lactulose mannitol ratio.[197]

Following the initial 24 week study, there was a 28 week extension study, with patients continued on the initial dose of 0.05 or 0.10 mg/kg/day of teduglutide. This study like many others was looking for a ≥20% reduction in PN volume from baseline, along with safety and tolerability.[198] By 52 weeks, 68% of the 0.05 mg/kg/day group and 52% of the 0.10 mg/kg/day had a ≥20% reduction in PN.[198] The efficacy appears to be able to be maintained to this point and is safe at 52 weeks of continuous use.

In longer-term studies, those patients that had been enrolled in the 24 week teduglutide original study, were extended for up to 30 months at 0.05 mg/kg/day.[199] Abdominal pain was the most common adverse event, with a decrease in PN volume from baseline of 66%.[199] 45% of the patients who were a part of the initial 24 week study were able to achieve enteral autonomy.[199]

3.2.3 Clinical Trials in Pediatrics

The doses used in pediatrics trials were largely extrapolated from the adult dosage of 0.05mg/kg/d.[182, 184] As will be seen, the clinical trials in pediatrics affirmed this dose in regard to pharmacodynamics. This is despite the few pharmacokinetic studies done in pediatrics that suggested the t1/2 would be shorter and the exposure longer with teduglutide as compared to adult studies, due to increased clearance.[178]

In a 12 week open-label study, pediatric patients with SBS who were TPN dependent, and had little to no advance in EN, ages 1-17 were given the GLP-2 analogue, teduglutide.[200] This first study was to determine safety and pharmacodynamics/efficacy of teduglutide.[200] Dosing was 0.0125 mg/kg/d, 0.025 mg/kg/d and 0.05 mg/kg/d.[200] The primary outcome for this study was reductions in PN, with an advancement in EN.[200] The most optimal dosing

determined from this study was 0.025 and 0.05 mg/kg/d, leading to trends toward a reduction in PN.[200] Side effects included GI-related adverse events, upper respiratory tract infection, catheter related complication, and pyrexia.[200] This study had a small sample size (groups ranging from n=8-15), along with the short duration of 12 weeks, especially when it comes to the process of adaptation.[200] This study was also open label, meaning that the clinical trial information wasn't withheld from the trial participants.[201]

Next, in a follow up study that addressed the two main limitations of the previous study, teduglutide was studied in a larger sample size and over 24 weeks.[200] The primary endpoint for this study was a 20% or greater reduction in PN volume.[201] The doses studied were based on the previous study trends, being 0.025 and 0.05 mg/kg/d, along with a standard of care group.[201] With the larger sample size and study duration, now the study was able to achieve statistical significance. There was a significant reduction in both doses in PN volume, PN calories, days per week and hours per day of PN infusions and an increase in EN.[201] Two patients out of the 24 in the 0.025 mg/kg/d and 3 patients out of the 26 in the 0.05 mg/kg/d groups achieved enteral autonomy.[201] Once again, the main side effects of teduglutide were pyrexia and vomiting.[201] Interestingly, side effects were seen in 95% of the teduglutide patients and 100% of the standard of care patients, with only 2 patients in the teduglutide groups determined to be treatment related, most of the side effects were similar between groups and arguably not due to the teduglutide treatment.[201] This study suggested that the dose-relationship in children (between 0.025 and 0.05 mg/kg/d) is relatively flat, and the authors suggested that doses above 0.05 mg/kg/d are unlikely to provide further benefit.[201] This study was still open-label, leading to this bias/limitation[201] still not being addressed.

In the next follow up study to this, patients in the 24 week study went directly into the follow up, while those in the 12 week study had a multiyear gap between studies.[202] Patients underwent long-term follow up, for ≤161 weeks.[202] Like as alluded to in the previous study, the most frequent side effects were not due to teduglutide, but due to complications of the underlying disease and typical illnesses seen in childhood.[202] Now, there were three serious side effects that were determined related to teduglutide treatment, with two or arguably all three of these being due to changes in gastric motility.[202] One patient experienced D-lactic acidosis, that was covered in chapter 2, but was most likely due to decreased intestinal motility, leading to increased contact time between the nutrition and microbiome leading to an overproduction of D-lactate from a bacterial overgrowth.[202] There was an ileus, and an intestinal obstruction due to hard stools.[202] There was 1 cecal polyp, a concern in adults given teduglutide due to this increase in cell proliferation, but it was not biopsied when visualized, and was not found on follow up colonoscopy.[202] Anti-teduglutide antibodies increased form 1.1% at baseline to 33.3% at 36 weeks, then plateaued from 36 weeks to 72 weeks.[202] Now, how this will effect patients given teduglutide long-term is unknown, but was similar to what is seen in adults.[202, 203]

In Europe a recent 48 week open-label single centre clinical trial was completed for teduglutide use in children, age 5-16.[204] To be included in this study, patients had to be on PN for ≥2 years and have small bowel length of <80cm.[204] Like the previous studies, patients had to have reached a plateau in PN and were dosed at 0.05 mg/kg/d, here for 48 weeks.[204] 32% of children were weaned from PN by 48 weeks, and by 24 weeks 96% of children had a >20% reduction in PN.[204] The number of infusions per week decreased from 6 to 4 days by week 48.[204] Fecal balance was also analyzed, looking at total energy absorption at baseline and 48

weeks.[204] The absorption rate went from 59% to 73%, a significant increase in energy absorption for these SBS children.[204] Like the previous studies, abdominal pain, stoma changes and redness at the injection site were the common side effects.[204]

3.3 Alternative Analogues in Development

The argument for alternative analogues would be for a reduced injection schedule through a longer half-life, which is especially relevant to pediatrics. Patients often get pain at the injection site,[202] and acceptance of a daily injection for children is poor. Regardless, in adult SBS patients teduglutide has been shown to improve quality of life scores, especially those with the highest starting PN volume.[205] However, it is possible to increase the drug half-life with further changes (amino acid substitutions, deletions, etc.) to decrease the efficiency of DPP-IV to degrade the analogue and so prolong clearance of the drug. This would allow for fewer injections and might be particularly welcome for children. Such alternatives in development include apraglutide and glepaglutide.

Apraglutide has a lipophilic amino acid substitution in positions 11 and 16, along with a change in the terminal group.[206] These changes allow slowed absorption from the subcutaneous site, very low clearance and high protein binding, along with it to be administered once weekly, instead of every day.[206] Glepaglutide has a polylysine moiety at the C terminus to reduce renal clearance along with amino acid substitutions.[206] Glepaglutide is administered once weekly as well. An interesting difference in cell based assays, is that while apraglutide and teduglutide are both selective for the GLP-2 receptor, glepaglutide is less selective for GLP-2 versus the GLP-1 receptor.[206]

Animal studies in rodents have shown a greater pharmacodynamic response to apraglutide over teduglutide and glepaglutide in terms of small intestine growth.[206] In adults with SBS, glepaglutide over 3 weeks during the phase 2 trial has reduced fecal output and increased intestinal wet weight and macronutrient absorption.[207] Glepaglutide is currently in phase 3 trials.

3.4 Long Term Efficacy of GLP-2 Analogues

With enteral autonomy being a major goal of SBS treatment, it is important to understand the degree of patients that reach enteral autonomy. The data is very inconclusive as some studies are looking at patients with lower PN dependence, while others are patients with high PN dependence that have very limited innate capacity for adaptation. For example, in the 24 week study for teduglutide in pediatrics, while 12% of patients achieved enteral autonomy in this timeline, with 69% of patients achieving a 20% or greater reduction in PN from baseline to week 24.[201] While 96% of the patients had remaining colon, 20% of patients had a stoma.[201] When teduglutide was given for 48 weeks in pediatrics, now 32% of patients achieved enteral autonomy.[204] Now, this study broke down these patients that reached enteral autonomy into type 1-3, interestingly, 50% of the patients that weaned off of PN had type 2 anatomy, with 25% with type 1 and 3 anatomy.[204] Of the patients that reached enteral autonomy, they had significantly decreased baseline PN volume compared to those that did not reach autonomy, along with significantly increased baseline EN volume.[204]

There are a few studies of note for when GLP-2 analogues are stopped and if the trophic effects are reversed. Starting in rats with a jejunoileal resection given sustained GLP-2 for 7

days, with increased markers of mucosal adaptation in animals terminated at day 7, but those that stopped at day 7 and terminated at day 18 the effects were reversed.[208] Adult patients in the 21 day teduglutide study we covered previously,[187] over the 21 days of treatment had improvements in intestinal absorption and decreased fecal output, but after the washout period these effects were reversed.[187] Adult patients treated with GLP-2 for two years, with an 8 week washout period, where once again the fecal wet weight reversed to baseline with GLP-2 being discontinued, along with creatinine clearance.[181]

In a study by Compher et al., when teduglutide was stopped after being administered for ≥24 weeks in adults, looking at body mass index and PN volume over 12 months, the change in BMI was predicted by colon and small bowel length, baseline BMI and the change in PN seen when on teduglutide.[209] This study highlighted around half the patients had no change in BMI by 12 months post treatment, and might be able to go off of teduglutide treatment while closely monitored, while other patients, especially those that were poor responders to teduglutide and had increased PN volume while on teduglutide had a further increase in PN volume after teduglutide stopped.[209] The levels of plasma citrulline decreased following discontinuation of teduglutide, indicative of some reversal of this increase in enterocyte mass producing citrulline.[199, 200, 210]

There have been similar results for apraglutide, when apraglutide was given in adults, after stopping apraglutide treatment for 4-6 weeks, the measure of urinary volume used to infer intestinal fluid absorption reverted back to baseline.[211] Now these results are often in adults, but in a neonatal piglet model of SBS, when either teduglutide or apraglutide were given to piglets for 7 days with a 7 day washout period to determine any reversal, after 7 days of

treatment cessation the mucosal adaptation like the previous studies regressed.[212] Conversely, the increased length seen with both GLP-2 analogues was further increased with apraglutide and maintained with teduglutide.[212] This is something that is difficult to measure in human studies, often citrulline is used as a marker of length via enterocyte mass, but animal studies allow us to directly measure the length of the remnant bowel with treatment.[212]

3.5 Safety Concerns

Due to the increased proliferation of cells with GLP-2 treatments, there has been concern for the potential of these analogues to promote cancer.[177] On myofibroblasts from colon cancer and the adjacent issue, Shawe-Taylor et al. investigated the effect of GLP-2 on these cells.[213] GLP-2 stimulated proliferation, migration and invasion of the myofibroblasts. The response was greatest for the cancer-associated myofibroblasts than the adjacent tissue, this was inhibited by an IGF receptor inhibitor.[213] Now, once again like the previous study and a common theme throughout lots of this work to follow, this was done on cancerous cells that are more prone to proliferation and are cells. This cell work isn't necessarily reflecting what would happen in vivo in normal cells. GLP-2 with a DPPIV inhibitor in human colon cancer cell lines lead to increased proliferation and increased migratory activity, the authors suggested this could promote existing intestinal tumours and potentially allow colon cancers to metastitize.[214]

With the GLP-2R being present in many other tissues and the nervous system, there is GLP-2R mRNA expression in gastrointestinal stromal tumors.[215] Now, this data is on mRNA, which does not necessarily mean that this mRNA will be translated into the protein and create a physiological change. Only 20% of human adenocarcinoma samples have GLP-2R expression,

with no expression in colonic adenomas, suggesting that in contrary to some of the previous results in rodents, GLP-2 expression is not a factor in adenoma-cancer pathogenesis.[216] This study used immunohistochemical staining for the receptor,[216] while another study that looked at GLP-2R mRNA expression using RT-PCR found expression in ileal carcinoid tumors and colon adenocarcinomas but not autoradiography.[215]

GLP-2 activating Akt phosphorylation and signalling has been established, but has been determined to be not be strictly dependent on IGF-1R/IGF-1 signalling, while β-catenin requires IGF-1.[217] This has been determined by both RT-PCR along with western blot, which strengthens the results as western blot visualizes the protein, showing the mRNA was translated to the protein, but this was in rodents which are not always an indication of what would occur in other animal models or humans. This becomes of concern as Akt is a proto-oncogene that is overexpressed in many human cancers as the kinase is responsible for cell cycle progression, cell survival and cell proliferation.[218]

One of the first studies to investigate the impact of GLP-2 on neoplasia in rodents, looked at mice treated with GLP-2 after treatment with methylating carcinogen 1,2-dimethylhydrazine (DMH).[219] While every animal developed colonic polyps, there was a significant increase in tumour load of mice treated with GLP-2.[219] Conversely, in nude mice with GLP-2R positive human colon cancer cells, chronic GLP-2 treatment had no effect on the growth of the colon cancer cells, and mice disrupted for the GLP-2R gene had no change in the number or size of polyps.[220] Due to this the authors concluded that GLP-2 did not impact intestinal tumor cell growth or survival.[220]

In rats fed the carcinogen 2-amino-1-methyl-6-phenylimadazo[4,5-b]pyridine and high fat diet, and mice with chronic dextran sodium-sulfate (DSS)-induced colitis administered azoxymethane, the authors investigated GLP-2 or teduglutide on cancer rates.[177] The results showed that teduglutide and GLP-2 promoted the development of preneoplastic rats in these animal models to a small degree, but when given a GLP-2 antagonist to block the actions of GLP-2, there was a reduction in cancer.[177] Now, it is important to note that although these results are concerning, these are in animal models that are extremely prone to cancer and might not reflect what would be seen in humans, or even other animal models.

Mice were treated with a GLP-2, a GLP-2R antagonist or PBS.[221] With chronic GLP-2 treatment had increased markers of adaptation as expected, but if given azoxymethane followed by GLP-2, adenocarcinomas developed in those receiving it.[221] This suggested that while chronic GLP-2 enhanced colon carcinogenesis, the GLP-2R antagonist decreased dysplasia.[221] Once again, these rodents were treated with azoxymethane that is carcinogenic so how this relates to humans is yet to be fully determined.

At 24 weeks of treatment with teduglutide in adults with SBS, there was no signs of malignancy or colonic adenomas in the subset of patients who had a colonoscopy performed.[222] In the extension study of this, where for 52 weeks total, patients were treated with teduglutide with colon in continuity, one patient was determined to have a 2 mm hyperplastic colonic adenoma and was withdrawn from the study, while no cancer or adenomatous polyps were found at the end of the study.[198]

In adults give teduglutide for up to 3.5 years, there were polyps in 9 out of 50 patients, with none that histology was available on being cancerous.[202, 223] One patient with a history of

Hodgkin lymphoma developed a metastatic adenocarcinoma in the liver during by 11 months into the study, while the origin of the tumor was unknown the histopathology suggested that metastasis most likely originated from a gastrointestinal tumor.[199] In 83 PN dependent patients with SBS on teduglutide at two doses (0.05 or 0.10 mg/kg/d) the study was not powered to detect significant differences in dysplasia, but there was no dysplasia or concerns for the mucosa at either of the two teduglutide doses over 6 months of teduglutide treatment.[210]

These results highlight how with different methods, animal models, cell lines, in vitro, in vivo, analysis techniques, dose, GLP-2 analogues, native GLP-2, etc. there is a drastic impact on answering the question of how GLP-2 analogues could impact neoplasia. It is difficult to determine if the current data, in animal or cell lines prone to cancer, or mRNA expression showing mixed results can tell us the true answer to this question. More investigation and for longer study periods in humans is warranted to determine the answer to this.

3.6 Cost Effectiveness and Quality of Life with GLP-2 Analogue Teduglutide

With the significant cost of teduglutide and other GLP-2 analogues, there is a need to better understand what patients will be most significantly impacted with GLP-2 treatment. With that, there are different cost and quality of life markers to assess. PN reduction, achievement of enteral autonomy, less nights on PN and other factors are assessed for quality of life. Along with the impact on quality of life for the patient and caregiver with less nights on PN and less hospitalizations. What cannot be forgotten is the impact of the high cost of GLP-2 analogues on the healthcare system or caregiver. Using a Markov model, in adults, teduglutide does not meet the cost-effectiveness threshold when aiming for PN reduction in adults with SBS compared to

the standard of care intestinal rehabilitation.[224] With a cost in adults of >$400,000/year, for teduglutide to be cost-effective it needs to be substantially reduced in cost to below what is traditionally acceptable at $100,000/quality of adjusted life years (QALY).[224] Now, this does not mean that teduglutide for adult SBS is not necessary, but instead puts an emphasis on looking at who it should be prescribed to in order to reduce health care expense. For example, patients with a high output jejunostomy might be impacted more significantly by teduglutide than other anatomies.[224] This also does not take into consideration the burden of care for both the individual and the family of the patient with SBS. While the cost of teduglutide would still need to be drastically decreased for it to be cost-effective with these measures accounted for, it cannot be underestimated how a single night off of PN per week could impact a patient and their family, let alone what achieving enteral autonomy could mean for both.

A similar Markov model was done looking at teduglutide in pediatric patients with SBS, with the same benchmark of $100,000/QALY. This study was able to determine that a reduction in the cost of teduglutide by 16% would allow that benchmark to be reached.[64] Similar to the previous study, the authors suggested the possibility of alternative dosing regimens to decrease cost, but this would need to be investigated.[64] Therefore, at the current cost in the US, teduglutide is not cost effective for adults or pediatric patients with SBS.[64, 224]

A recent publication into the cost effectiveness of teduglutide showed that teduglutide was cost-effective when started two and five years after intestinal resection once the natural process of intestinal adaptation is reduced.[225] This study used a cost-effectiveness criteria of $50,000/QALY gained in weaning PN support in children with SBS.[225] This provides a novel perspective and support of the current dosing of teduglutide and why it should not be initiated

until at least one year of age. This is also supported by the fact that the current literature shows that most children who are able to achieve enteral autonomy spontaneously will occur within the first two years after resection.[46, 225, 226] This data supports waiting for the process of adaptation to occur prior to considering trophic therapies like teduglutide. As well, when weight-based dosing was used with avoiding the wastage of the remaining 5mg dose vial designed for adults, teduglutide was less costly than the standard of care.[225]

As mentioned earlier improved quality of life using teduglutide in adult patients with SBS has been demonstrated over a 24 week study period.[205] This study used the disease specific short bowel syndrome quality of life scale.[227] Patients were also classified into subgroups, of PN volume, SBS etiology and bowel anatomy.[205] The study found a significant relationship between PN volume reduction and SBS improved quality of life, therefore hypothesizing that teduglutide can improve patients quality of life via reducing PN volume, i.e. by improving intestinal adaptation.[205] This study furthers the results of a previous study that did not reach statistical significance for improved quality of life, but had a reduction in PN volume associated with quality of life improvements in a similar cohort of adult patients.[228]

3.7 Mechanism of Action of GLP-2 Other Trophic Factors and Gut Adaptation

GLP-2 is only one trophic hormone involved in adaptation, other potential mediators include IGF-1 and EGF. Further, what is surprising about GLP-2 is the receptor, GLP-2R, isn't localized to the known target cells of the GLP-2 trophic effects, i.e. the intestinal epithelial cells, but instead to scattered enteroendocrine cells, enteric neurons and subepithelial myofibroblasts.[229] This has led to the hypothesis that GLP-2 exerts its trophic effects on

intestinal epithelial growth through paracrine and/or neural pathways.[229] The potential role of IGF-1 as a paracrine mediator of GLP-2 action will now be discussed.

3.7.1 Insulin-like Growth Factor-1 (IGF-1)

While GLP-2 is a very important postnatal hormone for gut development, in human milk and colostrum IGF-1 is one of the most abundant hormones.[230] IGF-1 is not only important postnatally provided in human breastmilk, but the placenta secretes IGF-1, and during gestation fetal circulating IGF-1 increases. At birth, levels of serum IGF-1 are positively correlated to the size of the infant and fat mass.[230] IGF-1 increases during the last trimester, when there is an exponential increase in gut growth.[230] Therefore, IGF-1 is a vital trophic factor for intestinal maturation.[230] IGF-1 is produced via the liver, and IGF-1 levels are reciprocally regulated via insulin-like growth factor binding proteins (IGFBP), which inhibit IGF-1 bioactivity.[230]

IGF-1 is expressed on smooth muscle cells, when piglets with a 75% bowel resection were given infant formula with colostrum protein concentrate, containing IGF-1 and IGFBPs, there was a significant increase in muscle width and the number of muscle cells.[231] This muscular hypertrophy and adaptation could lead to changes in transit time etc. that have not been evaluated throughout the literature. When rats underwent a 90% small bowel resection, and were given growth hormone (GH), IGF-1 or GLP-2 all given subcutaneously, after 14 days there were significantly increased weight gain in all hormone treated groups compared to control.[232] Villus height was also significantly increased for IGF-1 rats compared to the control resected group.[232]

As discussed throughout, TPN induces mucosal atrophy, and in mice maintained on TPN with or without an infusion of recombinant human IGF-1, dosed as 2.5 mg/kg/d IGF-1 was able

to reverse some of this atrophy, specifically the jejunal muscularis atrophy.[233] The TPN induced atrophy was reversed with IGF-1 for small bowel mass along with the reduction in small bowel protein and DNA concentrations.[233]A highlight from this study is that TPN decreased IGFBP-5 mRNA expression by 60%, while IGF-1 in TPN increased IGFBP-5 mRNA by 200%.[233] These results were all compared to enteral fed mice for reference in terms of mucosal atrophy.[233] There was significantly increased IGF-1 serum concentrations with the IGF-1 administration, along with a significant increase in spleen and kidney mass.[233]

IGF-1 has been postulated to be a potential mediator of the effects of GLP-2. In mice null for IGF-1 given GLP-2 they were unresponsive to GLP-2 in terms of adaptation metrics, but in mice with IGF-1 at the same dose now mice had adaptive changes.[229] GLP-2 also increased mRNA of IGF-1 in mouse small intestine.[229] These results confirmed the hypothesis that IGF-1 is an essential mediator for small and large bowel growth in response to GLP-2 treatment.[229] IGF-1 is expressed primarily in the subepithelial myofibroblasts and smooth muscle cells close to the GLP-2 receptors, along with the IGF-1 receptors being present in the epithelial crypt cells who are the drivers of villus lengthening in adaptation.[229] Therefore, it is proposed that GLP-2 stimulates the production of IGF-1 by intestinal subepithelial myofibroblasts and trophic effects of GLP-2 rely on the downstream signaling via the IGF-1 receptor (**Figure 3-2.**).

In mice given GLP-2, as expected, they had decreased permeability, measured with 4-kDa fluorescein isothiocyanate-dextran (FITC-dextran) and increased jejunal resistance.[234] However, when mice were null for the IGF-1R on intestinal epithelial cells, this effect was abrogated.[234] This suggests that IGF-1R is required for the effects of GLP-2 at least for intestinal permeability. An important aspect to remember when studying mRNA levels, is that the

presence of mRNA does not necessarily mean that it will become the protein. However, in mice, tight junction proteins regulate this jejunal paracellular permeability and can be assessed by western blot or immunoblotting, to directly measure that protein level.[234] GLP-2 induced an increase in tight junction proteins, but without the IGF-1R on intestinal epithelial cells this was again abrogated.[234]

In the 48 week teduglutide study, they did measure the plasma IGF-1 levels of all patients at baseline compared to at the end of 48 weeks of treatment.[204] Now there was no significant difference in IGF-1 levels, but this was for all patients not those stratified to anatomy or if achieved enteral autonomy which makes the results difficult to interpret.[204] In that being said, there was no significant difference in endogenous GLP-2, both fasting and postprandial while divided into patients weaned off PN and those not weaned.[204]

3.7.2 Epidermal Growth Factor (EGF)

Another potential mediator of the trophic actions of GLP-2 is EGF, encoded by the ErbB ligand and ErbB pathway.[235] EGF stimulates mitosis of epithelial cells, and has a significant role in growth, development and maturation of the GIT.[236] In mice given EGF via oral gavage or intraperitoneally following a 50% proximal small bowel resection, EGF stimulated the greatest adaptive response when dosed at 50 μg/kg/d via the oral route for one week.[236] There was not an additive effect when the dose was increased.[236] The EGF receptors are located primarily on the basolateral surface of the enterocyte.[236] Interestingly, serum EGF levels were higher when given via oral gavage than when given intraperitoneally.[236] In these mice the adaptive response is completed after 1 month, and when EGF was given after the adaptive response was completed the authors were unable to demonstrate a further adaptive response.[236] In this

same model it was also shown that when EGF was given via intraperitoneal injection, EGF not only enhanced intestinal adaptation, but also increased ileal expression of its own receptor, EGFR mRNA and in turn EGFR protein.[237] EGF in this model has been further shown when given via orogastric gavage to increase structural markers of adaptation like villus height and crypt depth, along with the proliferation index of the ileum.[238] This work showed a role for EGF in proliferation but also suppression of apoptosis following small bowel resection.[238] None of the work has demonstrated a role for EGF in well-adapted SBS.[239]

In mice given GLP-2, there was increased expression of multiple ErbB ligands, and gene expression in the small and large bowel.[240] In mice null for GLP-2, this was abrogated.[240] With an ErbB inhibitor, the effects of GLP-2 on crypt cell proliferation and bowel growth were also eliminated.[240] Interestingly, IGF-1 and keratinocyte growth factor both had no effect on the ErbB ligands.[240]

In wild type mice fasted, there is a significant reduction in small bowel mass, crypt and villus height and crypt cell proliferation as expected.[241] In the wild-type mice, refeeding increased GLP-2 and reversed the small bowel atrophy, while in mice that are GLP-2R null, refeeding did not reverse the small bowel atrophy, and mRNA for EGF, keratinocyte growth factor and IGF-1R were lower.[241] Interestingly, EGF restored markers of adaptation in GLP-2R null mice, while IGF-2 did not.[241] When ErbB inhibitor was given, this prevented markers of adaptation after refeeding in wild-type mice.[241] Exogenous EGF in rodents has improved adaptation in multiple studies.[236, 242] In fact, in a small pilot study of 5 patients given EGF dosed at 100 μg/kg/d given mixed in their EN for 6 weeks had improved carbohydrate absorption and increased EN tolerance.[243] There were no changes in intestinal permeability.[243]

EGF was given to piglets with a 75% resection and JC or JI anatomy enterally either alone or in combination with GLP-2 given intravenously.[235] EGF alone had increased small bowel weight per length and jejunal villus height in the JI group, this was not observed in the JC group.[235] The JC piglets with GLP-2 alone or in combination with EGF had increased intestinal weight and villus height.[235] The combination had decreased jejunal permeability of both mannitol and PEG in both JI and JC piglets measured via Üssing.[235] Combination treatment also lead to intestinal lengthening.[235] In JI piglets, GLP-2 and EGF alone increased claudin-7 measured via mRNA, while GLP-2 alone and in combination also increased claudin-15.[235] There were no significant differences for JC piglets.[235] Interestingly, with JC piglets IGF-1 expression was increased with EGF compared to GLP-2, while the JI GLP-2 had greater expression than the JC GLP-2 group.[235] There were no differences in GCG or ErbB1 mRNA (the main EGF receptor).[235]

3.8 Conclusion

Trophic factors have opened up a new era for SBS management, where adaptation can continue beyond the first 2-3 years following resection, aided by trophic factors, allowing patients who were PN dependent the possibility of achieving enteral autonomy.[12, 186] While these effects on decreasing PN volume by $\geq$20% are significant, the effects of teduglutide based on the current literature are reversible, meaning patients may need to stay on this costly treatment for life.[186] However, emerging data suggests that at least in adults, use of teduglutide might improve quality of life not just for those patients that achieve enteral autonomy, but also those that have decreased nights on PN. The downside is the ongoing cost of this treatment

and the potential for serious complications, most notably polyp or tumour growth. Hence, in pediatrics, given the longer potential exposure to drugs like GLP-2 analogues, optimizing their use to achieve enteral autonomy is a valid goal. The literature is growing in terms of dosing and when GLP-2 treatment should be initiated, with GLP-2 analogues providing the novel benefit of being able to stimulate adaptation and weaning from PN after the initial two years after intestinal resection when spontaneous adaptation occurs.

GLP-2 is not the only trophic factor of note, and investigating IGF-1, EGF, along with longer acting GLP-2 analogues is warranted. How these trophic factors work individually, along with in combination with GLP-2 is a novel area of study. As well, alternatives could allow less injections per day, an important goal especially for pediatrics

Alternative GLP-2 analogues, alternative dosing, ways to reduce cost or other trophic factors in combination with GLP-2 analogues could allow further adaptation for these patients, with increased weaning from PN and potentially the ultimate goal of enteral autonomy. These areas of study should aim to make trophic therapy use in SBS more cost-effective and safe while promoting enteral autonomy and improving quality of life.

Figure 3-1. Short Bowel Syndrome Anatomical Consequences for GLP-2.

Adapted from Suri, Lim, Tappenden. [28, 80, 81]

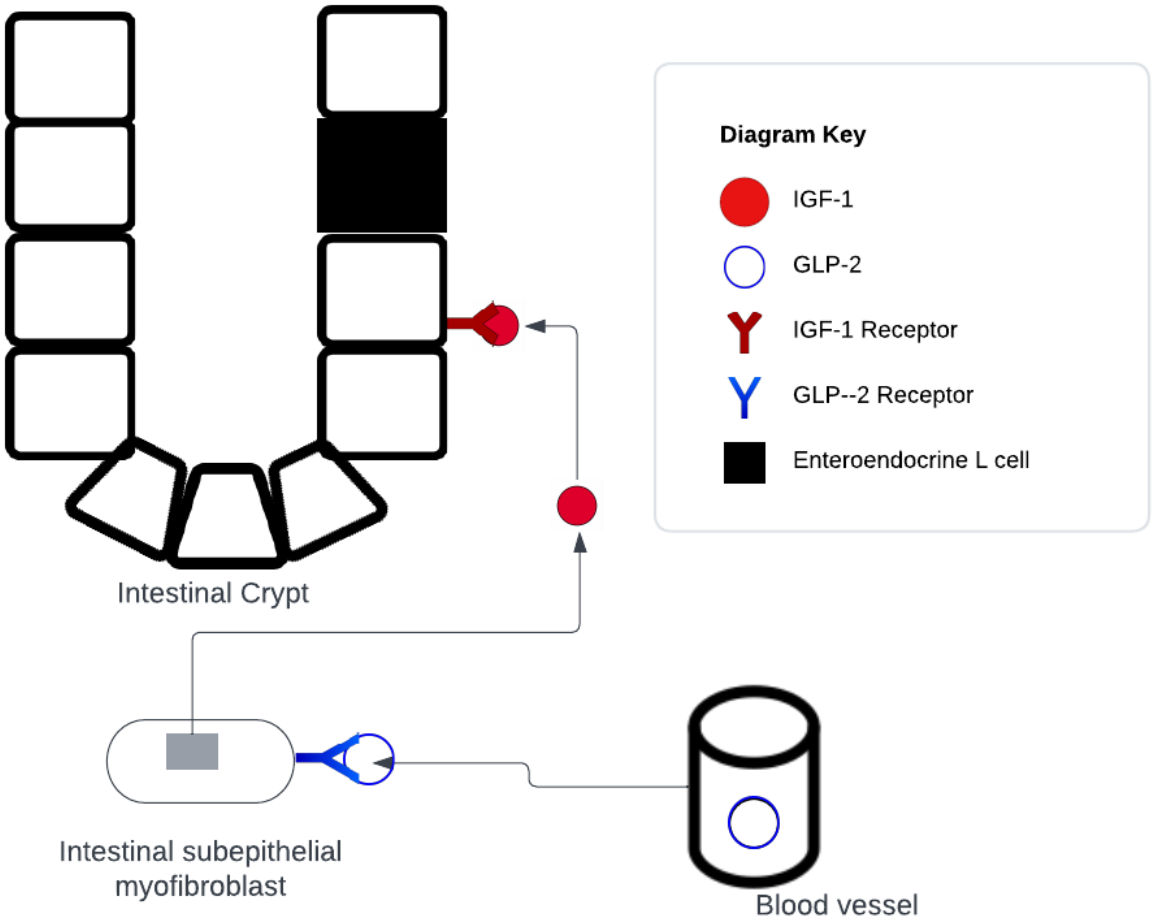


Figure 3-2. Downstream Effects of GLP-2 are Mediated by IGF-1.

Chapter 4. Use of the Piglet Model for Studying Novel Treatments for Pediatric Short Bowel Syndrome

4 Introduction

The prior chapters demonstrate the key role that animal models have played in the development of new treatments for SBS, notably trophic factors like teduglutide. In pediatrics the need for an animal model to study these new treatments is clear. Ethically novel treatments for human infants should be studied first in animal models and in adult humans. As stated previously, SBS is heterogenous, in not only anatomy, but also treatment, etiology and more. These patients can be extremely ill, and while treatment previously aimed to keep these patients alive, with improved mortality rates we need to prioritize improving quality of life for these patients. Animal models offer a way to study novel treatments, without increasing the risks for this vulnerable population. As well, they allow us to control anatomy, age, etiology, treatment and care when complications occur, all heterogenous and often uncontrollable factors in pediatric SBS. Another important factor covered previously is nutrition, with the use of different lipids, TPN formulations, and more in human infants, while in animal models we can control all these factors of nutrition, including a key clinical outcome, the advancement of EN. In studies in patients with SBS it would be entirely unethical not to advance EN. Finally, studies in adult humans or animals will not always translate to the developing human and using a developing animal model is key.

4.1 Common Animal Models of SBS

4.1.1 Rodents

Rodent models have been dominant in studies of SBS and development of treatments like GLP-2 analogues. Rodents hold a huge value in research due to relatively similar genes and

biochemical pathways as humans.[244] They are also widely available, have low cost and the ability to genetically modify rodents allows for key discovery research.[244] When comparing rodents to humans, the gestational length of rodents is 19-22 days, significantly shorter than humans at 37-40 weeks.[2] Premature infants are classified at 70-90% gestation, while prematurity occurs at 94-97% in rats.[2] Rodent research for SBS is limited by their small size, and their immature organs at birth.[245] Most gastrointestinal maturation in rodents occurs after birth with rapid gastrointestinal development during weaning.[2, 245] Neonatal rodents cannot handle the surgery required to create an SBS anatomical model.[2]

4.1.2 Piglets

In pigs, preterm delivery occurs at 88-95% gestation and so even at term they have GIT immaturity similar to preterm infants.[245] Piglets are also born with a similar weight at birth of 2kg to late preterm infants and have similar gastrointestinal anatomy and physiology.[2] Importantly for SBS research involving hepatic function, the piglet has similar hepatic features to humans along with a gallbladder, which is absent in rodents.[2] The development of the GIT, cardiovascular, central nervous system and the eye are similar in both piglets and humans, allowing further outcomes to be evaluated, like retina lipids for TPN studies.[246] Piglets do have rapid protein deposition, with similarities in intestinal development to humans allowing piglets to be used as an accelerated model of postnatal growth and development in comparison to humans.[244] Unlike rodents, due to its body size piglets can have surgical manipulation for SBS models, repeated blood sampling and long-term dietary studies like TPN studies.[244] TPN associated liver injury has been well documented in piglets similar to human neonates.[244] Importantly neonatal piglets have also been able to develop NEC and show similar pathological

changes to humans with just prematurity and formula feeding, like occurs in human infants.[244, 247] Another benefit is the large litter size, allowing litter blocking and littermates to be exposed to the different study treatments.[246] Piglets are also precocial animals, meaning they are able to be taken from the mother early, allowing them to be individually housed for microbiome or SBS studies.[246]

There are some differences that must be acknowledged, with the most obvious being that pigs have a spiral-shaped ascending colon, with humans having a square shaped colon. Piglets do differ with a low fat content body composition at birth and some digestive system differences.[244] Human infants are born as one of the mammals having the highest fat mass, while a piglet is born with very little fat content but with extremely rapid fat deposition in the first month.[248, 249] With this the piglet is slightly less mature than the human infant at birth with respect to its digestive system and liver, although this can be an advantage making term piglets a good representation of pre-term neonates.[2] Like preterm infants they have limitations in lipid digestion and absorption, specifically not absorbing long-chain fatty acids efficiently.[2]

As discussed in the previous chapter, there is a need for PK and PD studies for pediatric development of drugs. Drugs will not only be metabolized differently at different ages and developmental stages, but also vary in metabolism in different animal models.[246] In general, the smaller the animal the higher the metabolic rate and the larger the dose needed to be per body weight. For example, rodents will require a higher dose per body weight than piglets. As we know, the liver, small bowel and kidneys are the major sites of drug metabolism, yet are at varying stages of development as the patient or animal model ages.[246] Of note, development of the renal system occurs earlier in humans than piglets, and so for drugs metabolized by the

kidney this is an important consideration.[246] Along with the piglets nephrogenesis being different than humans.[246]

4.1.3 Other Animal Models of SBS

The first TPN studies were completed in beagle puppies and paved the way for TPN use in humans.[250] Beagle puppies on TPN demonstrate intestinal mucosal atrophy, like seen in human infants on TPN.[251] The ability to study the brain[252] and liver composition[253] made animal models vital in TPN development. However, human companion animals, like dogs are less available for current animal studies.

A very low order animal model of SBS is emerging in zebrafish, although largely used to study intestinal stem cell plasticity and drivers of adaptation like EGFR and IGF.[254] This models advantage is largely due to it allowing fast and less expensive studies, along with genetic modification.[254] Now, of course there are many differences, including the lack of stomach and drastic differences in intestinal anatomy and morphology, along with decreased ability to collect blood samples along with tissue sample size.[254] These studies are also only completed in adult zebrafish due to size, making inferences to human neonates difficult.

4.2 Animal Models of Intestinal Adaptation

Adaptation is a key outcome to target for drug therapy in SBS as it is really the ultimate key to autonomy from PN. Not surprisingly given the need for invasive sampling we know more about adaptation in animals then we do in humans. However, based on weaning from PN and sampling from small bowel stoma's in SBS patients we know that in humans adaptation starts almost immediately after resection and takes months to years for the process to complete.[2, 12]

The process has been much better characterised in animal models and occurs much faster allowing for viable short term intervention trails.

4.2.1 Rodents

Rodents are quite different, not only can they fully adapt within days with a 90% intestinal resection, but adult rodents have significant epithelial cell hypertrophy compared to humans and piglets.[2, 255] It has been well documented that adaptation in rats, much like in humans, is stimulated by EN.[24] One of the first examples of this was with the "functional workload" hypothesis, based on that there was increased intestinal adaptation with disaccharides over monosaccharides in rats.[256]

4.2.2 Piglets

Adaptation has been well characterized in piglets and the entire process takes weeks.[2] Similarly, growth of the intestine is stimulated by EN, specifically sucking of piglets, with increased motor activity and changes in digestive enzyme activity and nutrient absorption.[2]

Another advantage of pigs, especially for this thesis, is the similarity in trophic factors and their receptors. As well, there are primers and ELISA plates designed for these specific piglet receptors etc. This is something overlooked in animal models. Certain receptors do not have primers for them as they are species specific, and then endpoints cannot be measured. In piglets, the ability to use real-time PCR to measure mRNA GLP-2 receptor, IGF-1 receptor and EGF receptor has been well described throughout the literature.[235] As well, for adaptation function is a measure that is very difficult to measure in infants, yet function measures like Üssing chamber and fecal fat absorption can easily be performed in this model.

4.3 Animal models and the Microbiome

4.3.1 Introduction

After discussing how the microbiome can be both resistant to change but also so drastically altered with factors like age, sex, diet, environment, anatomy and other factors all having influence. Logically, different species, could have drastic differences in the microbiome. Especially when considering different ages of models, sexes, environment, treatments and more.

4.3.2 Rodents

Most broadly, when comparing differences in mice and rats to humans, there is the large diet differences; rats and mice are exclusively herbivores unlike humans.[257] Rodents also have a different circadian rhythm and eating habits than humans.[257] As well, there are differences in the GIT anatomy, with a forestomach in rodents used for food storage with little secretory activity, and therefore typically has higher levels of *Lactobacillus* forming a biofilm.[257] Therefore, often *Lactobacillus* make up a larger proportion of the GIT microbiome of rodents and bind to the stratified squamous epithelial of the rodent, unlike in the human.[258] Many of these changes are attributed to the higher abundance of *Bacteroides* and acetate in humans compared to rodents.[257] Rodents compared to humans also have a lower abundance of *Bacteroides, Bifidobacterium* and *Akkermansia* attributing to a lower growth rate and thickness of mucus in rodents compared to humans as well.[257, 259, 260]

In an SBS rat model compared to sham rats, alpha diversity was significantly decreased. Along with decreased Bacteroidetes and Firmicutes, large SCFA producers, with an increased abundance of *Lactobacillus, Enterococcus* and *Streptococcus.*[261] As covered in the microbiome

chapter, SCFAs are important not only in adaptation but are strongly related to the microbiome. They have been studied more in depth in rodents than piglet models. As expected, there was a decreased enrichment of the metabolome for SCFAs and amino acid metabolism.[261] Of note, these rats had a 75% bowel resection with loss of the ileum and ICV, but were 6 weeks old after being fed a nutrient fortified water gel for a week prior to surgery.[261] These rats were not kept on TPN postoperatively, and instead kept on this nutritionally fortified water gel for 1 week then solid chow for 3 weeks then euthanized.[261] All sequencing was performed on fecal samples. This study highlights some of the limitations when using rodent models for SBS work, being age, diet, sample size and sampling location.

4.3.3 Piglets

One of the first considerations for piglets as animal models is the lack of passive immunity at birth for piglets compared to humans, although otherwise the immune systems are very similar, with the human immune system developing slightly earlier than the piglets immune system.[246] Similarly to rodents, piglets have a stratified squamous epithelium that allows a more significant epithelial association and biofilm formation than that seen in humans, with humans having more bacteria living in the lumen not as firmly attached to the mucosa.[258]

The microbiome of pigs is dominated by Firmicutes and Bacteroidetes.[262] With this, at the genus level there is a dominance of *Prevotella, Blautia, Oscilibacter* and *Clostridium*.[262] An area of interest for this work is the influence of host genetics on the piglet microbiome. Landrace pigs have a more diverse microbiome than Duroc pigs,[263] along with increased Firmicutes at the phylum level for Landrace pigs over Duroc.[263] Conversely, Duroc were more dominated by *Blautia, Succinivibrio, Subdoligranulum* and *Phascolarctobacterium,* while

Landrace are more dominated by *Sphacrochaeta* and *Lactobacillus.*[263] Diet has a major impact on the piglet microbiome, while suckling there is an increase in *Enterobacteriaceae*, *Bacteroidaceae* and *Clostridiaceae*, while on feeds there is an increase in *Prevotellaceae, Ruminococcaceae, Lactobacillaceae* and *Veillonellaceae*. Similarly to humans, there is a rapid increase in alpha diversity with piglet age and decreased variability among individual piglets.[262, 264] Other factors to consider when looking at piglets as an animal model, is gilt versus sow milk composition on the piglet microbiome,[265] cross fostering piglets, litter blocking, sow diet, weaning and antibiotics.[262]

First, in 4 week old piglets with a 75% small bowel resection versus a sham operation or no operation, Pereira-Fantini et al. looked at the bacterial abundance in the colon and used liquid chromatography to determine bile acid concentration of the gall bladder, portal serum and fecal samples.[266] The objective of this study was to not only look at bile acid alterations within the gut-liver axis in a piglet model of SBS, but also to relate these changes to alterations in colonic bacterial composition.[266] Bile acid complexity and relative abundance were altered in the SBS piglet model, as early as 2 weeks post small bowel resection, and these changes persisted at 6 weeks which was when the animals were terminated.[266] For changes to the colonic microbiome, at 6 weeks there was a reduction in the relative abundance of the Clostridales order, including *Clostridium, uncultured Ruminococcus* and *Peptostreptococcus incertae sedis.*[266] There bacteria belong to the Firmicutes phylum. There was an increase in the relative abundance of *Acidaminococcus* and *Mitsuokella* in the SBS piglets at 6 weeks.[266] These are interesting findings as some of the bacteria that declined in the piglet model also have been shown to decline in humans, for example *Ruminococcus,*[130, 266] and bacteria from the phylum

Firmicutes in general.[71, 133, 266] Bile acids are very dependent on bacteria to deconjugate bile acids for conversion of primary bile acids into secondary bile acids via 7α-dehydroxylation.[266] Within all the SBS piglets at all time points, there was a shift to a primary bile acid dominance, and this is suggestive of a reduction in the bacteria that are responsible for 7α-dehydroxylation.[266]

In another piglet model, the authors use a 75% small bowel resection group, a sham group and a no surgery group to look at the colonic microbiota by high-throughput sequencing, 2 and 6 weeks post-surgery.[267] The authors also looked at gene expression of pro-inflammatory cytokines in the colonic mucosa via qRT-PCR to determine mucosal inflammation.[267] Lastly, they looked at the number of macrophages and percentage inducible nitric oxide synthase (NOS) staining in the colonic epithelium via immunochemistry.[267] They found that the total colonic bacterial number was not altered following small bowel resection at 2 or 6 weeks between any of the groups.[267] Conversely, at two weeks there was a trend towards decreased diversity in the colon for the short bowel syndrome piglets compared with the sham and no surgery groups.[267] By 6 weeks, there was a significant decrease in the microbial diversity in the colon for the SBS piglets compared with both control groups.[267] At 2 weeks, there was a significantly altered microbiome in the colon of the SBS piglets compared to both control groups, with a significant increase in *Veillonellaceae* and a significant decrease in *Ruminococcaceae*.[267] By 2 weeks *Peptostreptococceae* was undetectable in the SBS piglets and at the genus level there was a significant increase in *Acidaminococcus* in the SBS group compared to both controls.[267] By 6 weeks post-surgery, there was a significant decrease in the proportions of the phylum Bacteroidete*s* and significantly increased proportions of Fusobacteria in the SBS piglets

compared with both controls.[267] As well, the *Clostridium* proportions were significantly decreased in the small bowel resection group compared to the shams.[267] These results are very similar to the previous piglet model study.[266, 267] For the pro-inflammatory genes, at 2 weeks there were no differences among groups, but at 6 weeks there was a significant increase in the expression of IL-1β, IL-18, and TNF-α in the SBS piglets compared to the sham group, while IL-8 was significantly increased in the SBS group compared to both controls.[267] The increase in colonic pro-inflammatory cytokines was associated with an increase in the total number of macrophages present in the colonic epithelium of the SBS piglets compared to both control groups at 6 weeks.[267] At 6 weeks there also was a significant increase in the amount of NOS in the colonic epithelium of the SBS piglets compared to both control groups.[267] The results of this study interestingly did not show a change in *Lactobacillus* at the 2 week or 6 week timepoint.[267] This lack of change in *Lactobacillus* seems to have also been shown in pediatric microbiome studies.[130-132] The authors suggested that these differences could be differences in pediatric versus adult SBS in terms of the GIT microbiome.[267]

Next, in a study by Levesque et al. the authors aimed to evaluate the effects of antibiotics, surgical resection and enteral nutrition on the GIT microbiome using a piglet model of SBS.[268] The piglets underwent a 75% small bowel resection with either a JI anastomosis or a JC anastomosis.[268] An important distinction here is that with the JI anatomy, the ileum and the ICV are still present.[268] There were control groups of sham piglets and sow fed piglets.[268] In the colon and ileum on post-operative day 7 when the piglets were terminated, the bacterial genus diversity and relative abundance was greater in the sow fed controls compared to the JI, JC and sham piglets.[268] For the sow fed and the JI (with ICV still present) there was a difference

between the ileum and colon in terms of bacterial genus richness.[268] Conversely, for the sham and the JC model there was no difference between the ileum and colon bacterial genus richness.[268] This could suggest the possibility that without the ICV i.e. in the JC model, that without this "speedbump" that the microbiome becomes altered allowing the bacteria in the colon to colonize into the small bowel.[268] For relative abundance, the Firmicutes dominated both the ileum and colonic microflora in the sow fed controls, with Proteobacteria only representing around 5% in both the ileum and colon.[268] As well, Bacteroidetes represented 35% of the colonic microbiome.[268] This is an excellent example of a healthy infant/piglet microbiome, with the presence of bacteria able to metabolize the HMOs, a diverse microbiome for colonic energy salvage and SCFA production, and low levels of Proteobacteria. Conversely, regardless of surgical model (sham, JI, JC), there was a dramatic loss in the relative abundance of Firmicutes to below 20% in the ileum and 42% in the colon and an increase in the abundance of Proteobacteria to above 60% in the ileum and 25% in the colon.[268] With this dramatic shift in relative abundance, there was a loss of bacteria within the *Lactobacillaceae* and *Peptostreptococcaceae* families and an increase in the *Enterobacteriaceae* family.[268] The surgical animals were provided with antibiotics from day 0-4 and provided with TPN on day 0 through to termination.[268] These results lead to the conclusion that in this neonatal piglet model of SBS, administration of antibiotics and lack of enteral nutrition have a greater impact on the GIT microbiome than surgical resection alone.[268] This was a short study and with a small number of animals that could have caused the lack of statistical difference at the level of family, due to large between-animal variation.[268] This study is in contrast to the previous piglet study,[267] that did not show a change in *Lactobacillus* at 2 weeks or 6 weeks, while this study

showed an almost complete loss of bacteria from the genus *Lactobacillus* in 1 week.[267, 268] The reason for these inconsistencies in microbiome changes of the microbiome still need to be investigated, but potential explanations are antibiotics, EN, age (adult versus pediatric), study duration, fecal versus effluent samples and more.

4.4 Conclusion

In this thesis I have chosen the piglet model as it is ideal to create the experimental model of SBS in a neonatal animal. This I believe to be most justified for my research, based on anatomy, adaptation, similarities in trophic pathways, as well as for the microbiome. The anatomical model I have chosen to study is the type 2 or jejunocolic anatomy, because this represents the most common type of SBS (without an ICV) seen in the sickest human infants with SBS. This anatomy has the most limited capacity for adaptation and so provides the most challenges for development of new trophic therapies.

Finally, as reducing sepsis and CLABSI are key targets for new therapies the piglet is ideal given similarities in the microbiome. Animal models that utilize parenteral nutrition delivery face the same challenges in regards to CLABSI and sepsis as observed clinically in SBS.

Therefore, I will begin in Chapter 6 by exploring a novel solution to CLABSI in the piglet TPN and SBS model. Given data in piglets with SBS confirms that dysbiosis occurs and is similar to that described in infants and children with SBS I will then move on in Chapter 7 to explore a novel solution to this dysbiosis. Finally in Chapters 8 and 9 I will use the piglet model to explore new trophic therapies for SBS beyond teduglutide.

Chapter 5. Materials and Methods used throughout this Thesis: Surgery, Nutrition, Assessments of Adaptation, Permeability and Gene Expression

5.1 Surgical Model Technique

As discussed in Chapter 4, there are a few different anatomical animal models of SBS throughout the literature, in this thesis we will use the first published piglet model with a JC anastomosis, lacking the ICV and representing type 2 anatomy.[2] Type 2 anatomy is the most common anatomy seen in SBS children, yet the most understudied.[2] There are multiple additional advantages to using this model, including the lack of ICV for microbiome studies, along with limited innate potential for adaptation, which can potentially benefit studies of therapies to promote adaptation.[2] Another advantage of piglets is the ability to study IFALD in those receiving TPN, but without surgical resection, due to the similar hepatic features of neonatal piglets in comparison to human neonates.[2]

A limitation of this thesis that is discussed in Chapter 10 is that we did not use the JI SBS model with ICV, given that such anatomy can indeed occur in the clinical setting. Furthermore, studying both type 2 and type 3 anatomy allows us to compare results on the basis of anatomy. This is relevant to the microbiome, given the potential role of the ICV as a barrier to ascending bacteria, and also might better explain the actions of trophic treatments. This model has been useful to explain, for example, the role of higher endogenous levels of GLP-2 in type 3 anatomy in promoting adaptation as compared to type 2 anatomy.[2] However, having an anatomy where adaptation readily occurs could also mask the benefit of a trophic therapy like those studied in this thesis.

Overall, this unique neonatal piglet model of SBS with type 2 anatomy can be used to research novel treatments for SBS. This can have utility to translate to neonatal humans not readily achieving adaptation and enteral autonomy and so at greatest risk of complications of

long term PN, like IFALD. Practically, an advantage for this laboratory, is that an experienced neonatal surgeon, Dr. Wales, performs all the SBS surgeries, allowing for consistency in the experimental model as well as improved animal outcomes.

For 7, 10 and 14 day experiments, on day 0, under general anesthesia piglets underwent placement of a 5-Fr jugular central venous catheter for continuous TPN delivery.[269, 270] Briefly, the jugular catheter was placed in the left external jugular vein and advanced into the right atrium.[270] For 7 and 10 day SBS piglets, piglets also underwent a 75% distal small intestinal resection including the ileocecal valve, cecum and proximal colon, resulting in a jejunocolic anastomosis. A midline laparotomy was performed and the small intestine length was measured along the antimesenteric border using a 3-0 silk suture with minimal traction.[270] Length was measured from the Ligament of Treitz to the ICV.[270] The intestine was resected and the mesenteric vessels divided using dithermy (Valleylab, Boulder, CO) with a hand-sewn end-to-end bowel anastomosis.[270] A 10-FR gastrostomy tube was placed into the body of the stomach for EN.[270] Prophylactic antibiotics were given preoperatively. Both TPN and SBS piglets were given Florfenicol (15 mg/kg IM; Intervet Canada Corp. Kirkland, QB, Canada) and ampicillin (10 mg/kg IV; Sandoz, Boucherville, QB, Canada) and no additional rounds of antibiotics were given days 8-12 for TPN piglets.

Postoperatively piglets were housed in a temperature-controlled room in metabolic cages, with routine twice daily monitoring. The room was maintained at 25^{o}C, with a 12 hour light/dark cycle.[270] Piglets were monitored for signs of sepsis, including fever, vomiting and lethargy. As well, piglets hydration levels were assessed and urine output was measured to

calculate fluid balance daily.[270] Starting day 2 postoperatively, piglets were weighed daily to allow adequate nutrient intake and to assess piglet well-being.[270]

5.2 Nutrition

This model has been designed to allow delivery of both TPN and EN for nutrition. TPN administration is required for these piglets due to their type 2 anatomy and inability to absorb enough nutrients from EN for survival and growth.[270] The need for TPN delivery confirms that the SBS piglets with type 2 anatomy have intestinal failure, which has not been consistent with other animal models, raising concerns about their real relevance to this clinical problem. The TPN administration via a jugular central line mimics the clinical scenario in pediatric SBS, including the development of line complications. Using a TPN model without SBS, investigations of treatments to ameliorate complications like IFALD has also been undertaken, as reported in Chapter 6.[271] The goal of treatment of SBS is to increase EN and decrease TPN and this has been mimicked in the piglet model, demonstrating the role of trophic treatments in promoting the ultimate goal of enteral autonomy according to surgical anatomy.[184]

EN is also a key factor required for intestinal adaptation to occur and its provision is likely important in studies examining treatments to promote adaptation (as in Chapters 7-9 of this thesis).[270] Prior studies in TPN fed piglets without SBS have suggested at least 20-40% of EN should be provided for adaptation (overcoming the mucosal atrophy seen on TPN).[272] A rate of 20% is used in this thesis, given an assumption that a minimal trophic amount would be adequate to promote adaptation after surgery and resection, and assuming that higher EN rates might promote adaptation to a degree that a trophic treatment would add minimal

benefit.[184] This last point is important to allow the investigation of treatments to promote adaptation.[270, 272]

The TPN composition used in this research is based on prior research in the same facility, where the body composition of piglets fed TPN with the nutrient delivery used in this thesis was compared to that of sow-reared piglets at the same ages.[273] The EN composition is identical to the TPN, except polycose is substituted for glucose to reduce osmolarity and prevent osmotic diarrhea.[270]

As previously described, PN and EN were both prepared in our laboratory at target nutrient intakes of 1,100 kj/kg/d, 27% of energy from amino acids, 37% from carbohydrate and 36% from fat.[235, 273] The final solution is passed through a 0.22-μm filter that sterilizes both the TPN and the EN.[270] While sterile TPN is essential for intravenous delivery, this approach to also use sterile EN is specifically important for microbiome work as in Chapter 7, to reduce risks of contaminated EN confounding our results.

Both EN and PN are provided continuously using infusion pumps to strictly control the rate and volume of infusion. A potential confounder for both TPN and EN delivery is failure of pumps to infuse overnight. Both PN and EN volumes are measured in a 24-hour period so any differences between groups can be detected and reported in publications. Furthermore, tracking the infusions allows us to target 80% or more of TPN delivery as a quality metric and regularly service and calibrate pumps that are not meeting this target. However, for studies that focus on adaptation, ensuring all of the EN is delivered equally in all groups and meeting a minimal trophic amount is critical. As such, the rate of EN is adjusted to make up any missed diet. Potentially this variation in the EN rate is a confounder that could potentially change

outcomes for adaptation. However, as we are providing very minimal trophic nutrition, at only 20% of total nutrient requirements, this should be of minimal concern.[272] Overall, TPN and EN administration, composition and rate are heavily controlled.

Immediately after surgery all piglets received PN through the central venous catheter starting at 50% of their daily caloric intake to avoid refeeding syndrome and increased to 100% at intervals by day 1 post operative.[235] By day 2 EN was commenced at 20% of daily caloric intake via the gastrostomy tube.[235] While previously we wouldn't decrease TPN volume from 100% to 80% when EN started due to diarrhea and malabsorption concerns,[235] after changing the breed of piglets used we noted high rates of edema. Therefore, in all the trials included in this thesis we decreased PN from 100% to 80% with the start of EN.

5.3 Adaptation Measurements

The gold standard to assess adaptation in animal studies has been the collection of tissues and both macroscopic and microscopic measurements. Specifically, villus height measurement has become the gold standard for assessing structural adaptation.[274] Structural adaptation can be measured by intestinal lengthening, villus height, crypt depth, small bowel weight, mucosal scraping and more. In measuring length, it is important to have a consistent technique and not to stretch the bowel. The approach used consistently at baseline surgery by Dr. Wales, is to measure the antimesenteric border with a suture placed along the in situ intestine without stretching. Bowel weight in total is measured in the ex-vivo intestine drained of intestinal contents. This does have the potential limitation of variation in the intestinal contents drained, bowel wall edema or variation in submucosal tissues layers all potentially

impacting the weight measurements. The additional step of weighing a fixed length of scraped mucosa helps overcome this limitation. Additional steps to assure accuracy of these outcome measures in this thesis include consistent sampling from the same location in every animal, having the same staff perform the same roles at termination surgeries to decrease sampling variability and having a veterinary pathologist blinded to treatment undertake histological measurements.

All SBS piglets underwent general anesthesia at termination (day 7 or day 14) and the small intestine was measured in length along the antimesenteric border with minimal traction as done at baseline surgery. This involved measuring from the Ligament of Treitz to the anastomosis done at baseline surgery. Following euthanasia, the small intestine was removed and emptied of its contents, and the weight was measured. A mucosal scraping from a 20 cm segment of proximal jejunum was taken and weighed. Jejunal tissue 20 cm distal to the ligament of Treitz was preserved in 10% formaldehyde for histology. Jejunal segments 5 cm from the distal anastomosis were flash frozen in liquid nitrogen and stored at -80^{o}C for gene expression analyses for all studies. The liver was removed in total and weighed.

Paraffin mounted 5-μm sections of jejunum were stained with hematoxylin and eosin prior to assessment using a micrometer eyepiece (Nikon Eclipse 80i; Nikon, Tokyo, Japan). Mucosal hyperplasia was assessed by 10 measurements of villus height and crypt depth from longitudinal sections by a board-certified animal pathologist (P. N. Nation). Ideally, the crypt measurements were taken in the same area as the villus measurement.[270] As previously published,[270] a modified histology grading system was used for measuring liver pathology. This included 9 parameters: vacuolar degeneration, spotty necrosis, cholestasis, apoptosis, Kupffer

cell hyperplasia, sinusoidal dilatation, portal edema, tissue iron granules and polymorphonuclear leukocyte infiltrates.[270] A sum total of this was created with grading for each parameter of 0 for normal to 2 for severe.[270]

5.3.1 Fecal Fat

Assessment of fat absorption is a measure of functional adaptation that is very relevant to neonatal SBS, given dietary fat is the principal source of energy for growing infants and the major component of fecal energy. Malabsorption of fat is more significant than for protein and carbohydrates in SBS.[184, 275] This relatively inexpensive measure allows the determination of the amount of fat present in the piglet's stool compared to amount of fat delivered enterally during that time.[270] An advantage is that samples can be easily collected and stored for future analysis.[270] However, as we give only trophic amounts of enteral nutrition we might not expect to observe large losses of fat, compared to when higher amounts of EN are delivered, which could mean a large number of animals are needed for statistical difernces.[184, 276] Overall, it is a significant endpoint, which is clinically meaningful, and would be expected to represent functional adaptation, is inexpensive, easy to sample and to perform, and therefore this measurement was undertaken in Chapter 8, as follows.

The day before termination a 24 hour stool collection of piglets commenced, into stoma bags via fitted ostomy appliances (Hollister, Aurora, Ontario, Canada) to the perianal area. The enteral nutrition bags were weighed at the start and finish of the collection period to determine exact lipid delivery. The fat contents were determined on the fecal effluent, in paired freeze dried samples using the Folch method with an ether distillation.[277] Enteral fat absorption was calculated by subtracting the average fecal fat content from each sample from the total amount of lipid infused in the EN during the time the ostomy was applied.[235]

5.3.2 Permeability Measures

Assessment of intestinal permeability is performed both in vivo and ex vivo for determining the intestinal barrier function to both large and small molecules. There are many factors that go into generating the intestinal barrier and permeability. First, the lumen where antigens and bacteria are degraded by gastric acid and pancreatic enzymes and are moved along by motilty.[278] Next, as covered in Chapter 2 there is the commensal microbes of the microbiome that prevents colonization of pathogenic bacteria.[278] Close to the epithelium there is the unstirred water layer, glycocalyx and mucus layer.[278] Here, the Paneth cells secrete antimicrobial products and the enterocytes produce IgA to prevent bacterial adhesion.[278] Below this are the epithelial cells that are separated by tight junctions.[278] There are three different groups of proteins that make up the tight junctions. The integral membrane proteins, including the claudin family, measured in this thesis, forming the paracellular pore.[278] The claudins are associated with the occludins.[278] There are also junctional complex proteins and cell cytoskeleton structures.[278]

Intestinal permeability can be changed by neuroimmune modulation, with immune cell products like interleukins changing paracellular permeability by changing claudin expression.[278] These neuroimmune modulators can also increase macro-pinocytosis and effect the transcellular permeability.[278] These mechanisms include changes in tight junction proteins and function.

The microbiome, pathogenic bacteria, inflammation and the immune system play major roles in intestinal permeability and barrier function. Dysbiosis changes the signals like SCFAs for barrier function.[278] Inflammation and increased pro-inflammatory cytokines can alter permeability.[278] Increased epithelial cell shedding can impact barrier function.[278, 279]

Interestingly, bacteria endotoxin lipopolysaccharides (LPS) recognition by TLR4 on the basal side of the colon can increase paracellular permeability, while TLR2 recognition can decrease paracellular permeability.[280] Therefore LPS when crossing the intestinal barrier causes increased paracellular permeability while flagellin recognized by TLR2 would decrease this.[280]

There is both paracellular and transcellular permeability. Paracellular permeability is a passive transport process across an epithelium through the intercellular spaces between epithelial cells.[281] It is regulated by tight junctions for the movement of water, solutes and immune cells between the epithelial and endothelial cells.[282] The paracellular barrier restricts passage of solutes larger than 3 nm (up to 10-20 kDa) in radius, but transports ions, water and larger hydrophilic compounds.[283, 284] Transcellular transport is by transcytosis, selectively transports macromolecules across the endothelium, including albumin, albumin-bound ligands, PEG and hormones.[283] Transcellular transport is used by small hydrophilic and lipophilic compounds, like glucose.[284] LPS can potentially cross the leaky gut by endocytosis, with transcellular transport, but in general it is believed that bacteria cross between the epithelial cells utilizing paracellular transport.[284]

In this thesis we are using Üssing, which is often considered the gold standard ex-vivo method to assess intestinal permeability in animal studies. Üssing is expensive as it must be undertaken promptly to avoid tissue degradation, becoming labour intensive to both transport samples quickly and perform the chamber studies.

An important issue when looking at ex vivo studies is sampling location and tissue viability. As discussed in Chapters 1-3 there are variations in intestinal permeability for jejunum compared to ileum, specifically a decrease in intestinal permeability for ileum in comparison to

jejunum. It is very important that samples are taken from the same location. In this thesis, samples are always taken from the same location of the jejunum, and none of the piglets had ileum present to be sampled inaccurately. As mentioned above, tissue viability is a huge issue with Üssing, being that samples have to be transported instantly for analysis, along with issues with tissue sampling. When sampling the tissue, especially in post-surgical models, there can be high levels of adhesions that when broken up can impair the tissue viability. Another issue is the large amount of intestine needed, with SBS animals, especially those with low levels of intestinal lengthening occurring from the treatment, along with many other small bowel samples being taken, this can lead to minimal extra tissue remaining for Üssing.

FITC-dextran 4-kDa offers a less time intensive in vivo method that can be performed in piglets and has been published.[285-287] As well, the cost is about 60% cheaper compared to Üssing for our laboratory (unpublished data). As it only involves blood draws, samples can be frozen and measured in batches at a later date, saving time and money. Currently there are no studies comparing Üssing to FITC-dextran and preliminary research studies will need to be completed to determine the optimal dosing for SBS piglets with malabsorption. FITC-dextran at 40 dka gives us the trans-epithelial permeability after the FITC-dextran solution is administered orally, or in the case of SBS piglets via the gastric tube, and the amount that is in the blood is an indication of the trans-epithelial permeability.[286] Comparatively, 4kDA would be similar to the size of LPS and a marker of paracellular permeability.[287] These larger molecules, like 4 kDa FITC-dextran cross the intestinal epithelium via endocytic vesicles due to the large surface area of the enterocyte and shows this transcellular route.[284]

Currently the more commonly used method to measure permeability in vivo is the lactulose-mannitol ratio. This involves the administration of lactulose mannitol orally that is then quantified in urine samples.[288] In this case, mannitol is a marker of transcellular permeability and lactulose is a marker of gut damage and mucosal integrity.[289, 290] This is different than ex vivo studies like Üssing, where mannitol is a marker of paracellular permeability, but in vivo, mannitol is a marker of transcellular permeability.[291] However, this method is not suitable for SBS piglets due to the risk of causing osmotic diarrhea. Another issue would be ensuring accurate detection as uncontaminated urine collection is difficult in SBS piglets in metabolic cages.

5.3.2.1 Üssing Chamber

Üssing chambers have become a gold standard for measuring intestinal permeability in animal models, often with the use of radiolabeled mannitol and polyethylene glycol to determine permeability across a segment of tissue.[235] PEG is larger (M_r 380-420 respectively), showing permeability of larger molecules across the intestine, while mannitol is smaller (M_r 180).[235] The tissue is mounted in a chamber, with a donor chamber containing the radiolabelled markers, and second chamber to measure any of the radiolabeled markers that were able to go through the tissue when the current is applied.[292] Each segment is mounted on a segment holder and that is connected to the modified transport chamber.[293] This classic Üssing chamber tissues are mounted onto and compressed between two chamber halves. This modified system allows the investigation of multiple tissue types.[293] An asset of this modified Üssing chamber is that the investigator can look at not only different species tissues but also different sizes of tissues. One of the assets of Üssing is that there are multiple different parameters that can

measured. First, the apparent permeability coefficient (P_{app}, cm/s) that is calculated by P_{app} = dQ/dt X (1/(A X C_0)). It is easiest to understand what P_{app} is by breaking down the formula that produces it, dQ/dt is the appearance rate of the radiolabelled marker in the receiving chamber, in this case would be mannitol or PEG. A is the exposed surface area of the tissue and C_0 is the initial concentration in the donor chamber of that radiolabelled marker.[292] A pair of electrodes are placed beside each side of the tissue surface to measure the spontaneous transepithelial potential difference (PD).[292] The short-circuit current (Isc) is the current to reduce the spontaneous difference, i.e. the PD, across the tissue.[292] These two values are used to calculate the transepithelial electrical resistance (TEER).[235] TEER measures the nut flux of ions (cations and anions) across the epithelium and is the paracellular resistance, impacted by the tight junctions, the transcellular resistance to ions of the apical and basolateral membrane and the subepithelial resistance.[284] TEER is the resistance of the epithelium against ions to cross from the luminal to the basolateral side.[284] Mannitol being a small hydrophilic compound, is often used to demonstrate passive paracellular transport.[292] As the surface of the enterocyte is larger than the paracellular channels, transcellular diffusion across the enterocyte primarily occurs for absorption of larger molecules, like PEG.[292]

5.4 PCR

5.4.1 Real-time PCR

A real-time or quantitative PCR (qPCR) is the ability to detect amplification of small segments of DNA in real time as it occurs. The whole amplification profile is known, including the individual reactions amplification efficiency. This makes real-time compared to conventional PCR more precise and more reliable.[294] This technique is often used for looking at

gene expression. The delta-delta cycle method compares the difference in expression of the gene and reference gene compared to the control, instead of relating the PCR signal to the standard curve.[295] Being able to start with a very small amount of total RNA, the reverse transcription is able to copy the RNA and produce single-stranded, complementary DNA (cDNA).[296] This cDNA is less prone to degradation than RNA and can be amplified by PCR. This is semi-quantitative as it allows real-time PCR with comparison of relative mRNA levels.[296] This technique is used in Chapter 9, to analyze the relative mRNA levels of growth factors and their receptors of interest. A limitation of all PCR studies is the requirement to have primers specific to the mRNA of interest that is species specific.[297] This limitation is seen when investigating GLP-2, as we are unable to measure mRNA levels of GLP-2 as there is not a readily available porcine PCR primer.

Expression of genes related to growth factors, their receptors and tight junction proteins was assessed using total RNA isolated (RNeasy with QiaShredder; Qiagen, Toronto, ON, Canada) from the segments of jejunum taken 5cm from the jejunocolic anastomosis, and underwent reverse transcription (All-In-One Reverse Transcriptase MasterMix; Applied Biological Materials, Richmond, BC, Canada). Real-time semiquantitative PCR was performed using TaqMan Fast Advanced Master Mix (ThermoFisher Scientific, Mississauga, ON, Canada) using the primers (Life Technology, Burlington, ON, Canada). Relative mRNA expression was quantified using the delta-delta cycle threshold method[295] with 18S rRNA as the control, which has been previously validated as a stable porcine intestinal tract reference gene.[235, 298]

5.4.2 16S rRNA

The first step to prep samples for 16S rRNA analysis is to perform a DNA extraction. The widely used method in the literature is a Qiagen kit.[299] This kit allows fast, easy and high throughput isolation of DNA from both stool and tissue.[299] A major disadvantage of this technique is the high cost per sample,[299] along with sometimes needing multiple extractions for samples that have low levels of DNA (either a small sample or in low bacterial content samples). Aquastool is able to extract DNA or RNA with an ethanol precipitation, along with being much lower cost compared to Qiagen methods.[300] A disadvantage is this process takes much longer and involves more steps than the Qiagen methods.[300] In Chapter 7 Aquastool was used due to the low cost for this experiment.

As discussed in 2.2.1, there are conserved and variable regions of bacteria. Here, the primer is used for the conserved region present on bacteria and then we are able to use the variable and highly variable regions to identify the bacteria down to the species level.[86] Therefore amplicon libraries were constructed from these samples to amplify the V3-V4 region. The V3-V4 region is highly variable and used due to it being known to yield results for microbially diverse populations due to the maximum nucleotide heterogeneity yielding the maximum discriminatory power.[301] Briefly, the genomic DNA from the DNA extraction was sheared, enzymatic end repair, 5'-phosphorylation and 3'-extension of these resulting sheared fragments, adapter ligation, size fractionation on an agarose gel and PCR amplification of these adapter-ligated fragments in a thermal cycler.[302] A paired-end sequencing run was performed on an Illumina MiSeq platform. The raw sequence data obtained was then filtered through a quality control pipeline.[303]

A major limitation of the method used here is that this achieves a relative abundance of the microbes present, not the concentration of microbes per gram of sample.[304] This can lead to issues in interpreting data. For example, when comparing sample A to sample B. Sample A has 800 Proteobacteria and 200 Actinobacteria, while Sample B contains 80 Proteobacteria to 20 Actinobacteria. They have the same relative abundance (8:2) of Proteobacteria and Actinobacteria but a very large difference in the actual abundance. Now, while an advantage of 16S rRNA is that it can identify poorly described, hard to isolate or phenotypically aberrant strains, it also is limited to sometimes not being able to differentiate closely related bacteria.[305] For example, 16S rRNA cannot differentiate between *Shigella spp.* and *E. coli* at the species level due to their closely related sequences.[305]

In Chapter 7, DNA was extracted from samples using AquaStool solution (Multitarget Pharmaceutical LLC, Colorado Springs, CO, USA) as per the manufacturer's instructions.[300, 306] AquaRemove was also added to remove any Polymerase chain reaction (PCR) inhibitors via the manufacturer's instructions along with an ethanol/NaCL precipitation for better purification.[300, 306] 16S rRNA gene amplicon sequencing was performed amplifying the V3-V4 hyper variable regions of the 16S rRNA gene using primers.

5.4.2.1 Bioinformatics

Bioinformatics is the process in which the complex 16S rRNA data is analyzed and interpreted. An issue is differences in QIIME2 use to analyze data. For example, the creation of the classifier that can lead to differences in the ability to classify data down to the genus versus species.[307] Without a standardized method used by all laboratories to perform bioinformatics it

can be extremely difficult to compare results from different papers, especially in this rapidly advancing field with constant updates to the software.[307]

In this thesis, QIIME2 was used, with the adapted code as shown below.[307, 308] The data for this thesis was demultiplexed, denoised/clustered, the representative sequences and taxonomy classifier were created by experts (Dr. Fouhse and Jérémie Auger) the code for these steps will not be shown. Ideally, at some point in the future there will be a platform that imports sequences directly from Illumina and the data can be analyzed, with updates from QIIME, allowing the data to be manipulated consistently by all researchers. A major limitation of the taxonomy classifier/data being analyzed by multiple groups using different versions of QIIME is the inability to pool data in collaboration and this is time consuming to activate a different QIIME version.

For bioinformatics QIIME2 was used due to its wide use within the literature and readily available methods to adapt.[307, 308] The approach is as follows, first Conda and QIIME must be installed prior to completing this analysis. Briefly, first the function to be used must be activated (for example qiime feature-table), then the route to the input (i), metadata (m), and output (o) at a minimum must be inputed. Qza is the artifact and Qzv is the visualization.

Sorting File (removing treatment groups):

```
qiime feature-table filter-samples \
--i-table frequencytable.qza \
--m-metadata-file metadata.tsv \
--o-filtered-table id-filtered-table.qza
```

Feature Table from table.qza:

qiime feature-table group \

--i-table table.qza \

--p-axis sample \

--m-metadta-file metadta.tsv \

--m-metadata-column Treatment \

--p-mode sum \

--o-grouped-table Groupedtable.qza

Create Taxabarplot:

qiime taxa barplot \

--i-table table.qza \

--i-taxonomy taxonomy.qza \

--m-metadata-file metadata.tsv \

--o-visualization barplots.qzv

Diversity Metrics:

qiime diversity core-metrics-phylogenetic \

--i-phylogeny rooted-tree.qza \

--i-table table.qza \

--p-sampling-depth 1000 \

--m-metadata-file metadata.tsv \

--output-dir core metric results

Shannon/Simpson Alpha Diversity Measure: (Insert Shannon/Simpson for p metric)

qiime diversity alpha \

--i-table table.qza \

--p-metric shannon

--o-alpha-diversity core-metric-results/shannon.qza

Shannon/Simpson Alpha Diversity Measure Significance:

qiime diversity alpha-group-significance \

--i-alpha-diversity core-metric-results/shannon.qza \

--m-metadata-file metadata.tsv \

--o-visualization core-metric-results/shannon_significance.qzv

Diversity Alpha Significance Table:

qiime diversity alpha-group-significance \

--i-alpha-diversity core-metrics-results/faith_pd_vector.qza \

--m-metadata-file metadata.tsv \

--o-visualization core-metrics-results/faith_pd_group_significance.qzv

Diversity Alpha Significance Results/Graph: (Can insert ace, Shannon and Simpson metric when needed)

qiime diversity alpha-group-significance \

--i-alpha-diversity core-metrics-results/evenness_vector.qza \

--m-metadata-file metadata.tsv \

--o-visualization core-metrics-results/evenness_group_significance.qzv

Beta Diversity Distance Matrix:

qiime diversity beta-group-significance \

--i-distance-matrix core-metrics-results/unweighted_unifrac_distance_matrix.qza \

--m-metadata-file metadata.tsv \

--m-metadata-column Treatment \

--o-visualization core-metrics-results/unweighted-unifrac-significance.qzv

Beta Diversity Group Significance:

qiime diversity beta-group-significance \

--i-distance-matrix core-metrics-results/unweighted_unifrac_distance_matrix.qza \

--m-metadata-file metadata.tsv \

--m-metadata-column Sample \

--o-visualization core-metrics-results/unweighted-unifrac-subject-group-significance.qzv

Beta Diversity Significance: (Insert Jaccard or Bray-Curtis etc)

qiime diversity beta-group-significance \

--i-distance-matrix jaccard_distance_matrix.qza \

--m-metadata-file metadata.tsv \

--m-metadata-column Treatment \

--p-method permanova \

--p-pairwise \

--p-permutations 999 \

--o-visualization core-metrics-results/jaccard_significance.qzv

Relative Abundance Table:

qiime taxa collapse \

--i-table table.qza \

--i-taxonomy taxonomy.qza \

--p-level 7 (for species, 6 for genus etc.) \

--o-collapsed-table collapsed-table-7.qza

Relative Frequency:

qiime feature-table relative-frequency \

--i-table collapsed-table-7.qza \

--o-relative-frequency-table collapsed-frequency-table-7.qza

Convert Table from qza to tsv:

qiime tools export \

--input-path collapsed-frequency-table-7.qza \

--output-path col-table.biom

biom convert \

-I col-table.biom/feature-table.biom \

-o col-table.biom/7-table.tsv \

--to-tsv

Chapter 6. Benefits and Cost Saving Using 4%-Tetrasodium EDTA in Parenteral Nutrition Research Using Piglets

Adapted from:

Pauline M, Labonne E, Wizzard P, Turner J, Wales P. Association between 4%-tetrasodium EDTA and sepsis in neonatal piglets: A retrospective cohort study. Submitted to JPEN. Revisions submitted.

6.1 Abstract

Background: Central line associated bloodstream infections (CLABSI) are a major concern for children with intestinal failure and in animal research using parenteral nutrition (PN). In neonatal piglets on PN we compared sepsis, line occlusions, line replacements, mortality and costs with and without the use of a 4%-tetrasodium ethlenediaminetetraacetic acid (EDTA) locking solution (T-EDTA).

Methods: We performed a retrospective review of piglets with a central venous jugular catheter enrolled in 14-day Total PN (TPN) trials, or in 7-day short bowel syndrome (SBS) trials, pre and post initiation of T-EDTA. Lines were locked with a 1ml solution for 2hr daily (T-EDTATPN, n=17; T-EDTASBS, n=48) and compared to our prior standard of care using 1.5ml heparin flushes twice daily (CONTPN, n=34; CONSBS, n=48). Line patency and signs of sepsis were checked twice daily. Jugular catheters were replaced for occlusions whenever possible. Humane endpoints were used for sepsis not responding to antibiotic treatment or for unresolved catheter occlusions.

Results: Sepsis was reduced using T-EDTA compared to CON for both TPN (p=0.005) and SBS (p=0.046) studies. Line occlusions necessitating line changes were reduced 15% in TPN studies (p=0.1) and no line occlusions occurred for T-EDTASBS.

Conclusion:

In our neonatal piglet research, use of a 4%-tetrasodium EDTA locking solution decreased sepsis and while not statistically significant reduced occlusions requiring line replacements. Given the expense of animal research adding a locking solution must be cost effective and we were able to show that T-EDTA significantly reduced total research costs and improved animal welfare.

6.2 Introduction

Intestinal failure (IF) is defined as the inability to absorb adequate nutrients and fluid for survival and growth.[2, 309] Short bowel syndrome (SBS) is the leading cause of IF in infants, due to a reduction in functional mass for absorption.[310] While parenteral nutrition (PN) is necessary for these infants to survive, it comes with the associated risks of sepsis and cholestasis. Specifically, a major source of sepsis arises from central line associated bloodstream infections (CLABSI) from line contamination. Every time a child with SBS becomes septic, this negatively impacts their enteral tolerance and gut adaptation.[4] Ultimately, CLABSI risk can only be eliminated if the patient is able to achieve enteral autonomy, discontinue PN and remove the central line. With recent improvement in outcomes for children with IF who are dependent on PN, it has become vital to reduce morbidity and risk of mortality due to CLABSI, including maintenance of venous patency. The incidence of CLABSI for pediatric home parenteral nutrition (HPN) patients is reported at 2.1 per 1000 central venous catheter days (CVCD), and higher rates of line infections are observed in those children with the poorest outcomes.[1, 44] Worse neurodevelopmental outcomes have been associated with 2 or more episodes of sepsis in the first year of life.[42, 43] Sepsis can account for an alarming portion of mortality prior to transplantation, at approximately 20%.[45-47, 311] Managing these septic episodes in the first year of life has been suggested as a strategy to reduce some of the long-term neurocognitive risks.[42]

CLABSI risk has been managed previously with the use of antimicrobial locks, like 70% ethanol, but product availability, increased cost and worsening catheter patency remain concerns. There is currently an urgent need for novel alternatives. A new alternative to ethanol,

Kitelock™, that is 4%-tetrasodium ethylenediaminetetraacetic acid (EDTA) has been shown to significantly decrease CLABSI in children.[312]

Translational animal models of IF and SBS are used to advance the care of children who are dependent on PN. In these animal models, sepsis is also an issue, for both reliable research findings, as well as, compromising animal welfare. Ethically, we must constantly revise animal protocols to reduce animal use, morbidity and mortality.[6-8] For 18 years, we have used a neonatal piglet model of SBS and total PN (TPN) to study various aspects of intestinal adaptation and parenteral lipid solutions. The piglet is an ideal choice for this research due to its similar gastrointestinal physiology to the human and its rapid growth permitting assessment of outcomes in a short period of time.[2] In our neonatal piglet models, we are continually seeking methods to improve animal welfare and to decrease sepsis and costs.

The objectives of this study were to retrospectively compare sepsis, line occlusions, animal mortality, trial completion at expected endpoints and costs with and without the use of 4%-tetrasodium EDTA locking solution in our animal models. We hypothesize that 4%-tetrasodium EDTA is associated with decreased incidence of confirmed sepsis in neonatal piglets on continuous TPN for 14-days along with neonatal piglets with surgical SBS on both TPN and EN for 7-days.

6.3 Methods

All experiments were conducted in accordance with guidelines of the Canadian Council of Animal Care (AUP00000155 and AUP000003707). We performed a retrospective cohort study in neonatal piglets of our previous 14-day continuous TPN trials and 7-day SBS trials. Our exposure of interest was 4%-tetrasodium EDTA (T-EDTA) (Kitelock™). From 2020 onward, we

started to use the 4%-tetrasodium EDTA, commencing day 2, with central venous catheters locked for 2 hours daily with 1ml. In this retrospective study, our comparator (control/CON) was the laboratory previous standard of care twice daily 1.5mL heparin flushes of central venous catheters to maintain catheter patency, prior to 2020. We excluded trials in our laboratory that were studying treatments that we suspected could impact sepsis, such as studies aiming to alter the microbiome. Our primary outcome was confirmed sepsis, defined as a blood culture obtained from a peripheral vein or central venous catheter, positive for bacteria or yeast.

For both 7 and 14 day experiments, on day 0, under general anesthesia piglets underwent placement of a 5-Fr jugular central venous catheter for continuous TPN delivery.[269, 270] For 7 day SBS piglets, piglets also underwent a 75% distal small intestinal resection including the ileocecal valve, cecum and proximal colon, resulting in a jejunocolic anastomosis (JC). A 10-FR gastrostomy tube was placed into the body of the stomach for EN.[270] Prophylactic antibiotics were given preoperatively. Pre-4%-tetrasodium EDTA TPN piglets (2011-2012) were given ampicillin (10 mg/kg twice daily IV, Sandoz, Boucherville, Quebec, Canada) and trimethoprim-sulfadoxine (0.5mL, once daily IM, Intervet Canada Ltd, Whitby, Ontario, Canada) for 4 days post-operatively and on days 8-12 antibiotics were repeated. Pre-4%-tetrasodium EDTA (2017-2019) SBS piglets were given Florfenicol (15 mg/kg IM; Intervet Canada Corp. Kirkland, QB, Canada) and ampicillin (10 mg/kg twice daily IV for 48 hours postoperatively; Sandoz, Boucherville, QB, Canada). Post-4%-tetrasodium EDTA (2020-2023) both TPN and SBS piglets were given Florfenicol (15 mg/kg IM; Intervet Canada Corp. Kirkland, QB, Canada) and ampicillin (10 mg/kg IV; Sandoz, Boucherville, QB, Canada) and no additional round of antibiotics were

given days 8-12 for TPN piglets. Postoperatively piglets were housed in a temperature-controlled room in metabolic cages, with routine twice daily monitoring.

The piglets on TPN trials[269, 313] were randomized SMOFlipid® (n=8) and Intralipid® (n=9) at a dose of 10 g/kg/d (equivalent to 2 g/kg/day in a human) via TPN. TPN commenced immediately postoperatively, as previously described increasing to a dose of 100%.[269] For SBS trials,[276] the only lipid used was Intralipid®. In addition, SBS piglets had trophic enteral nutrition (EN), commencing on post operative day 2 at 20% of the nutrient requirements and the PN was reduced to 80%.[270]

Line patency was checked twice daily, along with monitoring for signs of sepsis and recording of these events was maintained twice daily. Sepsis was suspected on the basis of fever, lethargy and/or vomiting. If suspected, aerobic and anerobic blood cultures were taken, and an empiric treatment course of antibiotics started. Confirmed sepsis is based on a positive blood culture, while presumed sepsis is based on the symptoms of sepsis listed above, leading to a blood culture being taken. The jugular venous catheters could be replaced once per trial if there was loss of line patency that would impact trial procedures. Humane endpoints were used if sepsis or catheter patency was determined to require withdrawal from the trial.

6.3.1 Cost Analysis

Cost estimates were based on the cost of 4%-tetrasodium EDTA and costs of replacing lines or replacing animals due to failure to meet research endpoints. Additional costs were related to treatment of sepsis, including cost of culture vials, blood culture analysis and antibiotics. Animal replacement costs included the cost of the piglet, initial jugular venous catheter surgery, consumables and TPN costs prior to removal from the trials. Line replacement included cost of the catheter, personnel overtime and surgery costs. No additional personnel

costs were included. Cost analysis was based on the return-on-investment calculations used previously.[314]

6.3.2 Statistical Analysis

Data was analyzed with Pearson's Chi Square or Mann Whitney U test where appropriate. Results for Mann Whitney U test are presented as median [interquartile range (IQR)]. Results for Pearson's Chi Square are presented as n (%). Analysis was performed in SPSS (version 28; SPSS Inc, an IBM Company, Chicago, IL, USA). An alpha value <0.05 was deemed significant.

6.4 Results

6.4.1 Total Parenteral Nutrition Piglets

Altogether, 17 piglets had 4%-tetrasodium EDTA (T-EDTATPN, 1mL lock SID; n=17) compared to 34 piglets having standard catheter care with heparin (CONTPN, 1.5 mL BID; n=34). There was no significant difference in median [IQR] piglet age (T-EDTATPN: 4d [3, 4], CONTPN: 4d [3, 4]; p=0.89) or weight (T-EDTATPN: 2.4kg [2.2, 2.6], CON TPN: 2.2kg [2, 2.4]; p=0.05) at trial initiation. There was a significant decrease in confirmed sepsis for 4%-tetrasodium EDTA compared to heparin flushes in the T-EDTASBS and T-EDTATPN data (**Table 6-1**). There was no significant difference in the number of piglets euthanized early due to sepsis or loss of line patency (T-EDTATPN: n=2/17 (12%), CONTPN: n=12/34 (35%); p=0.08). There was no significant difference in median [IQR] number of occlusion events per piglet between groups (T-EDTATPN: 0 [0, 1], CONTPN: 0 [0, 6]; p=0.50) or the number of lines replaced due to unresolved occlusions (T-EDTATPN: n=0/17 (0%), CONTPN: n=5/34 (15%); p=0.1); however, no piglet receiving 4%-tetrasodium EDTA required central venous catheter replacement for

occlusion. Considering the costs related to confirmed sepsis, the cost of 4%-tetrasodium EDTA and costs of replacing animals due to early mortality, before research endpoints, there was a 58% cost reduction with the use of 4%-tetrasodium EDTA (**Table 6-2**).

6.4.2 Short Bowel Syndrome Piglets

We compared 48 piglets given 4%-tetrasodium EDTA (T-EDTASBS, 1mL lock SID) to 48 piglets having standard venous catheter care (CONSBS, 1.5ml heparin BID). Piglets given 4%-tetrasodium EDTA were slightly older and heavier median [IQR]at baseline (T-EDTASBS: 4d [3, 4] CONSBS: 3d [3, 4], p=0.03; T-EDTASBS: 2.4kg [2.3, 2.5] CONSBS: 2.3kg [2.2, 2.4], p<0.001). There was a significant difference in confirmed sepsis for 4%-tetrasodium EDTA compared to heparin flushes (T-EDTASBS: n=1/48 (2%), CONSBS: 6/47 (13%) p=0.046) (**Table 6-1**). There was no significant difference in median occlusion events per piglet (p=0.38), trial ended early due to sepsis or loss of line patency (T-EDTASBS: n=0/48 (0%), CONSBS: 1/48 (2%) p=0.320) and no lines were replaced for either group. There was a 16% cost reduction with the use of T-EDTA (**Table 6-3**).

6.5 Discussion

This retrospective study demonstrates that PN fed neonatal piglets, with and without SBS, given a 2-hour lock of 4%-tetrasodium EDTA had lower rates of confirmed sepsis with a reduction in research costs compared to piglets treated with heparin flushes. For TPN piglets, while there was not a statistically significant difference for piglets euthanized early due to sepsis or loss of line patency, we believe there was a meaningful clinical difference (12% T-EDTATPN versus 35% CONTPN, respectively (p = 0.08)), that improved animal welfare and was

cost effective for our research program. The longer the study, 14 days versus 7 days, the greater the expected cost reduction. Similarly, while with both models there was no significant differences in median line occlusions or line replacements, no line replacements were necessary in the 4%-tetrasodium EDTA treated groups. For line replacements, that are both a cost and can negatively impact animal welfare and research findings, given the piglets undergo additional surgery on trial, a 0% replacement for T-EDTATPN piglets versus 15% for CONTPN piglets is also very meaningful.

While systematic reviews have repeatedly shown that ethanol locks in children with intestinal failure reduces the risk of CLABSI,[62, 63] there is considerable concern about the increased cost of this therapy. Raghu et al., recently evaluated ethanol lock prophylaxis in children with IF and did not find this to be cost effective, despite a 40% reduction in CLABSI frequency.[311] Ethanol locks currently cost around $1000/day, while to be cost-effective Raghu et al. study suggested the cost would need to be $68/day.[311] In a previous cost analysis in adults receiving hemodialysis, Hill et al., examined the cost of 4%-tetrasodium EDTA versus standard of care (saline, heparin and taurolidine), with endpoints of the cost of CLABSI, line occlusions and catheter replacement.[314] The authors found there were no occlusions post treatment, and a significant reduction in CLABSI, that was associated with cost effectiveness over time (66% at 12 months, 63% at 24).[314] A recent cost-utility analysis using a Markov cohort model found a savings of $88,277 with 4%-tetrasodium compared with heparin and $52,120 compared with taurolidine from the healthcare payer perspective.[315] The study by Gattini et al. is very relatable to the current study as this model was designed to show the cost-effectiveness of 4%-tetrasodium EDTA in use in children with IF followed by an intestinal rehabilitation center.[315]

Parameters included in this model similar to the current study included the monthly cost of locking solution, cost per CLABSI admission, alteplase cost, and central line removal and insertion costs.[315] Further to this, the study included costs to the caregiver for productivity loss with CLABSI admissions, PN and after achieving enteral autonomy.[315] This allowed the unique perspective of the societal perspective savings, with 4%-tetrasodium EDTA saving $90,696 compared to heparin and $36,973 compared with taurolidine.[315] Kitelock™ used to be called a different brand name of Cathasept™, when used in adults on hemodialysis that were followed for 8 months there was a significantly reduced microbial colonization of the catheter by 87%, but failed to achieve statistical significance for reduced CLABSI, and was associated with more thrombotic complications compared to heparin standard of care.[316] There was reduced CLABSI events, although not significant, perhaps due to the small sample size.[316] The 4%-tetrasodium EDTA solution was also only instilled three times weekly and there was no documentation of how long the lines were locked with heparin or the 4%-tetrasodium EDTA solution.[316] Future studies have not shown the increased thrombotic complications with 4%-tetrasodium EDTA.[312, 314]

Furthermore, the ideal locking solution prevents both CLABSI and thrombosis and there is a concern that ethanol locks are actually associated with increased occlusion risk.[317-319] In the meta-analysis by Oliveira et al. the rate of catheter occlusion was approximately 15%.[63] Biofilms not only are a source of bacteria in the catheter, but also a site for thrombus and fibrin sheath formation. Kitelock™, contains tetrasodium salt of EDTA that is able to disrupt and destabilize biofilms.[312] EDTA, a calcium and iron chelator with anticoagulant activity, has been shown to prevent colonization of certain bacteria and fungi.[320] 4%-tetrasodium EDTA has been shown to

kill all microorganisms that are clinically relevant at concentration of 4% or less within 24 hours.[321] Locking therapies need to not only be able to prevent biofilm formation, but disrupt intact biofilms, as is seen with EDTA ion chelators.[41]

Antibiotic locks are limited by the susceptibility of the biofilm to that antibiotic, with a reminder that biofilms are heterogenous with different species of bacteria present, leading to different antibiotic and antimicrobial susceptibilities.[41] Antibiotic locks don't have direct impact on biofilm disruption or prevention.[41]

Antibiotic locking therapies may also contribute to antibiotic resistance. In children with IF receiving taurolidine-citrate prophylactic lock, the median CLABSIs per 1000 CVC days decreased from 2.07 to 1.23.[66] In neonates, 70% ethanol locks come with the risk of catheter occlusion, and other mechanical complications such as breakage when used with polyurethane catheters.[322, 323] The prevention of clotting in the venous catheter with the use of heparin has been our standard of care. In children with IF, studies have found a 93% thrombosis free survival per 1000 CVC days with use of heparin, versus 48% without heparin.[56] Unfortunately, there is no evidence of heparin preventing infection.[57] Heparin also has a low half-life of 60-90 minutes.[58] In addition, there is the potential for heparin induced thrombocytopenia along with stimulating biofilm formation at high doses.[60, 61, 314]

A limitation of this animal study was the retrospective nature, with animals from different experiments over a period of time in our laboratory that included different personnel (although consistent line handling protocols and senior technical staff). We chose to examine two different animal models (TPN studies and SBS piglet studies) as our research program encompasses both areas and we wanted to evaluate the impact of this lock solution in both

settings. Although the research conditions are homogeneous with animals of similar size, age, anatomy and genetics, we have pooled data from different trials that could potentially confound results. The TPN studies from 2011-2012 used a different breed of piglet, specifically without the addition of Duroc to the piglet cross. While this is an acknowledged confounder, the piglets' genetics are still more homogenous than clinical studies in humans. In the TPN studies, some piglets received soybean based lipid while others received a composite lipid emulsion, which theoretically could alter susceptibility to inflammation. Also, we changed our prophylactic antibiotic protocol prior to starting use of 4%-tetrasodium EDTA in our lab. Mainly, the change from trimethoprim-sulfadoxine to florfenicol from earlier TPN studies to more recent SBS studies. However, all the SBS piglets were on the same antibiotic regimen and still had significantly decreased confirmed sepsis with the use of 4%-tetrasodium EDTA. For our TPN piglets, the main reason for not requiring additional prophylactic antibiotics at day 8-12 since 2020, was a decrease in sepsis after we started routine use of 4%-tetrasodium EDTA. This study confirms that our laboratory subjective observation was indeed significant. This means there was decreased antibiotic use at the same time as decreased confirmed sepsis in TPN piglets given 4%-tetrasodium EDTA. We also chose a dwell time of 2 hours for 4%-tetrasodium EDTA, while clinical studies in humans have used a minimum of 4 hours. Due to the size and age of the piglets utilized, their TPN is delivered continuously. We decided we could adjust the infusion rate and safely interrupt the infusion for 2 hours without causing dehydration or hypoglycemia. It is possible that the results would be even more compelling with a longer dwell time. Finally, SBS studies are only conducted for 7 days and so, although we showed a decrease in confirmed sepsis, we did not show a difference in occlusions or replacements. Line replacements typically

happen after day 7 in our studies, presumably the time for biofilm and thrombus formation. However, we were still able to show a cost saving in the SBS model.

6.6 Conclusion

Reduction of catheter related sepsis in experimental models is important for reducing research costs and improving animal welfare and we have experienced a significant decrease in sepsis in our research laboratory since starting to use 4%-tetrasodium EDTA. Sepsis is a clear confounder of key outcomes we seek to study, including intestinal failure associated liver disease and the role of the microbiome in intestinal failure complications. Finally, given the translational utility of neonatal piglets as a model for pediatric intestinal failure, these results suggest further investigation into the benefits of 4%-tetrasodium EDTA solution for use in human patients is warranted and timely given current lack of access to ethanol locks and recent evidence that those locks are not cost effective.

Table 6-1. Catheter Outcomes for Piglets in both TPN and SBS Groups

	CONTPN (n=34)	T-EDTATPN (n=17)	P-value	CONSBS (n=48)	T-EDTASBS (n=48)	P-value
Occlusion Events Median [IQR]	0 (0-6)	0 (0-1)	0.5*	0 (0-0)	0 (0-0)	0.38*
Lines replaced No. (%)	5 (15%)	0 (0%)	0.1	0 (0%)	0 (0%)	0.38
Confirmed Sepsis No. (%)	16 (47%)	2 (12%)	0.005	6 (13%)	1 (2%)	0.046
Piglet Loss No. (%)	12 (35%)	2 (12%)	0.08	1 (2%)	0 (0%)	0.32

Data presented as No. (%) unless otherwise indicated. P-values determined by Pearson's chi-square unless designated by (*) then P-value determined by Mann-Whitney U test.

TPN- Total parenteral nutrition

SBS- Short bowel syndrome

Table 6-2. Cost Analysis of Standard of Care versus a 4%-Tetrasodium EDTA Locking Solution in Neonatal Piglet Model of Total Parenteral Nutrition

	Cost Per	No Locking Solution (n=34)		4% T-EDTA Locking Solution (n=17)	
	Piglet	Count	Total Cost	Count	Total Cost
Locking Solution	66.00	34	0	17	1122.00
Confirmed Sepsis[a]	100.95	16	1615.2	2	201.9
Piglet Loss[b]	1175.7	12	14108.4	2	2351.4
Line Replacement[c]	337.92	5	1689.6	0	0
Total Cost			**17413.2**		**3675.3**
Cost Per Piglet			**512.2**		**216.3**
Cost Savings Piglet Percentage Cost Reduction					**58%**

[a]Confirmed sepsis takes into account cost of culture vials, blood culture analysis and antibiotics.

[b]Piglet loss takes into account cost of the piglet, initial jugular catheter surgery, consumables and TPN to the median day when piglets died or were euthanized (11 days). Does not account for personnel cost.

[c]Line replacement takes into account cost of the catheter, personnel overtime and surgery costs.

Table 6-3. Cost Analysis of Standard of Care versus a 4%-Tetrasodium EDTA Locking Solution in Neonatal Piglet Model of Short Bowel Syndrome

	Cost Per	No Locking Solution (n=48)		4% T-EDTA Locking Solution (n=48)	
	Piglet	Count	Total Cost	Count	Total Cost
Locking Solution	27.5	0	0	48	1320
Confirmed Sepsis[a]	100.95	6	605.7	1	100.95
Piglet Loss[b]	1083.88	1	1083.88	0	0
Line Replacement[c]	337.92	0	0	0	0
Total Cost			**1689.58**		**1420.95**
Cost Per Piglet			**35.2**		**29.6**
Cost Savings Piglet Percentage Cost Reduction					**16%**

[a]Confirmed sepsis takes into account cost of culture vials, blood culture analysis and antibiotics.

[b]Piglet loss takes into account cost of the piglet, initial jugular catheter surgery, consumables and TPN to the median day when piglets died or were euthanized (6 days). Does not account for personnel cost.

[c]Line replacement takes into account cost of the catheter, personnel overtime and surgery costs.

Chapter 7. Probiotic Treatment Versus Empiric Oral Antibiotics for Managing Dysbiosis in Short Bowel Syndrome: Impact on the Mucosal and Stool Microbiota, Short Chain Fatty Acids and Adaptation

Adapted from:

Pauline M, Fouhse J, Hinchliffe T, et al. Probiotic treatment vs empiric oral antibiotics for managing dysbiosis in short bowel syndrome: Impact on the mucosal and stool microbiota, short-chain fatty acids, and adaptation. JPEN. Journal of parenteral and enteral nutrition. Nov 2022. Volume 46. Pg:1828-1838. doi: 10.1002/jpen.2377.

7.0 Abstract

Background: Infants and children with short bowel syndrome (SBS) are presumed to be at risk of gut microbial dysbiosis with potential sequelae of bacterial overgrowth that include sepsis, D-lactic acidosis, mucosal inflammation and malabsorption. In neonatal piglets with SBS, we compared intestinal microbial composition, short chain fatty acids (SCFA) and adaptation given probiotic treatment (*Lactobacillus* and *Bifidobacterium* spp.) versus oral metronidazole.

Methods: Following 75% distal small intestinal resection, piglets were allocated to: probiotic (PRO, 500mg BID n=7), metronidazole (MET, 15mg/kg BID n=8) and placebo (PLA, 500mg BID n=8). After 10 days of parenteral and enteral nutrition, 16S rRNA gene amplicon sequencing (colon tissue and stool) were undertaken and SCFA analysis (stool and colon effluent) performed using gas chromatography.

Results: In colon, Shannon diversity was higher for PRO compared to MET and PLA ($p=0.002$). PRO and PLA increased abundance of Bacteroidetes species (e.g. *Bacteroides fragilis*), compared to MET ($p<0.001$). PRO compared to PLA increased abundance of Firmicutes species (e.g. *Lactobacillus fermentum*) ($p<0.001$). MET increased abundance of Proteobacteria members, predominately *Enterobacteriaceae* compared to PRO ($p=0.004$). In stool, microbial findings were similar and SCFA (butyrate) concentrations were highest for PRO ($p=0.003$) compared to MET.

Conclusion: In pediatric SBS, the empiric use of oral antibiotics, such as metronidazole, is common for presumed clinical consequences of microbial dysbiosis. In this study of SBS piglets, that approach was associated with decreased microbial diversity and increased abundance of

potentially inflammatory Proteobacteria. In contrast, a probiotic treatment using *Lactobacillus* and *Bifidobacterium* spp. increased both diversity and SCFAs.

7.1 Introduction

Short bowel syndrome (SBS) is the leading cause of pediatric intestinal failure, with reduction in functional intestinal mass for absorption below that which can sustain life.[7] Parenteral nutrition (PN) is essential, but is associated with risks of sepsis and cholestatic liver disease.[2] Historically, SBS had a 25-50% mortality rate and while this has improved significantly with strategies that ameliorate hepatic dysfunction, sepsis remains a significant cause of morbidity and mortality.[324] Sepsis negatively impacts enteral tolerance and gut adaptation in children with SBS, and adversely impacts time to achieving autonomy from PN.[4]

Gut adaptation involves structural and functional changes of the intestine that enhance nutrient absorption. However, resulting bowel dilatation and dysmotility can promote luminal stasis impacting the resident microbiome, causing dysbiosis. This may have clinical consequences, including small bowel bacterial overgrowth (SBBO), D-lactic acidosis, mucosal inflammation, malabsorption and growth impairment.[325] The current clinical practice to manage SBBO, one possible manifestation of microbial dysbiosis in SBS, is use of broad-spectrum oral antibiotics.[326] This treatment is often empiric, rather than guided by small bowel cultures, and there are concerns this could potentially increase antibiotic resistance and decrease microbial diversity and the abundance of beneficial bacteria in the remnant intestine.[326] Bacteria produce metabolites, including short chain fatty acids (SCFAs), shown to modulate colonic energy salvage, the intestinal barrier, immune function and adaptation in animal studies.[144, 327]

Probiotics are an alternative to antibiotics to alter the microbiome; however, a major concern with their use is the potential for sepsis from either bacterial translocation across an

unhealthy mucosa or contaminating the central venous catheter. As a result, even though there is interest in this therapy for SBS patients, few formal studies have been performed and the literature largely consists of isolated case reports, with conflicting results.[162, 328-330] In a small trial using a *Lactobacillus* probiotic there were no change in stool microbiota or the patients growth.[330] In contrast, in a small series four children treated with a synbiotic (*Bifidobacterium breve*, *Lactobacillus casei* and galacto-oligosaccharides) appeared to have improved growth.[328] Two children with SBBO treated with *Lactobacillus plantarum* demonstrated reduced diarrhea, enabling one to discontinue antibiotics and advance enteral nutrition.[329] In another case report a child treated with *Lactobacillus casei, Bifidobacterium breve* and galacto-oligosaccharides had improved motility and absorption, associated with decreased pro-inflammatory bacteria (specifically *Escherichia coli*) and increased plasma SCFAs.[162]

In our neonatal piglet model of SBS, we have shown that the intestinal microbiota is impacted adversely by the administration of parenteral antibiotics and nutrition.[268] Our objectives for this study were to evaluate if SBS piglets treated with a combination probiotic (*Lactobacillus* and *Bifidobacterium*) demonstrated differences in small intestinal microbial composition, SCFAs and intestinal adaptation as compared to piglets treated with an oral broad-spectrum antibiotic or placebo. This model allows for preliminary safety and efficacy evaluation of probiotics compared to the effects of oral antibiotics or no treatment on the microbiota in SBS.

7.2 Methods

All experiments were conducted in accordance with guidelines of the Canadian Council of Animal Care (AUP00000155). Newborn male Duroc Cross piglets were obtained from the Swine Research and Technology Centre (SRTC) and randomized (blocked by litter) to three experimental groups: probiotic (PRO, 500 mg twice daily), metronidazole (MET, 30 mg/kg/day divided into two doses) and placebo (PLA, 500 mg twice daily). All were delivered enterally via a gastrostomy tube. The placebo contained the equivalent maltodextrin base present in the probiotic powder. Both PRO and PLA powders were dissolved in 3mL sterile water prior to administration. The probiotic (Renew Life Canada, Brampton, ON) contained 600 million CFU of *Bifidobacterium breve,* 500 million CFU *Lactobacillus rhamnosus,* 400 million CFU *Bifidobacterium bifidum,* 300 million CFU *Bifidobacterium longum infantis* and 200 million CFU *Bifidobacterium longum*. An intravenous formulation of metronidazole (Baxter, Mississauga, ON, Canada) was given via the gastrostomy tube at volumes ranging from 5-15mls.

On Day 0 under general anaesthesia, piglets underwent a 75% distal small intestinal resection including the ileocecal valve (ICV), cecum and first few centimetres of colon, with a resulting jejunocolic anastomosis (JC). Colon effluent was collected at baseline, during initial surgery. The small intestinal length was measured from the Ligament of Treitz to the cecum using a 3-0 silk suture along the anti-mesenteric border.[270] A 10-Fr gastrostomy tube was placed into the body of the stomach for trophic enteral nutrition (EN) and a 5-Fr central venous catheter was placed for PN delivery.[270] Prophylactic parenteral antibiotics were given preoperatively as a single dose: ampicillin (Sandoz, Boucherville, QB, Canada) and florfenicol (Intervet Canada Corp., Kirkland, QB, Canada).

Piglets were housed for 10 days in metabolic cages, in a heat and light controlled room with daily care as previously reported.[270] Nutrition commenced immediately postoperatively with PN. On day 2, isocaloric isonitrogenous EN commenced at 20% of nutrient requirements, with PN reduced from 100 to 80%. All experimental treatments commenced day 2, at the time as EN commenced. If piglets had suspected sepsis (based on fever, lethargy or vomiting) aerobic and anerobic blood cultures were taken to confirm if bacteremia was present. These piglets were not treated with antibiotics but were closely monitored. If piglets deteriorated humane euthanasia was indicated.

A baseline stool sample on day 2 was collected prior to commencing treatments, along with a stool sample day 10. Stool samples were collected using stoma appliances (Hollister, Aurora, Ontario, Canada) as previously reported.[270] At terminal surgery day 10, the small intestine length and weight were measured, tissue was collected for phylogenetic characterization of mucosal associated microbiota and histology and colon effluent was collected for SCFA analysis. Tissue was collected from jejunum (30cm proximal to the anastomosis) and colon (15cm distal to the anastomosis). Tissue samples were snap-frozen with liquid nitrogen, and all samples were stored at -80^{o}C.

7.2.1 DNA Analysis

DNA was extracted from day 2 and day 10 fecal samples and day 10 colon tissue using AquaStool solution (Multitarget Pharmaceutical LLC, Colorado Springs, CO, USA) as per the manufacturer's instructions.[300, 306] AquaRemove was also added to remove any Polymerase chain reaction (PCR) inhibitors via the manufacturer's instructions along with an ethanol/NaCL precipitation for better purification.[300, 306] 16S rRNA gene amplicon sequencing was performed

amplifying the V3-V4 hyper variable regions of the 16S rRNA gene using primers as shown in **Table 7-1**.

7.2.2 Bioinformatics

The QIIME2 pipeline (2021.2) was used for analysis of the 16S libraries.[331] Quality filtering was performed using DADA2.[332] Alpha diversity measures, Shannon and Simpson were determined using the alpha-group-significance pipeline.[307] Beta diversity measures and principal coordinate analysis was determined using weighted UniFrac with the core-metrics-phylogenetic and the beta-group-significance pathways with a sampling depth of 9000 sequences for fecal samples and 1000 sequences for colon tissue.[307]

7.2.3 Structural Adaptation Assessment

A board-certified veterinary pathologist (P.N.) blinded to treatments performed histological measurements on formalin-fixed and paraffin-embedded hematoxylin and eosin-stained sections, as previously described.[270] Villus height and crypt depth were measured via a micrometer eyepiece (Nikon Eclipse 80i; Nikon, Tokyo, Japan).

7.2.4 SCFA Analysis

All stool and effluent samples were prepared for gas chromatography (GC) SCFA analysis via added 5% phosphoric acid at a 1:4 ratio (v:v) prior to freezing and storage. Centrifuged, supernatant was transferred to a GC vial and combined with internal standard in a 5:1 ratio. Samples were then analyzed using a Varian model 3400 Gas Chromatograph (Varian, Walnut Creek, CA) with Stabilwax-DA column (30-meter x 0.53 mm ID; Restek Corp, Bellefonte, PA).[300] Flame-ionization detection was used with an injector temperature of 170^{o}C and detector temperature of 190^{o}C.[300]

7.2.5 Statistical Analysis

Data is expressed as median/interquartile range and mean/standard deviation as appropriate for data distribution. Analysis was performed in SPSS (version 28; SPSSS Inc, and IBM Company, Chicago, IL, USA). Between group comparisons used Kruskal-Wallis or ANOVA according to data distribution. Diversity measures were tested using permanova or independent samples Kruskal-Wallis. Microbial composition was analyzed using relative abundance results. Differences were considered significant at an alpha value ≤ 0.05.

7.3 Results

7.3.1 Animal Outcomes

In total, we randomized 24 piglets to the 3 groups (8 probiotic: PRO; 8 metronidazole: MET; 8 placebo: PLA); 23 reached the endpoint, 1 PRO was excluded due to a partial bowel obstruction. At baseline piglets across groups were not different in age, weight, total small bowel length (SBL), and post-resection SBL (**Table 7-2**).

There were 5 piglets with suspected sepsis (1 PRO, 2 MET, 2 PLA) and 2 confirmed sepsis (1 PRO, 1 MET). Organisms cultured included *Escherichia coli,* and *Enterococcus hirae.* No cases of sepsis were caused by bacterial species present in the probiotic. No piglets were removed from the study due to clinical deterioration from sepsis. There was no significant relationship between treatment groups and the frequency of presumed (p=0.85) or cultured sepsis (p=0.55). Eight piglets developed bloody stool during the trials (2 PRO, 1 MET, 5 PLA). No relationship across treatment groups with the frequency of bloody stool was noted (p=0.10); however, combining the intervention groups (MET and PRO) compared to PLA there was a significant

increase in bloody stool in piglets given the placebo (p=0.042). There was no association between presumed sepsis and bloody stool (p=0.78).

7.3.2 Alpha Diversity Metrics

Colon tissue alpha diversity metric Simpson (p=0.002) was increased for PRO compared to PLA and MET with no difference between PLA and MET (**Figure 7-1a**). Alpha diversity metric Shannon (p=0.002) was increased for PRO compared to PLA and MET, with PLA being increased compared to MET (**Figure 7-1b**).

Day 2 stool had no differences in alpha diversity metrics (data not shown). Stool day 10 results were similar with alpha diversity metric Simpson (p=0.003) and Shannon (p<0.001) increased for both PRO and PLA compared to MET, with no difference between PLA and PRO (**Figure 7-2**).

7.3.3 Beta Diversity Metrics

Day 10 colon tissue beta diversity metrics Bray Curtis (p<0.001) and Jaccard (p<0.001) were significantly different for PRO and PLA compared to MET, no difference between PRO and PLA (**Figure 7-3**). In **Figure 7-3c**, showing weighted UniFrac distance, MET was different to PRO and PLA (p<0.001), with no difference between PRO and PLA (p=0.08).

In stool day 2 there were no differences in beta diversity. In stool day 10 beta diversity metrics Bray Curtis (p<0.001) and Jaccard (p<0.001) were significantly different for PRO and PLA compared to MET with no differences between PRO and PLA (Figure 6-3). In **Figure 7f**, by weighted UniFrac distance, MET was different to PRO and PLA (p=0.002), with no differences between PRO and PLA (p=0.18).

7.3.4 Colon Mucosa Microbial Composition

There was a significant increase for MET compared to PLA and PRO of *Enterococcus* (p=0.012) (**Figure 7-4**). PRO compared to MET and PLA had increased *Lactobacillus fermentum* (p<0.001) and *Anaerotruncus colihominis* (p=0.016). PLA compared to PRO had increased *Pseudomonas* (p=0.027), with MET not different to either PLA (p=1.00) or PRO (p=0.24). PLA compared to MET had increased *Prevotella* (p=0.022), with PRO not different to PLA (p=0.14) or MET (p=1.00). PRO compared to MET had increased *Bacteroides thetaiotaomicron* (p=0.012), *Desulfovibrio piger* (p=0.005), *Akkermansia muciniphila* (p=0.021) and *Blautia* (p=0.007), with PLA not different to either. PRO and PLA compared to MET had increased *Bacteroides fragilis* (p<0.001) (**Figure 7-5**) and *Veillonella caviae* (p=0.006).

7.3.5 Stool Microbial Composition

At baseline (Day 2), there were no differences in stool microbial composition measured at the phylum, family, genus and species level. As seen in **Figure 7-6**, Day 10 stool, MET treated piglets had increased *Enterococcaceae* members (p<0.001), which include bacteria involved in lactic acid production compared to PLA and PRO and *Enterobacteriaceae* members (p=0.004) compared to PRO. PRO compared to PLA and MET had increased *Bifidobacterium breve* (p=0.006) and *Bifidobacterium longum* (p=0.005), both members of the probiotic cocktail, along with *Lactobacillus zeae* (p<0.001). PLA compared to MET had increased *Prevotella stercorea* (p=0.021) (**Figure 7-7**), PRO was not different than PLA (p=0.13) or MET (p=1.00). PRO compared to MET had increased *Blautia producta* (p=0.012), *Ruminococcus gnavus* (p=0.003), *Butyricimonas* (p=0.037), *Clostridium* (p=0.002) and members of *Desulfovibrionaceae* (p=0.025),

PLA was not different to either. PRO and PLA compared to MET had increased *Bacteroides fragilis* (p=0.007) and *Veillonella* (p=0.004).

7.3.6 SCFA Results

In general, SCFA concentrations on day 10 were highest with PRO (**Table 7-3**). There were no differences in baseline stool or colon effluent SCFAs. Butyrate was increased for both PRO and PLA in day 10 stool compared to MET (p=0.003). In colon effluent, acetate (p=0.009) and propionate (p=0.002) were increased for PRO compared to MET, with PLA not different than either.

7.3.7 Structural Adaptation

At termination, there was no difference in body weight, change in SBL, total SB weight, jejunum mucosal scraping weight in 20cm, villar length or crypt depth between any group (**Table 7-2**). Total jejunum weight was derived from the jejunal scraping weight per cm, corrected for the total remnant SBL. Total jejunal weight was increased for both PRO and MET compared to PLA (p=0.008). Of note, there was a trend toward increased villus length for both PRO and MET compared to PLA (p=0.053).

7.4 Discussion

The objectives of this study were to determine if a combination probiotic (*Lactobacillus* and *Bifidobacterium*) would lead to improved bacterial abundance and diversity, increase SCFA concentrations and improve structural adaptation in SBS piglets, compared to an oral broad-spectrum antibiotic and placebo. It was also important to determine if probiotic therapy would have any detrimental effects on the health of SBS piglets, specifically with sepsis occurring from the probiotic.

Probiotic use was associated with increased relative abundance of beneficial bacteria in both the mucosal and luminal microbiota, along with increased alpha and differences in beta diversity metrics compared to the antibiotic, metronidazole. Probiotic use increased concentrations of SCFAs in stool and colon effluent. Metronidazole was associated with a decrease in beneficial bacteria and diversity in both the mucosal and luminal microbiota. In fact, it was associated with an increase in potentially pro-inflammatory members of Proteobacteria. In contrast, both probiotic and metronidazole use had a potential impact on adaptation compared to placebo, along with a decrease in observed bloody stool. No detrimental effects to piglet health were observed by the probiotic in this model, including no increase in presumed or confirmed sepsis.

In our study, both alpha and beta diversity metrics appeared to be most impacted by PRO at the colon mucosal level. An abundance of Proteobacteria and low diversity of the gut microbiota are both regarded as negative factors in adaptation.[133, 326] MET led to a decrease in all measured metrics of alpha diversity compared to PRO. As well, MET lead to lower relative abundance in Bacteroidetes and an increase in Protobacteria, which has been characterized as a dysbiosis typical of SBS.[326] A decrease in diversity can lead to decreased interspecies competition, allowing certain species, in this case members of the phylum Proteobacteria, to become dominant.[326] There were signals of changes to structural adaptation. Both MET and PRO compared to PLA had increased total jejunal weight, along with a trend for greater villus height ($p=0.053$). This suggests that changes to the microbiome could be contributing to mucosal inflammation and bloody stool in this model and that this in turn could lead to changes in adaptation. There were significant inflammatory changes in the mucosal and serosal layers in

the colon of some of the piglets experiencing bloody stool noted by our veterinary pathologist. This supports the need for a microbiome targeted intervention, although did not differentiate between this probiotic combination or metronidazole as both seem to be superior to PLA for structural adaptation. Further confirmation in a larger sample size would help clarify this potential finding.

Probiotic treatment led to increase in all measures of alpha diversity, as well as an increase in relative abundance of bacteria like *Bifidobacterium breve, Lactobacillus fermentum* and *Bifidobacterium longum.* Such Firmicutes can harvest energy from substrates such as SCFAs. SBS patients with a microbiome deficient in Firmicutes may have a lowered capacity to energy harvest, which may be very important given growth complications of pediatric SBS that have been linked to the microbiome.[71]Different bacteria are involved in the production of SCFAs, although this is also dependent on type and composition of the fermentable fibre present in the colon.[326]

The mucosal microbiome is important for epithelial growth, repair and inflammation, while the luminal microbiome (represented by stool) is involved in metabolic processes, like SCFA production. While the mucosal microbiome has a close relationship with the hosts immune response and inflammation, the luminal microbiome is more impacted by diet, specifically fibre.[333] It is likely that the mucosal microbiome has a role in the bloody diarrhea observed in our model, presumably related to mucosal inflammation. Metronidazole and probiotic treatment appeared protective of this bloody diarrhea. We hypothesize that this could be due to the presence of potentially pro-inflammatory mucosal bacteria *Prevotella* that was increased in both mucosa and stool for PLA compared to MET. The family *Prevotellaceae*

was increased for PLA compared to both MET and PRO at the mucosal level. *Prevotella* spp. have been shown in mice to exacerbate intestinal inflammation, along with reducing acetate production.[334] Mucosal inflammation in a piglet model has been shown to be associated with decreased bacterial diversity of the colon.[267] Mucosal inflammation is also a concern in children with SBS due to dysbiosis.[133] PRO had the potentially protective benefit compared to PLA of reduction in bacteria from *Prevotellaceae*, as well as increased SCFA concentrations and overall microbial diversity. In comparison, while MET lead to a dramatic decrease in diversity and SCFAs, at the mucosal and stool level it did have the potential benefit of a reduction in *Prevotella*. This does highlight the complexity of microbiome targeted therapies and the need to understand the potential benefits of different probiotic and antibiotic interventions.

There has been much interest in clinical practice to use probiotics in SBS patients, but this enthusiasm has been dampened by concern over the potential for probiotic bacterial translocation across the intestinal mucosa causing sepsis. It has been found that the overall risk of sepsis related to probiotic bacteria like *Lactobacilli* and *Bifidobacterium* is small, and most importantly is similar to that caused by commensal strains of these bacteria.[335, 336] It is important when choosing a probiotic to use species that rarely cause infection in humans, do not have intrinsic antibiotic resistance, come from the commensal microbiome and, in the case of SBS, do not produce D-lactate.[336] In our piglets we did not observe any sepsis that was associated with culture of the probiotic *Lactobacillus* or *Bifidobacteria spp*; however, given probiotic bacteremia is likely a rare event, this requires confirmation in larger study sample sizes. Equally, a larger sample size might help confirm if probiotic use might actually decrease bacterial translocation of other gut derived bacteria (dominant in the blood cultures of SBS

piglets as in this study). Of note, MET treated piglets had an increase in *Enterococcus* (one MET piglet culturing *Enterococcus hirae*). In addition, our study design did not explore the intestinal barrier or do intestinal cultures (e.g. lymph nodes or spleen) for gut bacterial translocation. Going forward, the impact of probiotic versus antibiotic use on intestinal barrier and mucosal immune function warrants clarification. While we monitored for sepsis, future studies could include routine blood cultures at termination or blood markers of infection to better understand any potential detrimental or beneficial effects on piglet health.

Another potential treatment for this altered microbiome is the use of FMT for SBS, which has undergone limited studies in animal models. First, in SBS rats with JC anatomy, FMT did not alter the microbiome or alpha/beta diversity metrics, while it did alter the microbiome of sham rats.[337] This was postulated to be due to the unique luminal environment of SBS rats, specifically favouring often aero-tolerant or facultative anaerobes, over the donors FMT being more optimal for an anaerobic colon.[337] These rats had an 80% bowel resection, and did receive post operative antibiotics specifically to deplete the microbiome.[337] The FMT was from rats fed a high fat diet. This study postulated that possibly for SBS, more targeted treatments are required, like bacteria strains specific for the disease state that would be able to establish in this unique GIT.[337] Similarly, in piglets from our research group using the JC surgical SBS anatomy, FMT only altered the microbiome transiently and did not lead to long-term changes.[338] The study did not use antibiotics specifically to deplete the microbiome prior to surgery and the FMT donor was an adult sow, which may not be a developmental appropriate donor.[338] These two studies do not discount FMT from possibly having a role in SBS care, but highlight how difficult it can be for bacteria to outcompete the species already present in the

gut, especially when an altered diet, surgery, anatomy, antibiotics and more are introduced. Much of this revolves around the changes in oxygen levels throughout the SBS gut and these opportunistic facultative anaerobes dominating the microbiome and needing a cocktail of bacteria to be able to not only outcompete the resident bacteria but be metabolically active to yield benefits to the host. In contrast the use of probiotics as in the present study does seem able to result in meaningful changes in the microbiome at least when probiotics continues to be delivered.

Limitations of this study include the requirement to give prophylactic surgical antibiotics, an acknowledged confounder in our SBS microbiota studies.[268] However, this is an accepted approach for humans undergoing laparotomy with intestinal resection and has not prevented meaningful results in our prior microbiota studies.[268] While the study duration was only 10 days, we also note that in our previous microbiota studies (and other animal studies) substantial changes are seen in a few days.[268] The neonatal gut microbiome, especially the SBS gut, is quickly changing and during this time dysbiosis can occur.[82] Conversely, during this rapid development, microbiome changes could potentially last a lifetime and so we believe studying short term changes in the vulnerable neonatal model are not without relavence.[82] While there was a potential impact on adaptation, mucosal hyperplasia could be measured via proliferation or apoptosis immunohistochemistry. As stated previously, fibre is important for SCFA production and energy salvage,[333] but our EN did not contain any sources of fibre and so different diet sources should be investigated. Finally, this preliminary study may have been limited by too small a sample size to detect potential differences in structural adaptation.

We chose to study metronidazole as one antibiotic used clinically in SBS (for treatment or prevention of SBBO). In rats, metronidazole increases the intestinal mucus layer thickness and has a direct immune (anti-oxidant) function.[339] Metronidazole has activity mainly against anaerobes.[49] So when metronidazole is used as monotherapy we likely selecting out only specific anaerobes. There will be a need to study other oral antibiotics commonly used in SBS patients and their impact on the microbiome. Future studies using this model should evaluate intestinal barrier and immune function, metabolomics and expression of trophic factors that might be influenced by the microbiome and explain the findings regarding adaptation. We should not only include other antibiotic and probiotic combinations, but also prebiotics.. It is noteworthy that studies in piglets using *Lactobacillus* spp. alone showed detrimental effects on intestinal adaptation, while a synbiotic combination had positive effects.[340]

7.5 Conclusion

In pediatric SBS, the current standard of care for presumed clinical consequences of dysbiosis like SBBO is empiric use of broad-spectrum oral antibiotics, like metronidazole. In this study, that approach was associated with decreased diversity and increased potentially proinflammatory bacteria. While there has been reluctance to use probiotics in humans with SBS, this data suggests potential benefits to both the mucosal and luminal microbiota, supported by increased SCFAs. We should continue to investigate the use probiotics as an alternative to empiric antibiotics to modify microbial composition and diversity in SBS and seek to clarify benefits, safety and mechanisms.

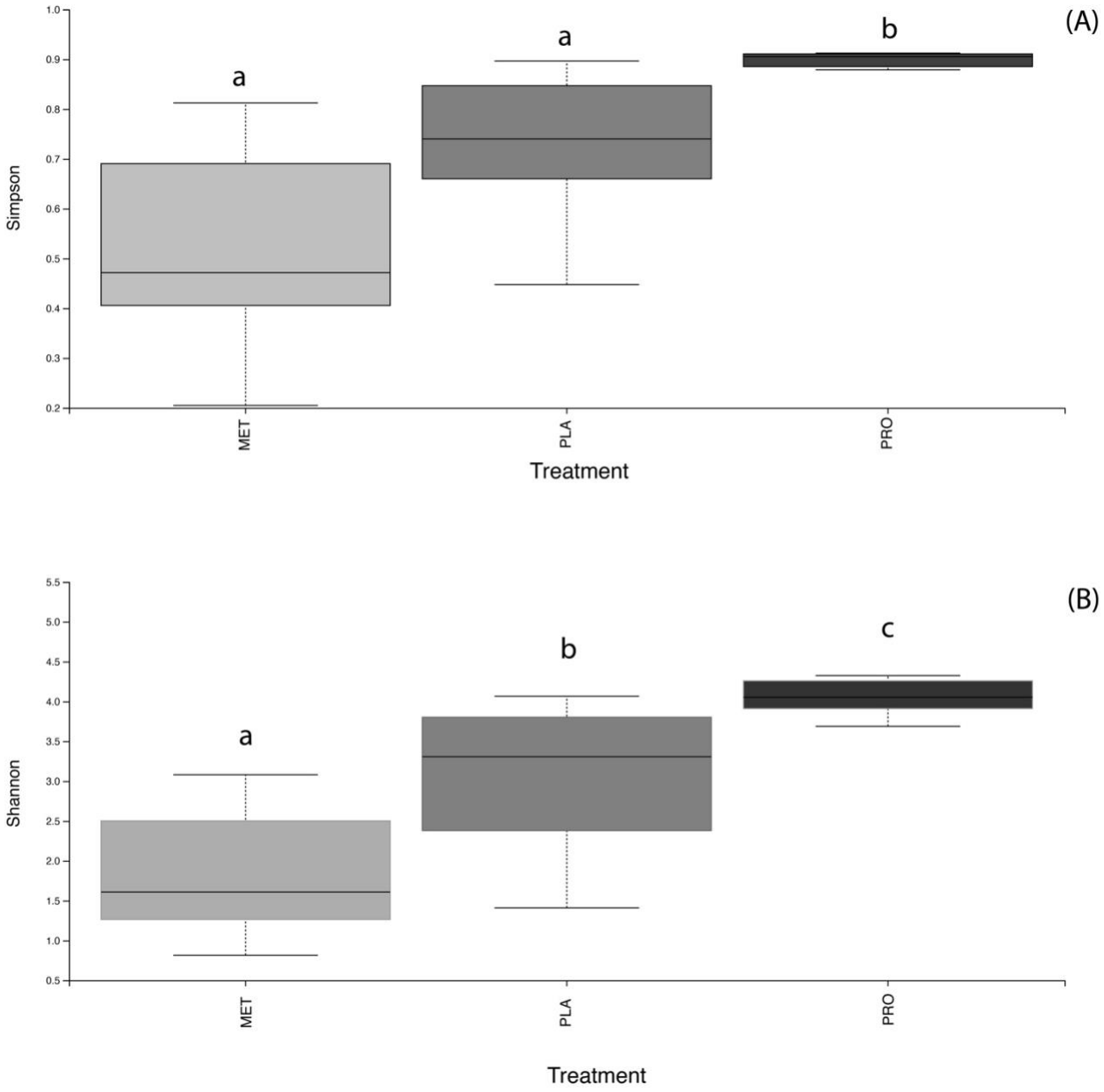


Figure 7-1. Alpha Diversity Metrics.
(A) Simpson (B) Shannon of Day 10 Colon Tissue. A) Simpson diversity. Light grey is MET (n=7), dark grey is PLA (n=8), black is PRO (n=6). Kruskal Wallis test was used with p=0.002 B) Shannon diversity. Light grey is MET (n=7), dark grey is PLA (n=8), black is PRO (n=6). Kruskal Wallis test was used with p=0.002 .Superscripts a,b,c represent post hoc differences.

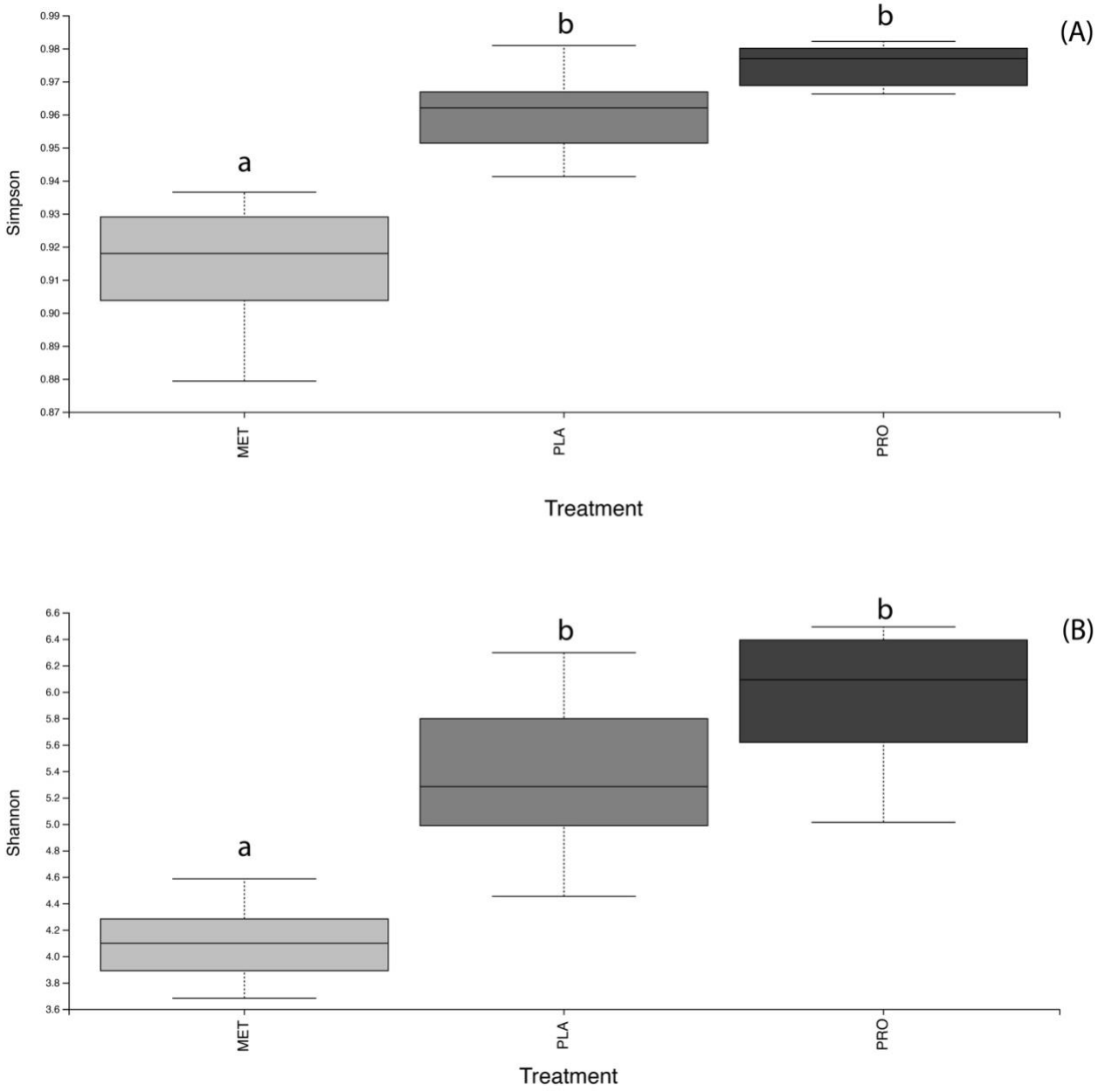


Figure 7-2. Alpha Diversity Metric Simpson and of Day 10 Stool.
A) Simpson diversity. Light grey is MET (n=7), dark grey is PLA (n=6), black is PRO (n=6). Kruskal Wallis test was used with p=0.003 B) Shannon diversity. Light grey is MET (n=7), dark grey is PLA (n=6), black is PRO (n=6). Kruskal Wallis test was used with p<0.001 .Superscripts a,b represent post hoc differences.

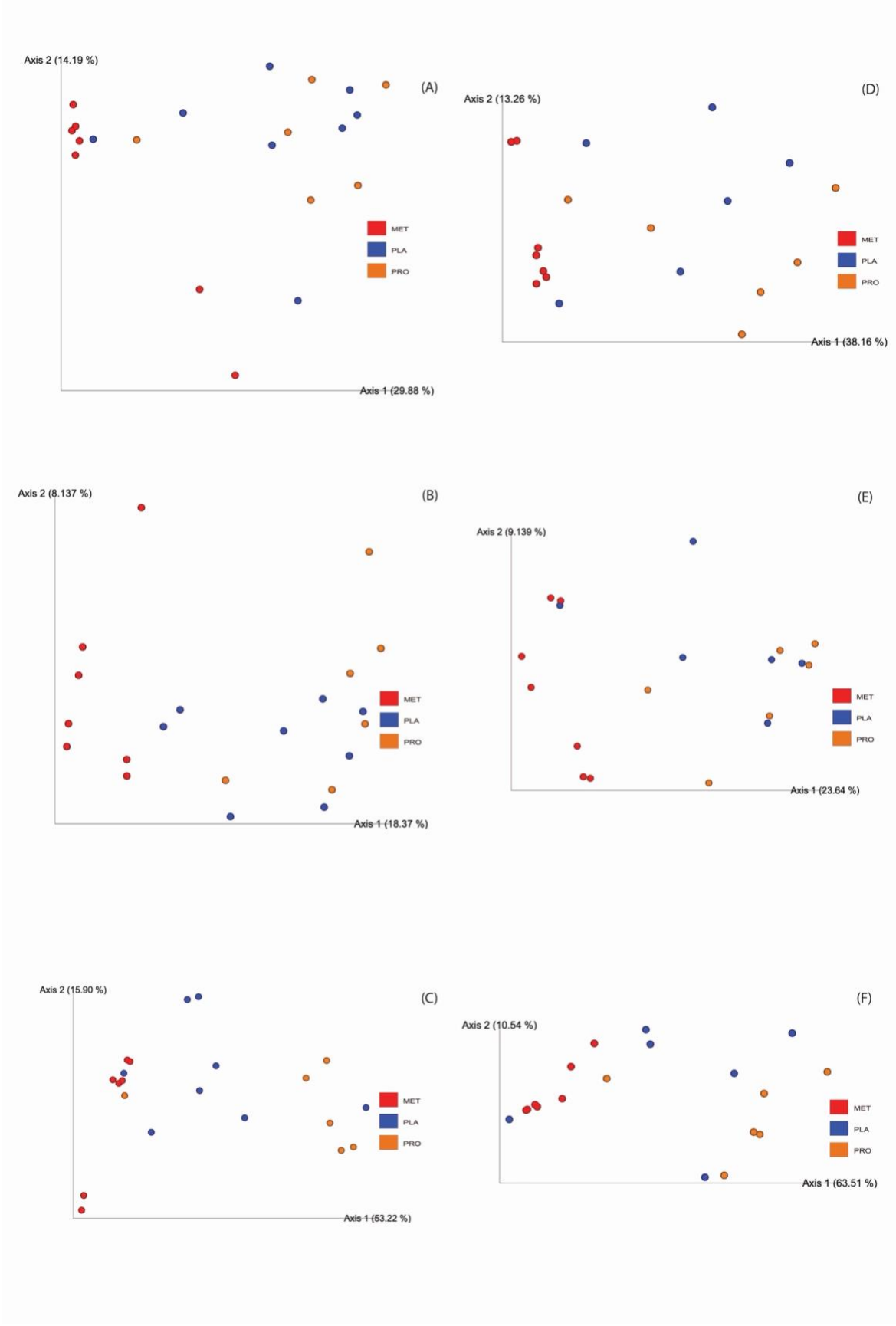

Axis 2 (14.19 %)
(A)
MET
PLA
PRO
Axis 1 (29.88 %)
Axis 2 (13.26 %)
(D)
MET
PLA
PRO
Axis 1 (38.16 %)
Axis 2 (8.137 %)
(B)
MET
PLA
PRO
Axis 1 (18.37 %)
Axis 2 (9.139 %)
(E)
MET
PLA
PRO
Axis 1 (23.64 %)
Axis 2 (15.90 %)
(C)
MET
PLA
PRO
Axis 1 (53.22 %)
Axis 2 (10.54 %)
(F)
MET
PLA
PRO
Axis 1 (63.51 %)

Figure 7-3. Bray-Curtis, Jaccard and Weighted UniFrac PCoA Emperor Plots of Day 10 Colon Tissue and Stool.

A) Colon Tissue Bray-Curtis PCoA. Red is MET (n=7) blue is PLA (n=8), orange is PRO (n=6). Permanova test was used with $p<0.001$. B) Colon Tissue Jaccard PCoA. Red is MET (n=7) blue is PLA (n=8), orange is PRO (n=6). Permanova test was used with $p<0.001$. C) Colon Tissue Weighted UniFrac PCoA. Red is MET (n=7) blue is PLA (n=8), orange is PRO (n=6). Permanova test was used with $p<0.001$ D) Stool Bray-Curtis PCoA. Red is MET (n=7), blue is PLA (n=6), orange is PRO (n=6). Permanova test was used with $p<0.001$. E) Stool Jaccard PCoA. Red is MET (n=7), blue is PLA (n=6), orange is PRO (n=6). Permanova test was used with $p<0.001$. F) Stool Weighted UniFrac PCoA. Red is MET (n=7), blue is PLA (n=6), orange is PRO (n=6). Permanova test was used with $p=0.002$.

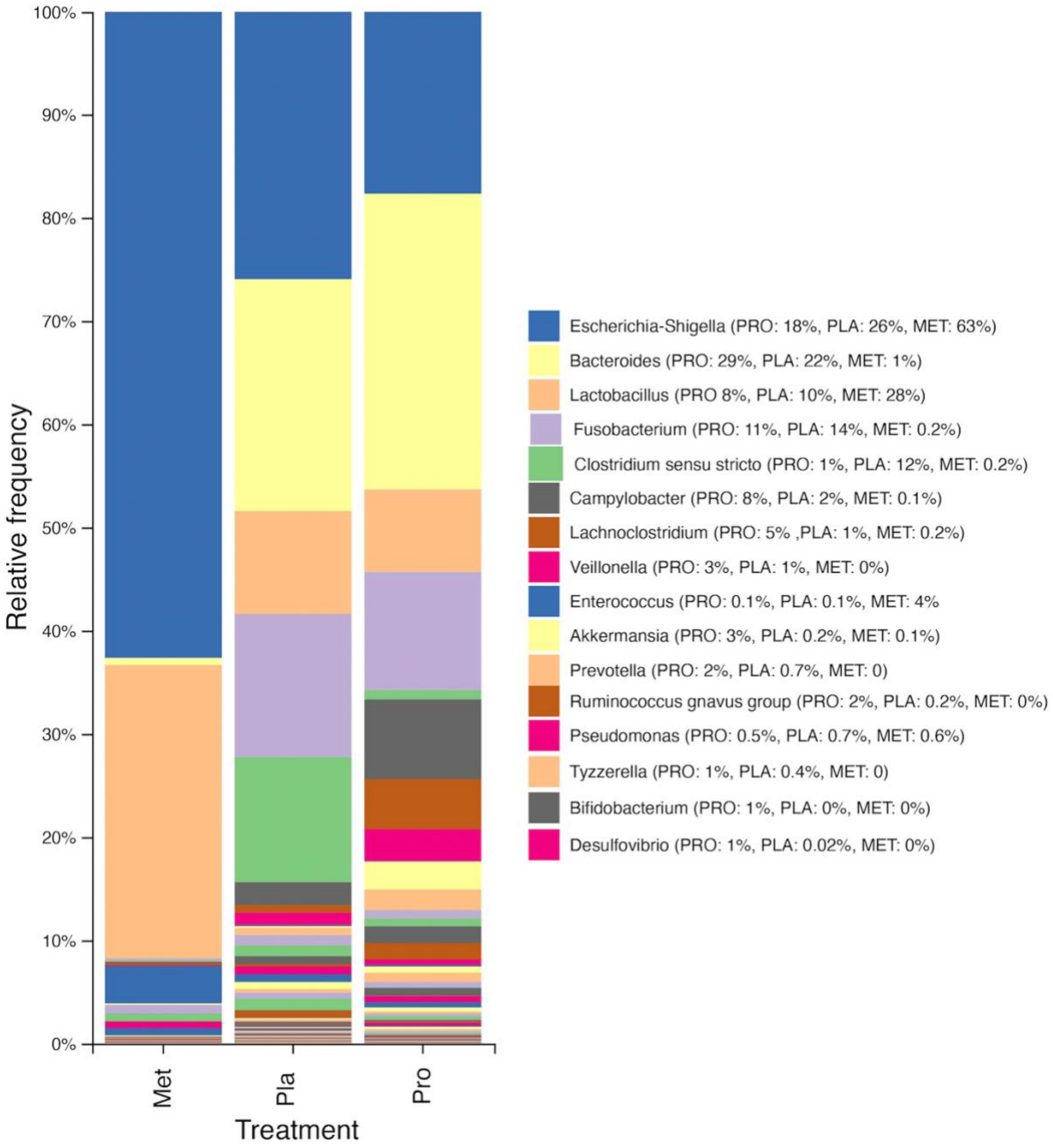


Figure 7-4. Day 10 Colon Tissue Taxonomy Relative Abundance at Genus Level.

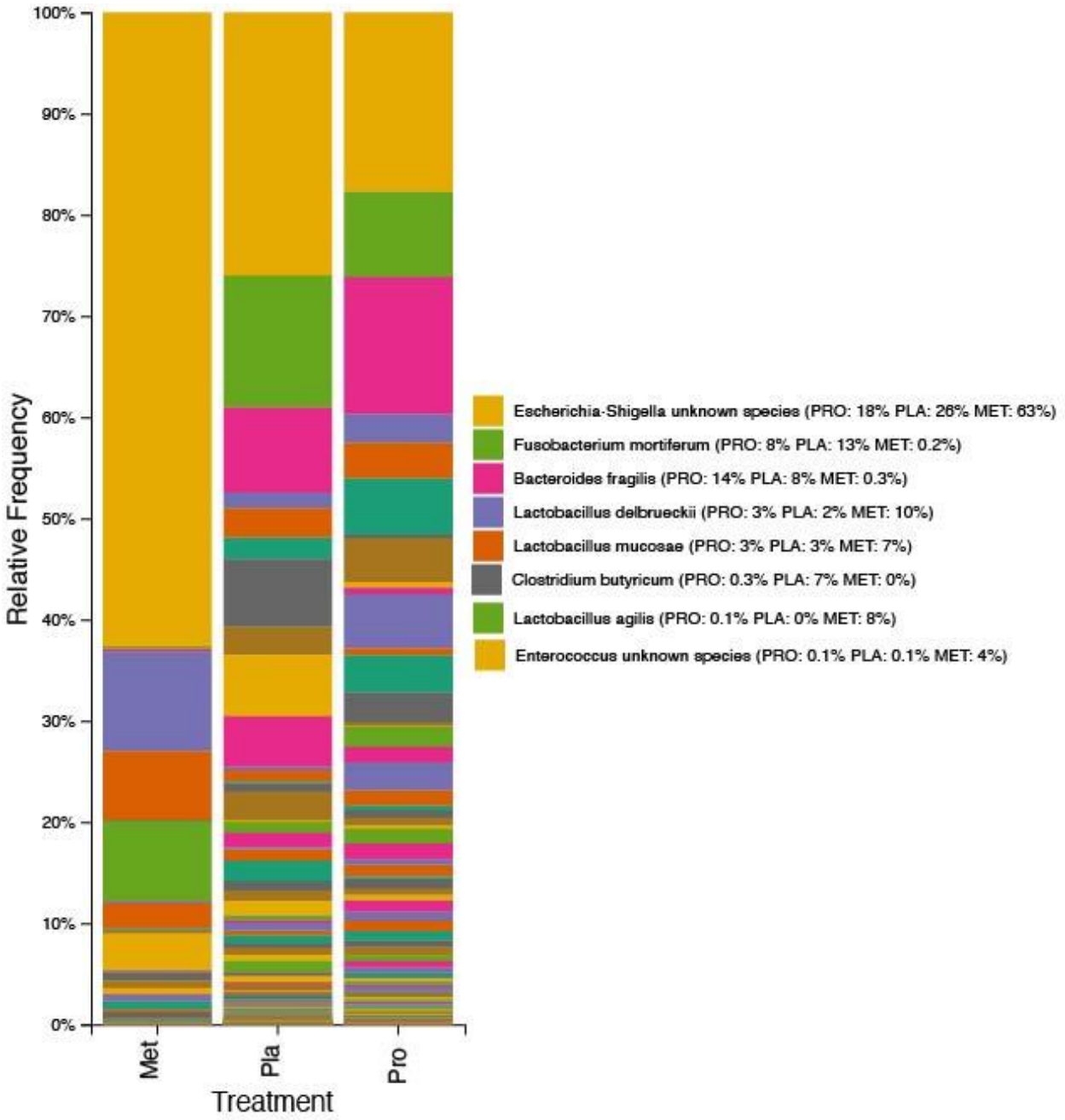


Figure 7-5. Day 10 Colon Taxonomy Relative Abundance at Species Level.

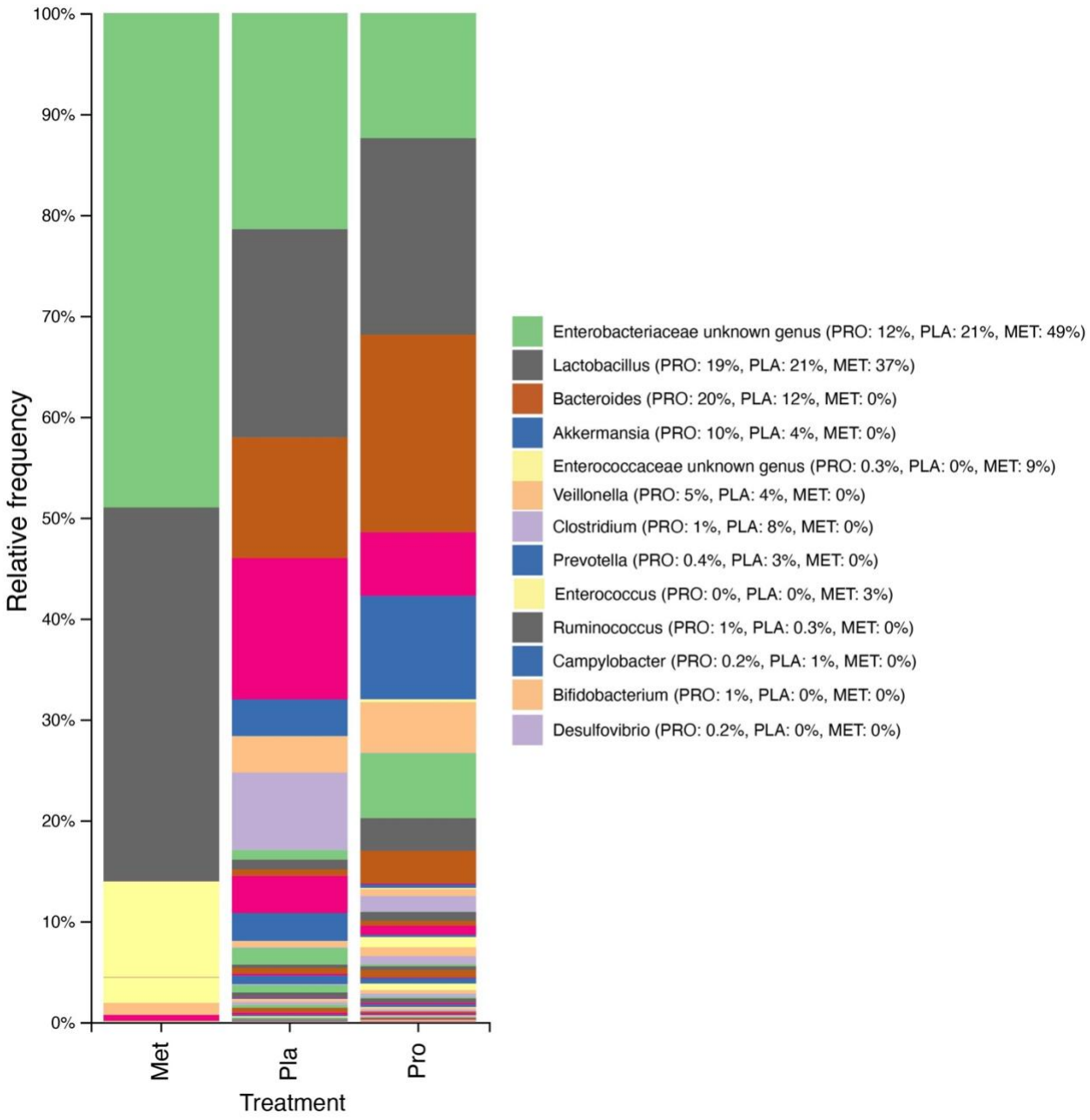


Figure 7-6. Day 10 Stool Taxonomy Relative Abundance at Genus Level.

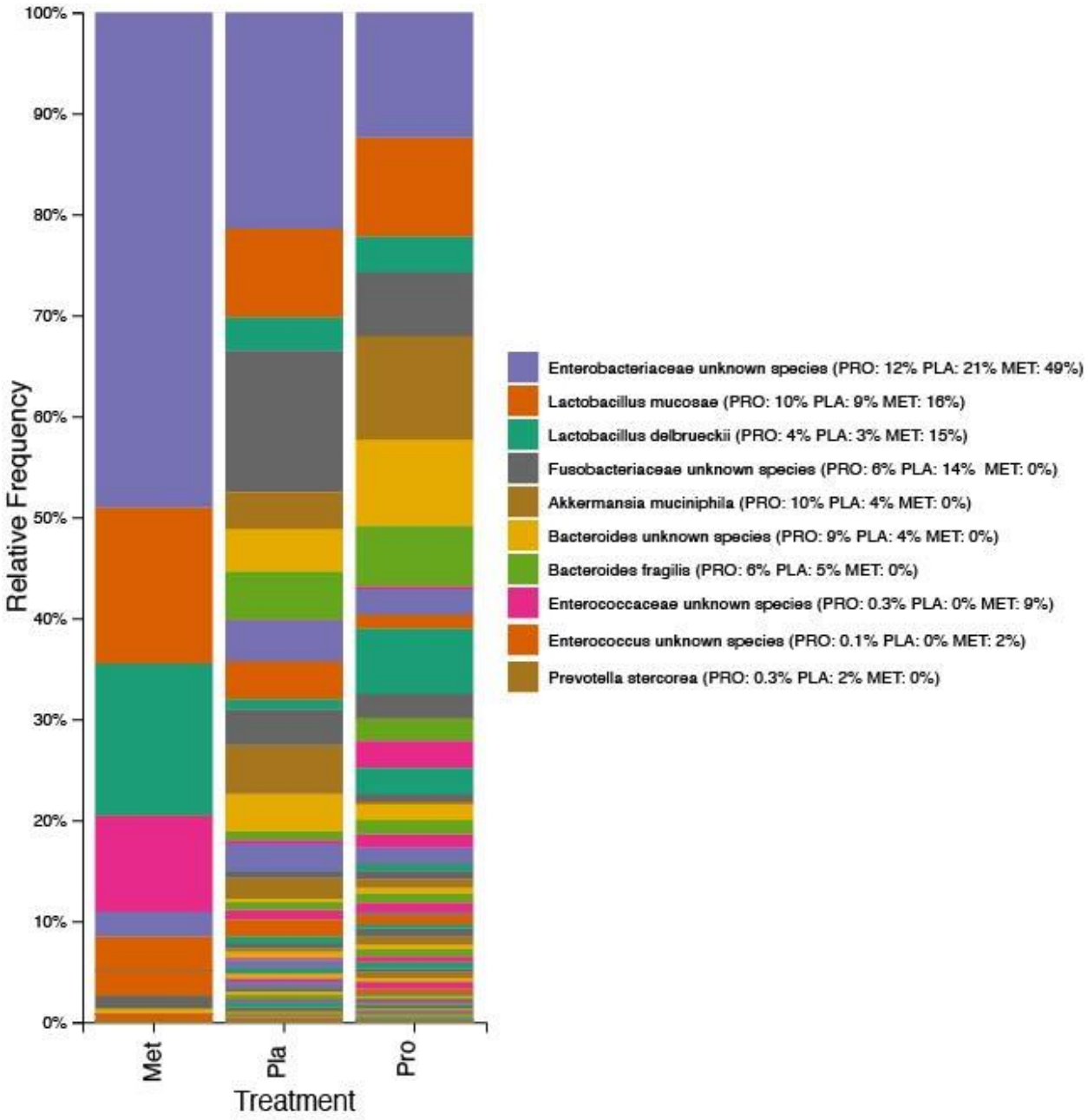


Figure 7-7. D10 Stool Taxonomy Relative Abundance Species Level.

Table 7-1. Primers

Sample Type	Primer	Sequence (5'-3)
Colon	Forward	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG
	Reverse	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC
Stool	Forward	CCTACGGGNGGCWGCAG
	Reverse	GACTACHVGGGTATCTAATCC

Table 7-2. Structural Adaptation.

	Metronidazole (n=8)	Placebo (n=8)	Probiotic (n=7)	Significance
Age (d)	3.88 (0.99)	4.25 (0.89)	4.00 (0.82)	0.71
Wt Baseline (kg)	2.39 (0.18)	2.35 (0.14)	2.45 (0.15)	0.49
Wt Termination (Kg)	4.04 (0.27)	3.82 (0.50)	4.10 (0.36)	0.33
Post-Resection SBL (cm)	142 (8.8)	135 (10)	135 (8.2)	0.27
Change in SBL (cm)	8 (17)	-7 (12)	9 (15)	0.10
SB Wt (g)	34 (10)	31 (11)	30 (7)	0.62
Jej Scraping Wt (g/20cm)	2.23 (0.42)	2.05 (0.36)	2.36 (0.20)	0.24
Total Jej Wt (g)	16 (1.9)[b]	13 (1.8)[a]	17 (3.4)[b]	0.008
Villar Length (mm)*	0.67 (0.14)	0.49 (0.18)	0.60 (0.08)	0.053
Crypt Depth (mm)	0.15 (0.02)	0.17 (0.02)	0.16 (0.01)	0.09

Data is represented as mean and (standard deviation) and analyzed using ANOVA. Superscripts a, b represent post hoc differences. *Placebo n=7

Jej, jejunum

SBL, small bowel length.

Wt, weight

Table 7-3. Day 10 Short Chain Fatty Acids.

Sample	SCFA	Metronidazole	Placebo	Probiotic	Significance
Stool	Acetic Acid	7.7 (4.3-11.3)[a]	9.6 (6.1-13.3)[a,b]	17.95 (13.9-40.7)[b]	0.014
(μmol/mL)	Propionic Acid	0.22 (0.08-0.38)[a]	1.5 (0.24-8.6)[a,b]	13.36 (5.8-18.9)[b]	0.016
	Butyric acid	0.005 (0.0.0-0.04)[a]	1.5 (0.14-4.5)[b]	2.4 (0.17-5.0)[b]	0.003
Colon Effluent	Acetic Acid	2.71 (1.3-3.5)[a]	5.1 (3.4-6.6)[a,b]	10.65 (6.0-15.2)[b]	0.009
(μmol/mL)	Propionic Acid	0.11 (0.05-0.16)[a]	0.68 (0.30-1.1)[a,b]	5.97 (3.1-7.3)[b]	0.002
	Butyric Acid	0.00 (0.00-0.009)[a]	0.53 (0.02-0.83)[b]	1.39 (0.15-2.4)[b]	<0.001

Data is represented as median and (interquartile range) analyzed using Kruskal Wallis with Bonferroni correction. Concentrations are in μmol/mL. Superscripts a, b represent post hoc differences.

Chapter 8. Comparing the Intestinotrophic Effects of Two Glucagon-like Peptide-2 Analogues in the Treatment of Short Bowel Syndrome in Neonatal Piglets

Adapted from:

Pauline ML, Nation PN, Wizzard PR, et al. Comparing the intestinotrophic effects of 2 glucagon-like peptide-2 analogues in the treatment of short-bowel syndrome in neonatal piglets. JPEN. Journal of parenteral and enteral nutrition. March 2021. Volume 45 issue 3. Pg 538-545. https://doi.org/10.1002/jpen.1853

8.0 Abstract

Background: A priority goal in treating short bowel syndrome (SBS), is autonomy from parenteral nutrition (PN). This relies upon intestinal adaptation, which can be augmented by glucagon-like peptide-2 (GLP-2) analogues. In neonatal piglets with SBS, we compared intestinal adaptation following treatment with two GLP-2 analogues: teduglutide (TED) and apraglutide (APRA).

Methods: Following 75% distal small intestinal resection, neonatal piglets receiving 80% PN were allocated to four treatments by subcutaneous injection: daily saline (CON: n=8), twice weekly APRA (5mg/kg/dose; n=8) and teduglutide once daily (TED, 0.05mg/kg/dose; n=8) or twice daily (TEDBID, 0.05mg/kg/dose; n=7). Pharmacokinetic studies were undertaken and on day 7, small intestinal length and weight were measured and jejunal tissue collected for histology.

Results: Pharmacokinetic profiles were different between the two analogues. To achieve a comparable exposure to apraglutide, teduglutide requires twice daily injection (TEDBID). Compared to saline, APRA and TEDBID increased small bowel length (cm) [CON: 141, APRA: 166, TED: 153, TEDBID: 165; p=0.004], while APRA increased small bowel weight (g) [CON: 26, APRA: 33, TED: 28, TEDBID: 31; p=0.007] and villus height (mm) [CON: 0.59, APRA: 0.90, TED: 0.58, TEDBID: 0.74; p<0.001].

Conclusion: Apraglutide injected only twice during the 7 consecutive days demonstrated a superior intestinotrophic effect compared to teduglutide injected once daily. Even at more comparable drug exposure, when teduglutide was injected twice a day, apraglutide showed superior trophic activity at the mucosal level. This is highly relevant for the treatment of

pediatric SBS given the markedly lower dose frequency by subcutaneous injection of apraglutide.

8.1 Introduction

Short bowel syndrome (SBS) is the leading cause of neonatal intestinal failure.[2] Parenteral nutrition (PN) is required to support survival and growth, but comes with the associated risks of liver disease and sepsis.[341] Historically, SBS had a >25% mortality rate[341, 342] in infants, although this has improved more recently with development of multidisciplinary care and new lipid management strategies that have reduced liver disease.[341-343] Long term PN therapy is associated with significant health related costs and negative impact on the child and family's quality of life.[1] Autonomy from PN is dependent on adaptation of the remnant intestine. Intestinal adaptation is a compensatory process that involves both structural and functional changes, including lengthening of the gut, mucosal hyperplasia, bowel dilation, slowing of motility and increased nutrient transporter expression.[344] All these changes ultimately enhance nutrient absorption allowing weaning off PN support. In children this process takes months to years.[345]

A recent advance in the management of SBS is the advent of glucagon-like peptide-2 (GLP-2) analogues as trophic therapies that aim to enhance adaptation.[79] Teduglutide (Gattex®/ Revestive®; Takeda, Japan) (0.05 mg/kg by daily subcutaneous injection) has been approved for adult use in the US and Canada, with clinical trial data showing a ≥ 20% reduction in PN following 20 weeks of teduglutide treatment.[186] Teduglutide has recently been approved in the US and Canada for children ≥ 1 year of age (same dose, same regimen), with pediatric trials also demonstrating significant reduction in PN volume.[200, 201]

A key difference between adults and young children with SBS is the potential for linear gut growth; however, intestinal lengthening has not always been a treatment outcome of interest, including in animal studies of GLP-2 analogues. Nevertheless, clinical studies show that residual length of both small and large intestine at the time of initial diagnosis are major predictors of autonomy.[4, 346] Logically, a therapy that increases the length of the small bowel for SBS patients would be ideal in the pediatric setting. GLP-2 treatment in rodents has not consistently shown small bowel lengthening.[208, 347] In piglet models, both native GLP-2 and teduglutide treatments have not shown intestinal lengthening.[18, 184] Recently we studied, apraglutide, a GLP-2 analogue with an even longer half-life than teduglutide.[78] Apraglutide was associated with intestinal lengthening in our piglet model of SBS with total ileal resection (jejunocolic or JC anastomosis); a model wherein consistently we have not seen linear intestinal growth without trophic peptide treatment.[184]

Apraglutide has amino acid substitution in position 11 and 16, with a change in the terminal OH to NH_2 leading to low clearance via the kidneys and decreased enzymatic cleavage by DPP-IV, along with high plasma protein binding.[206] Apraglutide has slower absorption from the subcutaneous site than teduglutide, leading to a longer time before the T_{max} is observed.[206] A PK study was performed on rats, monkeys and minipigs of apraglutide, teduglutide and glepaglutide.[206] Renal clearance of human GLP-2, teduglutide, glepaglutide and apraglutide in rats was 25, 9.9, 2.8 and 0.27 ml/kg per minute respectively, demonstrating this reduced renal clearance of GLP-2 analogues compared to GLP-2, with apraglutide have the lowest renal clearance.[206] The elimination half-lives were longer for the GLP-2 analogues as expected, with apraglutide's half-life substantially longer than the other analogues.[206] There were similar PK

profiles for minipigs and monkeys.[206] As discussed in dosing, it is important that a drug has high selectivity for its receptor, in this case GLP-2R, both teduglutide and apraglutide were determined to be highly selective for GLP-2R, with very little selection for the GLP-1 receptor.[206] Interestingly, Glepaglutide had high selectivity for the GLP-1 receptor.[206] For pharmacodynamics, i.e. the effect of the drug on the body, apraglutide had the greatest impact on intestinal adaptation measured by intestinal growth and weight in rats.[206]

In piglets given apraglutide compared to saline for 7 days with a 75% bowel resection and jejunocolic anastomosis.[78] Apraglutide was given twice weekly at a dose of 5mg/kg subcutaneously.[78] As discussed previously, with loss of ileum with a jejunocolic anastomosis these piglets have reduced endogenous GLP-2, with the receptor still present and presumably would be amenable to GLP-2 replacement therapy. Compared to the saline piglets, apraglutide treated piglets had intestinal lengthening, increased small bowel weight, increased villus height and crypt depth.[78] Importantly from a functional standpoint these piglets when measuring fecal fat had lower fecal fat and decreased energy losses.[78]

Like teduglutide, apraglutide has been studied for intestinal fluid absorption. In 8 adults, apraglutide was given at a dose of 5 mg/kg/d for 4 weeks, than at 10 mg/kg/d for 4, or placebo was given.[211] There was no significant differences in how patients responded to the 5mg/kg/d or 10mg/kg/d doses.[211] At both doses, urine volume output that was used to infer intestinal fluid absorption was significantly increased.[211] The higher dose also had significantly increased urinary sodium excretion, another measure of intestinal fluid/sodium absorption.[211] For PN volume, the primary endpoint of many of the teduglutide studies (specifically a decrease in PN

volume by ≥20%), the 10 mg/kg/d apraglutide significantly decreased the relative PN volume compared to placebo by 28%, while there were no significant changes for 5mg/kg/d group.[211]

The aim of this study was to compare the effect of two GLP-2 analogues, apraglutide and teduglutide, on linear intestinal growth and mucosal hyperplasia (structural adaptation). Given apraglutide has a half-life of 30 hours and is administered twice weekly, compared to teduglutide with a half-life of 2 hours and administered daily, we performed pharmacokinetics to ensure the doses were in fact comparable. We began this study using a daily dose of teduglutide, as has been studied in piglets previously,[18] and the same as the FDA approved dosing regimen for human adults and children. Subsequently, a second dose group was added based on PK findings.

8.2 Methods

All procedures in this study were approved by the University of Alberta Animal Care and Use Committee for Livestock and were conducted in accordance with guidelines of the Canadian Council of Animal Care.

8.2.1 Animals and Treatments

Male neonatal Duroc piglets were obtained from the Swine Research and Technology Centre (SRTC) and allocated to the following: saline control (CON), apraglutide 5 mg/kg/dose twice per week (APRA), teduglutide 0.05 mg/kg/dose either once daily (TED) or twice daily (TEDBID). Apraglutide was supplied by the manufacturer in kind (VectivBio, Basel, Switzerland). Teduglutide was manufactured by Toronto Research Chemicals Inc. (Toronto, Canada). All treatments commenced immediately post operatively. Saline and apraglutide treatments were administered on days 0 and 3. Teduglutide single daily dose treatment was administered daily

in the morning and teduglutide twice-daily treatment was administered every 12 hours, both for 7 consecutive days. All treatments were given by subcutaneous (sc) injection.

8.2.2 Surgical Procedures and Aftercare

Under general anesthesia piglets had insertion of a 5 French central venous catheter for PN infusion.[78] All piglets had a laparotomy to measure the small-intestine length using a silk 3-0 suture along the antimesenteric border with minimal traction.[78] Intestinal resection was the same for all piglets: 75% distal resection with jejunocolonic (JC) anastomosis, removing the entire ileum and a small portion of the right colon.[78] A 10 French Stamm gastrostomy tube was inserted for enteral nutrition.[78]

Piglets were maintained in the laboratory as previously reported.[270] Pain management and antibiotics, single dose administration of florfenicol (15 mg/kg/d IM; Intervet Canada Corp. Kirkland, QB, Canada) at surgery and ampicillin (10 mg/kg twice a day IV; Sandoz, Boucherville, QB, Canada) for 48 hours were provided postoperatively. Additional standardized care included further treatment with ampicillin, baytril (5 mg/kg/d iv; Bayer Animal Health Mississauga, ON, Canada) or gentamicin (3 mg/kg 3 times a day iv; Sandoz, Boucherville, QB, Canada) for sepsis presumed on the basis of fever, lethargy or vomiting. We provided Lasix (2 mg/kg/d iv; Intervet Canada Corp. Kirkland, QB, Canada) to animals that appeared to be fluid overloaded with excessive weight gain for 2 consecutive days.

Nutrition commenced immediately postoperatively using a PN solution formulated in this laboratory.[273] From day 2, PN was reduced to 80% of usual target rates until study end to avoid excessive fluid retention and edema observed in previous studies. Trophic enteral nutrition (EN) to support adaptation was also provided at 20% of target nutrient requirements.[270] On Day 7, piglets were put under general anesthesia and the small intestine

was again measured in length, prior to being removed, emptied and weighed. Jejunal tissue for histology and mucosal scrapings (measured on a standardized 20cm length board) were also collected.

8.2.3 Fecal Fat Collections

On Day 6, a 24 hour stool collection of piglets commenced, into stoma bags via fitted ostomy appliances (Hollister, Aurora, Ontario, Canada) to the perianal area. The enteral nutrition bags were weighed at the start and finish of the collection period to determine exact lipid delivery. The fat contents were determined on the fecal effluent, in paired freeze dried samples using the Folch method.[277]

8.2.4 Assessment of Structural Adaptation

Sections of the jejunum 20 cm distal to the ligament of Treitz were obtained for histology. Paraffin mounted 5-μm sections from each intestinal site were stained with hematoxylin and eosin prior to assessment using a micrometer eyepiece (Nikon Eclipse 80i; Nikon, Tokyo, Japan). Mucosal hyperplasia was assessed by measurement of villus height and crypt depth, performed by a board-certified animal pathologist (P. Nation) blinded to treatment group. Height was taken from the villi in longitudinal section; crypt depth was taken from the same area. Ten measurements per villus-crypt axis were used to calculate the mean (SD).

8.2.5 Pharmacokinetic studies

Pharmacokinetic (PK) studies were undertaken on a subset of 6 treated surgical piglets to determine pharmacologically equivalent doses: 3 treated with apraglutide and 3 treated with teduglutide. Following injection with apraglutide blood draws were performed at time 0, 6, 12, 24, 48, 72, 96 and 168 hours. Following injection with teduglutide, blood draws were

performed at time 0, 2, 6, 12, and 24 hours. Blood was collected into EDTA tubes and, following centrifugation, plasma was collected, frozen in dry ice and stored at -80°C until analysis.

8.2.5.1 Detection and Analysis of Plasma Peptide Concentrations

Following solid-phase extraction of apraglutide or teduglutide from the plasma samples, the compounds were identified and quantified using reversed-phase high-performance liquid chromatography with tandem mass spectrometry (HPLC-MS/MS) detection. For apraglutide, extracted samples were injected into an XBridge Protein BEH C4, 50 X 2.10 mm, 3.5µm HPLC column (from Waters) coupled to an HPLC system and the analyte was eluted using a gradient method with a mobile phase (A: 0.2% Formic Acid in H2O; B 5% TFE in acetonitrile) at a flow rate of 0.6 mL/min. For teduglutide, extracted samples were injected into a Halo peptide ES C18, 50 X 2.10 mm, 2.7 µm HPLC column (from Advanced Materials Technology) coupled to an HPLC system and the analyte was eluted using a gradient method with a mobile phase (A: 0.1% Formic Acid in H2O; B Acetonitrile) at a flow rate of 0.5 mL/min. Both analytes were detected using an API-5000 triple quadrupole mass spectrometer (Applied Biosystems SCIEX, Ontario Canada) using a Turbo V ion source and operating in the positive electrospray ionization mode. Analyte concentrations were calculated by linear regression analysis using the peak area ratio of analyte to the internal standard on the Applied Biosystems Analyst software version 1.6 and 1.6.3.

Pharmacokinetic parameters were determined by best fitting of compound concentration-time curves using a non-compartmental curve stripping method (PK Solutions 2.0™ software, Summit Research Services, Montrose, CO). The PK parameters assessed were the area under the curve from time zero to infinity ($AUC_{0-\infty}$) and from time zero to the last

measurable timepoint (AUC_{0-t}), the maximum observed plasma concentration (C_{max}), the elimination half-life ($T_{1/2}$) and the time of the maximum observed plasma concentration (T_{max}).

8.2.6 Statistical Analysis

Results are expressed as a mean and standard deviation following confirmation of normal distribution. One way ANOVA was performed with Post hoc tukey for all comparisons. Statistical tests were performed using SPSS for Windows (version 24; SPSS Inc, and IBM Company, Chicago, IL, USA). Differences were considered significant at $P < 0.05$.

8.3 Results

8.3.1 Pharmacokinetics

The PK profiles of apraglutide and teduglutide were compared following administration by single dose bolus subcutaneous injection. In neonatal piglets, average terminal elimination $T_{1/2}$ and T_{max} were notably longer for apraglutide than for teduglutide (**Table 8-1**). The observed long value of T_{max} post dose (12 h) is the reflection of the long half-life ($T_{1/2}$=12.4 h) of the peptide in the circulation and its slow absorption from the sc injection site. In contrast, teduglutide was absorbed fairly rapidly (T_{max} = 0.5 h) and had an average terminal elimination $T_{1/2}$ of 0.54 h.

Average plasma concentrations of apraglutide and teduglutide over time after s.c. administration, are shown in **Figure 8-1**. Average C_{max} of apraglutide and teduglutide were 4.1 and 37.1 □g/ml respectively, while the $AUC_{0-\infty}$ of apraglutide was 1.7-fold higher as compared to a single dose of teduglutide. Therefore, a subsequent group of animals was added to the study design to undergo twice a day treatment with teduglutide, in order to reach equivalent exposure.

8.3.2 Clinical Outcomes

Target sample size was 8 per group, altogether 38 piglets underwent surgery and 31 piglets completed the study. One saline piglet was excluded due to sepsis. In the apraglutide treated group there were three exclusions: dislodged gastric tube (1), bowel obstruction (1) and sepsis (1). No teduglutide once daily treated piglets were excluded. In the teduglutide twice daily treated group there were three exclusions: severe intestinal adhesions (1) and sepsis complicated by marked fluid overload (2). Final groupings were CON (n =8), APRA (n=8), TED (n=8), and TEDBID (n=7).

At baseline, piglets were not different according to age (CON: 3.75d [0.7], APRA: 3.5d [0.8], TED: 3.6d [0.7], TEDBID: 4.6d [1.0]; P = 0.066); weight (CON: 2.4kg [0.2], APRA: 2.2kg [0.2], TED: 2.2kg [0.2], TEDBID: 2.4kg [0.1]; P = 0.152); or small bowel length (CON: 559cm [40], APRA: 553cm [54], TED: 520cm [68], TEDBID: 588cm [26]; P = 0.097). Over the study period, weight gain was the same between all groups (CON: 1.3kg [0.3], APRA: 1.2kg [0.2], TED: 1.3kg [0.2], TEDBID: 1.3kg [0.2]; P = 0.811).

8.3.3 Fecal Fat Collections (Table 8-2)

Fat delivery was equivalent between groups, while fat absorption was not different; however, it tended to be higher in the treated groups (e.g. percentage fat absorbed: saline: 80.6% [17.1], apraglutide: 94.9% [3.1], teduglutide daily: 87.3% [16.4], teduglutide BID: 94.3% [5.1]; P = 0.134).

8.3.4 Small Intestine Linear Growth (Table 8-3)

On Day 1, after 75% bowel resection, remnant small bowel length was not different between groups [CON: 139 cm [10], APRA: 138 cm [14], TED: 134 cm [13], TEDBID: 147 cm [6]; P = 0.209) (**Figure 8-2A)**. On Day 7, the mean length of the small bowel of animals treated with

saline showed a minimal increase of +2cm [12] (or +1.5% [8.5] over remnant length). This contrasted with the increased length or linear intestinal growth noted for piglets treated with the GLP-2 analogues. Treatment with APRA resulted in a significant increase of +28cm [16] (+20.8% [12.6]), with TED the increase in length was +19cm [8] (+14.4% [6.6]) the same as with TEDBID, was +19cm [12] (+12.9% [8.1]). Neither TED nor TEDBID were different from CON or from APRA, however, APRA was superior to CON (**Figure 8-2B**).

8.3.5 Small Intestinal Mass (Table 8-3)

Absolute small bowel weight was increased by treatment with APRA compared to CON, while both teduglutide treated groups were not statistically different from either CON or APRA (CON: 26g [3], APRA: 33g [6], TED: 28g [3], TEDBID: 31g [2]; P = 0.007).

Following treatment with APRA, jejunum scraping weight was increased compared to CON, while TED and TEDBID were not statistically different between CON or other treatments (CON: 0.10g/cm [0.02], APRA: 0.13g/cm [0.03],TED: 0.11g/cm [0.03], TEDBID: 0.11g/cm [0.01]; P = 0.030).

8.3.6 Small Intestine Histology (Table 8-3)

Jejunum villus height was greater for APRA compared to CON and TED, while TEDBID was not statistically different from either CON, APRA, or TED (CON: 0.59 mm [0.08], APRA: 0.90 mm [0.13], TED: 0.58 mm [0.13], TEDBID: 0.74 mm [0.19]; P < 0.001] (**Figure 8-2C**). Jejunum crypt depth was not different between any of the treatments (CON: 0.16 mm [0.34], APRA: 0.15 mm [0.14], TED: 0.16 mm [0.19], TEDBID: 0.14 mm [0.17]; P = 0.421).

8.4 Discussion

Glucagon-like peptide 2 has become the focus for trophic therapy for SBS. GLP-2 is a 33-amino acid peptide produced in the enteroendocrine L cells that are localized primarily to the ileum and colon.[348] GLP-2 administration inhibits gastric acid secretion and motility,[349, 350] stimulates intestinal blood flow,[349, 350] increases nutrient absorptive capacity of the intestinal mucosa, and enhances crypt cell proliferation (CCP) and villus height.[348] The native 33-amino acid peptide GLP-2 has a half-life of only 7 minutes, being rapidly degraded by dipeptidyl-peptidase IV.[351] For clinical purposes, to improve the half-life of native GLP-2, amino acid or hydrophobic substitutions allow GLP-2 analogues to be poorly recognized by dipeptidyl-peptidase IV.[352] Teduglutide, differs from native GLP-2 by one amino acid and in adult humans has a half-life of 2 hours. It is the only GLP-2 analogue approved for use in patients in Canada and the US and has been demonstrated to enhance both structural and functional integrity of the remaining intestine in SBS.[187] Apraglutide differs from both native GLP-2 and teduglutide mainly by 3-amino acids, resulting in an extended half-life of 30 hours.[352] This is the first head to head study comparing the impact of the pharmacokinetic profile of these GLP-2 analogues, administered at pharmacologically comparable doses, on various structural parameters defining intestinal adaptation.

The results from the study clearly show that in an SBS neonatal animal model mimicking the common anatomy seen in infants and children, both the level and duration of exposure to a GLP-2 analogue can impact significantly on clinically relevant outcomes of intestinal adaptation, including small bowel weight, villus height, small bowel linear growth and fat absorption. It should be noted that to date only limited pharmacokinetic data are available for GLP-2

analogue studies, including in children.[192] In addition, prior studies in piglets[18] and in human infants[200] have adapted dosing and regimen of GLP-2 analogues based on preliminary data from rodents[78] or from adult human studies.[353] Similar to previous work in piglets[18] our PK data shows that teduglutide was excreted faster with a shorter half-life as compared to adult humans (approximately 30 minutes in piglets versus 2 hours in adults).[18] Similarly, the half-life of apraglutide is shorter in both neonatal (current study) and juvenile piglets as compared to adult humans (approximately 12 hours in piglets versus 30 hours in adults).[352]

The present study confirms as well that apraglutide and teduglutide, when given at appropriately comparable doses, can promote small bowel linear growth and adaptation. Previous studies of teduglutide in piglet models, using doses of 0.2 mg/kg, have shown a trend towards increased villus height, but with no statistically significant improvement and no evidence of linear intestinal growth.[18] In this study the only treatment that caused a significant increase in villus height over CON, was APRA. Previous studies of teduglutide in piglets have shown an increase in mucosal mass, but this adaptation was only seen in the ileum.[354] Within our model, these piglets had their ileum completely removed so could not be measured. Consistent with our findings regarding villus hyperplasia, APRA was also the only treatment that increased absolute small bowel weight and jejunum scraping weight over CON. Altogether, considering both the increased small intestinal length in the APRA group and the mucosal findings, greater overall benefit for structural adaptation was observed with apraglutide.

It is important to also consider the impact of structural adaptation on function. In this regard, we measured fat absorption, as we had undertaken in prior studies, using the same animal model, where APRA decreased fat losses per gram of stool compared to control.[78] While

no treatment produced a statistically significant increase in fat absorption, TEDBID and APRA showed a trend in increased fat absorption from 80.6% for CON animals, to 94.9% for APRA and 94.3% for TEDBID. Given in this experiment we only provided 20% enteral nutrition, it may be that we are not allowing for major functional differences in fat absorption to be observed as we would with increased enteral delivery that would overcome the short bowel absorptive capacity. While crypt depth did not change, it is possible that crypt proliferation was increased, as it was not specifically measured in our study design.[347] Furthermore, the short time frame in our model may have been insufficient to show changes in crypt size, which has been observed in SBS piglets studied 2-6 weeks post resection.[355]

We do recognize short study duration as one potential limitation of this research. However, due to their very rapid growth rates, short term studies in piglets translate to longer durations in humans.[2] Piglets have similar adaptive responses to humans[355] and have a similar developmental potential for gut lengthening.[78] Furthermore, the jejunocolic piglet model with 75% intestinal resection is ideal for studies of trophic therapies as it has consistently demonstrated limited potential for adaptation in the absence of such treatments.[2] Neonatal piglets with JC anatomy have had their ileum completely removed and have lower endogenous GLP-2 production, limited adaptation, and more severe intestinal failure compared to other animal models that have ileum present.[182] As most causes of neonatal SBS are associated with congenital loss or resection of the ileum and ileocecal valve, the JC model is the most clinically relevant for neonatal SBS.[2] Infants with JC anatomy have also been shown to have reduced endogenous GLP-2 production.[356]

Given the innate ability for intestinal growth in neonates and young children, we regard this outcome as important to consider; more readily done so in neonatal animal models, like the piglet. When teduglutide was previously studied in piglets there was not a statistically significant increase in small bowel length.[18] This may have related directly to the model itself (only 50% mid-intestinal resection with residual ileum), or to the doses given (from 0.01 to 0.2 mg/kg/d) or relate to the short half-life of teduglutide as the authors suggested.[18] Further studies in piglets with teduglutide, combined with EN, have shown there is a synergistic effect on markers of adaptation, including mucosal surface area and acute nutrient processing capacity, compared to either treatment or EN alone.[354] In that study, similar to our results, structural adaptation preceded functional adaptation.[354] In humans, both adults and now pediatric studies, teduglutide has been shown to improve intestinal absorption and reduce days on PN.[79, 186] However, data on structural adaptation and in particular lengthening is lacking in humans. This mainly reflects the difficulties in doing such measurements in vivo.

With increases in small bowel length, overall surface area available for absorption increases.[255] In piglets, it has been shown that this leads to increased nutrient absorption.[255] Another adaptive mechanism to increase surface area is to increase the height, width, and density of the villi.[255] Equally, an increase in villus surface area leads to an increase in mucosal surface area.[255] However, in rats given exogenous GLP-2, mucosal hyperplasia regressed with cessation of treatment.[208] In adult humans, it also appears that reduction in PN volumes may not be sustained after discontinuation of teduglutide.[209] We postulated that linear growth in neonates and young animals enhanced by GLP-2 therapies will not regress. In a follow up study when JC piglets in our laboratory were either maintained on apraglutide or teduglutide for 7

days then treatment was stopped but piglets were terminated on day 14 and compared to the current study.[276] We aimed to investigate if intestinal lengthening persists following the discontinuation of GLP-2 analogue treatment.[212] Interestingly, intestinal length was maintained with teduglutide treatment, while it was furthered with apraglutide treatment.[212] Conversely, mucosal adaptation measured by villus height was not maintained by either treatment.[212]

8.5 Conclusions

In this study, the subcutaneous GLP-2 analogues apraglutide and teduglutide, when given at pharmacologically equivalent doses, both enhanced intestinal growth and adaptation in a neonatal model of SBS with ileal resection, as compared to saline control. Insufficient dosing conferred questionable, if any, benefit of teduglutide over saline control. This is a relevant clinical issue, given the costs of these GLP-2 therapies. Apraglutide with a substantially longer half-life compared to teduglutide warrants further investigation for the pediatric population with SBS as it does not require daily subcutaneous dose administration and in this study had superior benefit, certainly for mucosal hyperplasia and potentially also for lengthening. Apraglutide has the potential to augment intestinal adaptation to a greater degree than teduglutide with less frequent injections, which is likely to be better tolerated in the pediatric population.

There is emerging interest in the development of newer GLP-2 analogues for the treatment of intestinal failure in SBS and we strongly recommend that appropriate pharmacokinetic studies be undertaken linked to key clinical outcomes, ideally including intestinal growth in neonates and young children. However, given difficulties in measuring

intestinal growth in vivo we also recognize the important ongoing role for preclinical animal studies to clarify the importance of intestinal growth in achieving autonomy from PN in pediatric SBS and in further understanding the molecular mechanisms.

Table 8-1. Comparison of Pharmacokinetic Profile Between Apraglutide and Teduglutide

	APRA n=3	TED n=3
Dose (mg/kg)	5.0	0.05
C_{max} (mg/ml)	4.1	37.1
AUC_{0-t} (mg*h/ml)	119.6	70.0
$AUC_{0-\infty}$ (mg*h/ml)	119.6	71.3
T_{max} (h)	12.0	0.5
$T_{1/2}$ (h)	12.4	0.5

Data is the mean. APRA: 5 mg/kg apraglutide administered once; TED: 0.05 mg/kg teduglutide administered once.

Table 8-2. Fecal Fat Absorption

	CON n=6	APRA n=8	TED n=7	TEDBID n=6	ANOVA p value
Fat delivery (g/kg/d)	1.99(0.11)	1.95(0.54)	1.93(0.53)	2.00(0.64)	0.994
Fat absorbed (g/kg/d)	1.61(0.39)	1.86(0.55)	1.74(0.63)	1.90(0.62)	0.801
% Fat absorbed (%)	80.6(17.1)	94.9(3.1)	87.3(16.4)	94.3(5.1)	0.134

Data is the mean and (standard deviation). CON: saline; APRA: 5 mg/kg apraglutide twice weekly; TED: 0.05 mg/kg daily teduglutide; TEDBID: 0.05 mg/kg teduglutide twice daily.

Table 8-3. Structural Adaptation

	CON n=8	APRA n=8	TED n=8	TEDBID n=7	ANOVA p value
Baseline SBL (cm)	559(40)	553(54)	520(68)	588(26)	0.097
Remnant SBL (cm)	139(10)	138(14)	134(13)	147(6)	0.209
End SBL	141(15)[a]	166(14)[b]	153(13)[a,b]	165(13)[b]	0.004
SB weight (g)	26(3)[a]	33(6)[b]	28(3)[a,b]	31(2)[a,b]	0.007
SB weight (g/kg)	11.0(1.2)[a]	14.8(2.4)[b]	12.6(1.3)[a]	12.8(0.6)[a,b]	0.001
Jej weight(g)/cm	0.10(0.02)[a]	0.13(0.03)[b]	0.11(0.03)[a,b]	0.11(0.01)[a,b]	0.030
Villus height (mm)	0.59(0.08)[a]	0.90(0.13)[b]	0.58(0.13)[a]	0.74(0.19)[a,b]	<0.001
Crypt depth (mm)	0.16(0.34)	0.15(0.14)	0.16(0.19)	0.14(0.17)	0.421

Data is the mean and (standard deviation). CON: saline; APRA: 5mg/kg apraglutide twice weekly; TED: 0.05 mg/kg daily teduglutide; TEDBID: 0.05 mg/kg teduglutide twice daily. Superscripts denote post hoc tukey significant differences. Jej, jejunum; SB, small bowel; SBL, small bowel length.

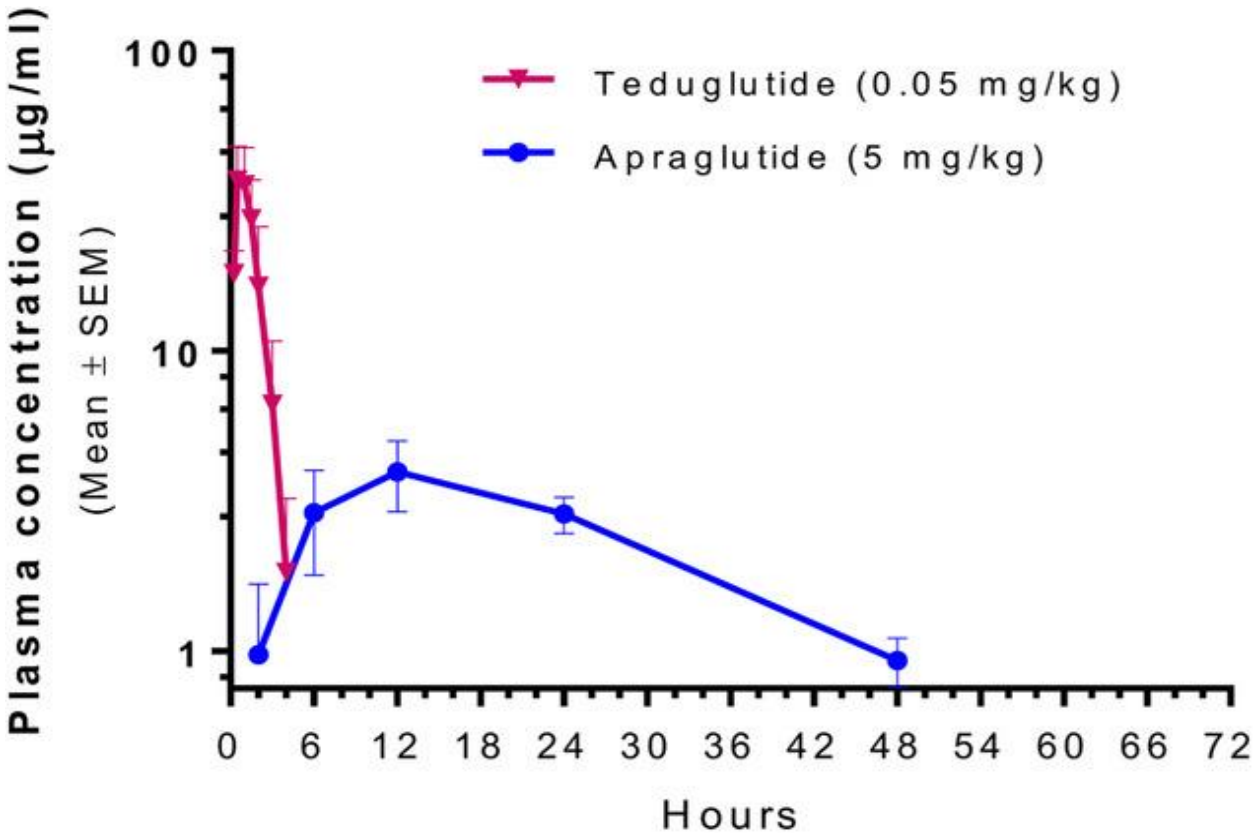
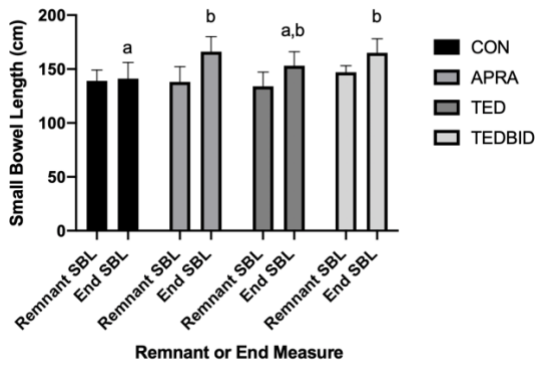
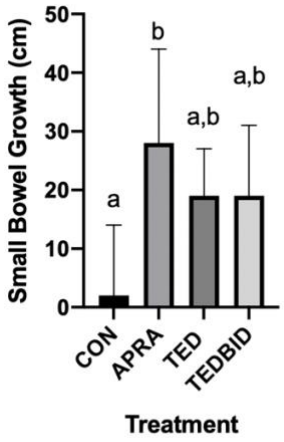


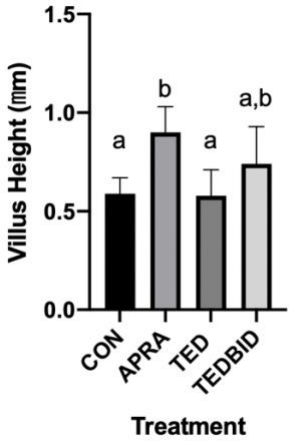
Figure 8-1. Plasma Concentration of Apraglutide and Teduglutide Following Subcutaneous Administration.


Small Bowel Length (cm)
200
150
100
50
0
a
b
a,b
b
CON
APRA
TED
TEDBID
Remnant SBL
End SBL
Remnant or End Measure

(a)


Small Bowel Growth (cm)
50
40
30
20
10
0
a
b
a,b
a,b
CON
APRA
TED
TEDBID
Treatment

(b)


Villus Height (mm)
1.5
1.0
0.5
0.0
a
b
a
a,b
CON
APRA
TED
TEDBID
Treatment

(c)

Figure 8-2. Gross Intestinal Morphology.

(a) Remnant versus Post-Treatment Intestinal Length:

The length of the small bowel (cm) between GLP-2 analogue treatment groups before and after treatment are presented. CON: saline; APRA: 5mg/kg apraglutide twice weekly; TED: 0.05 mg/kg daily teduglutide; TEDBID: 0.05 mg/kg teduglutide twice daily. A and B superscripts denote post hoc Tukey significant differences. SBL, small bowel length.

(b) Change in Intestinal Length:

The linear growth of the small bowel (cm) between GLP-2 analogue treatment groups are presented. CON: saline; APRA: 5mg/kg apraglutide twice weekly; TED: 0.05 mg/kg daily teduglutide; TEDBID: 0.05 mg/kg teduglutide twice daily. A and B superscripts denote post hoc tukey significant differences.

(c) Jejunum Villus Height:

The mean villus height of jejunum (mm) between GLP-2 analogue treatment groups are presented. CON: saline; APRA: 5mg/kg apraglutide twice weekly; TED: 0.05 mg/kg daily teduglutide; TEDBID: 0.05 mg/kg teduglutide twice daily. A and B superscripts denote post hoc Tukey significant differences.

Chapter 9. Independent of its Potential Role in Glucagon-like Peptide-2 Signaling, Insulin-like Growth Factor-1 Therapy has No Additional Benefit for Intestinal Adaptation in Short Bowel Piglets

9.0 Abstract

Background: Short bowel syndrome (SBS), has prompted development of glucagon-like peptide-2 (GLP-2) therapy with the goal of autonomy from parenteral nutrition (PN). Insulin-like growth factor-1 (IGF-1), an intestinotrophic factor present in mammalian milk, has been proposed to be a key mediator of GLP-2 action. Therefore, in neonatal piglets with SBS, we explored the potential for combined therapy with GLP-2 and IGF-1.

Methods: Following 75% distal small intestinal resection, neonatal piglets receiving 20% enteral nutrition and 80% TPN were allocated to four treatments: saline (CON: n=10), twice daily teduglutide given subcutaneously (TED, 0.05mg/kg/dose n=9), IGF-1 given via gastric tube in three divided doses (IGF, 200μg/Kg/day: n=11), and teduglutide and IGF-1 combined (TED/IGF: n=10). Piglets were maintained for 7 days. At termination small intestinal length and weight were measured and jejunal tissue collected for histology. Intestinal permeability was measured via Üssing chamber and gene expression via real-time quantitative PCR. Data are analyzed via one-way ANOVA with post-hoc Tukey for comparisons between all groups where appropriate or Kruskal-Wallis.

Results: There was no difference between groups in piglet age at surgery (p=0.7), weight at surgery (p=0.06), pre-resection intestinal length (p=0.5) or post-resection length (p=0.5). Intestinal lengthening was only observed with TED and TED/IGF compared to IGF or CON (p=0.001). Small bowel weight (g) was increased for TED compared to CON, with IGF and IGF/TED not different (p=0.007). Small bowel weight (g/kg) was increased for TED and TED/IGF compared to CON and IGF (p<0.001). There was no significant impact for any of the treatments on mucosal-to-serosal permeability of mannitol (p=0.7) or polyethylene glycol (0.5). There were

no differences in gene expression of tight junction proteins, IGF-1 or its receptor, or GLP-2 receptor between treatment groups. IGF treatment alone increased the expression of proglucagon (*GCG*) compared to all groups (p=0.029).

Conclusion: At the dose we studied, IGF-1 did not seem to have independent benefits for mucosal hyperplasia or to have a role in intestinal lengthening. Furthermore, there was no apparent synergistic action when combining both treatments, at the doses tested. It was noted that IGF-1 treatment was associated with an increase in tissue proglucagon (*GCG*) expression. Therefore, it is plausible that the benefit of IGF-1 treatment on mucosal hyperplasia is exerted, at least in part, by increased tissue release of the proglucagon-derived hormones, and its actions are entirely mediated via GLP-2.

9.1 Introduction

Short bowel syndrome (SBS), is the leading cause of pediatric intestinal failure, leading to dependence on parenteral nutrition (PN) for a minimum of 60 days.[7] Historically, SBS had a 25-50% mortality rate; this has improved significantly due to multidisciplinary care teams, and new lipid strategies to minimize liver disease, along with trophic factors to promote adaptation.[3, 341-343] Adaptation is a compensatory process, with both structural and functional changes to improve nutrient absorption, including lengthening of the small intestine, mucosal hyperplasia, bowel dilation, slowing of motility and increased nutrient transporter expression.[344] These changes aid in decreasing PN volume while increasing nutrient absorption in the remnant intestine, aiming for enteral autonomy in these patients and decreasing the long term risks of PN, including sepsis, cholestasis, health care expense, and negative impact on the child and family's quality of life.[1]

Glucagon like peptide-2 (GLP-2) is the first clinically available trophic factor for SBS, with teduglutide (Gattex®/Revestive®; Takeda, Japan), a once daily subcutaneous (sc) injection (0.05 mg/kg sc) recently being approved for adults and in children 1 year of age or older with SBS in Canada and the US.[186] The primary endpoint of these studies has been a ≥20% reduction in PN volume, which has been shown to occur in both pediatric patients,[201, 202, 204] and adults.[195, 198, 199] Indeed, 12% of children will achieve autonomy[201] with GLP-2 therapy in the short term and up to 32% in the longer term.[204] These studies are impacted by the anatomy of SBS being treated, different SBS anatomies will benefit to different degrees with GLP-2 therapy.[357]

Studies into the actions of GLP-2 analogues in animals have shown that other intestinal peptides are necessary for the actions of GLP-2 and suggest that combination therapies may be

more effective than GLP-2 alone.[235, 358, 359] Previously in our laboratory we investigated epidermal growth factor (EGF), which has a significant role in growth, development and maturation of the gastrointestinal tract.[236] We found that, in neonatal piglet models of different SBS anatomies, EGF alone increased bowel weight per length and villus height in piglets with ileum present, but not without ileum present.[235] However, notably, GLP-2 and EGF had synergistic effects, especially on decreasing intestinal permeability in both models, with or without ileum.[235]

Insulin-like growth factor-1 (IGF-1) is one of the most abundant hormones in human milk and colostrum and, importantly, the placenta secretes IGF-1 and the levels increase in the infant at birth.[230] IGF-1 is a vital trophic factor for intestinal maturation, and increases during the last trimester when there is an exponential increase in gut growth.[230] IGF-1 has been shown to be a mediator of the effects of GLP-2, as mice null for IGF-1 were unresponsive to the expected intestinal growth and barrier effects of GLP-2 observed in wild type mice.[229, 234] IGF-1 is expressed in the subepithelial myofibroblasts which express the GLP-2 receptor, with the IGF-1 receptor being present in the epithelial crypt cells, which are the drivers of villus lengthening in adaptation.[213, 360, 361] GLP-2 has been shown to stimulate the production of IGF-1 from these subepithelial myofibloblasts.[213, 229, 358, 361] Therefore, it is proposed that GLP-2 stimulates the production of IGF-1 by intestinal subepithelial myofibroblasts and that the trophic effects of GLP-2 rely on the downstream signalling via the IGF-1 receptor.[362]

It is clearly relevant to study the role of IGF-1 treatment in addition to GLP-2 treatment on intestinal adaptation and to propose that there may be synergistic action. However, to date there has been conflicting data from prior studies looking at IGF-1 treatment in SBS, somewhat

confounded by different doses and routes of delivery. Furthermore, no prior studies have examined IGF-1 treatment in combination with GLP-2 in the setting of SBS. Hence, the aim of this study was to compare the effects of enteral IGF-1 alone or in combination with GLP-2 analogue treatment on structural and functional adaptation in our neonatal piglet model of SBS without ileum.

9.2 Methods

All experiments were conducted in accordance with the guidelines of the Canadian Council of Animal Care at the Swine Research and Technology Centre (SRTC) (AUP00000155). Newborn male Duroc cross piglets were obtained from the SRTC and allocated to the following treatments: saline control (3mL given via the gastric tube 3 times daily), teduglutide 0.05 mg/kg/dose subcutaneously (sc) twice daily, IGF-1 200μg/kg/d divided into three doses per day given via the gastric tube, or GLP-2 and IGF-1 given in combination at the same doses as the other treatments. Teduglutide was manufactured by Toronto Research Chemicals Inc. (Toronto, Canada), *E. coli* derived recombinant human IGF-1 was produced by R&D Systems (Minneapolis, USA). GLP-2 treatments commenced immediately post operatively, and IGF-1 and saline started on day 2 post operative when enteral nutrition (EN) commenced.

As we have previously reported, on day 0 under general anesthesia, a 5-French central venous catheter was placed for PN delivery.[270] All piglets had a laparotomy to measure the small-intestine length using a 3-0 silk suture from the Ligament of Treitz to the cecum along the antimesenteric border with minimal traction.[270] Piglets underwent a 75% distal small intestinal resection including the ileocecal valve (ICV), cecum and first few centimetres of colon, with a

resulting jejunocolic anastomosis (JC). A 10-Frech Stamm gastrostomy tube was placed into the body of the stomach for trophic EN.[270] Prophylactic parenteral antibiotics were given preoperatively as a single dose: ampicillin (Sandoz, Boucherville, QB, Canada) and florfenicol (Intervet Canada Corp., Kirkland, QB, Canada) and 4% tetrasodium-EDTA (Kitelock™, 1.5 mL) was used as a prophylactic line lock for prevention of central line associated bloodstream infections (CLABSI) and catheter occlusions as we have shown (submitted and under revision).

Piglets were housed for 7 days in metabolic cages, in a heat controlled room with a 12 hour light cycle, and daily care was completed as previously reported.[270] Nutrition commenced immediately postoperatively with PN formulated for these piglets.[273] From day 2, PN was reduced to 80% of target rates until end of study to avoid excessive fluid retention and edema. On day 2, EN was commenced at 20% to reach target nutrient requirements and to support adaptation.[270] If sepsis was suspected with fever, lethargy and/or vomiting, piglets were treated with ampicillin and baytril (5mg/kg/day intravenous (iv); Bayer Animal Health Mississauga, ON, Canada) and/or gentamicin (3mg/kg 3 times a day iv; Sandoz, Boucherville, QB, Canada).

9.2.1 Justification of Dose of IGF-1

The dose of IGF-1 was based on targeting a dose within the range of that delivered in swine colostrum. Neonatal pigs should ideally drink around 300mls of colostrum in the first 24 hours, containing a range of 500-1000 μg/L IGF-1, estimating an IGF-1 intake in the range of 150-300 μg.[363, 364]

9.2.2 Tissue Collection and Assessment of Structural Adaptation

On Day 7, piglets underwent general anesthesia and the small intestine was measured in length along the antimesenteric border as done at baseline surgery. Following euthanasia, the small intestine was removed and emptied of its contents, and the weight was measured. A

mucosal scraping from a 20-cm segment of proximal jejunum was taken and weighed. Jejunal tissue 20 cm distal to the ligament of Treitz was preserved in 10% formaldehyde for histology. Jejunal segments 5 cm from the distal anastomosis were flash frozen in liquid nitrogen and stored at -80oC for gene expression analyses.

Paraffin mounted 5-μm sections of jejunum were stained with hematoxylin and eosin prior to assessment using a micrometer eyepiece (Nikon Eclipse 80i; Nikon, Tokyo, Japan). Mucosal hyperplasia was assessed by 10 measurements of villus height and crypt depth from longitudinal sections by a board-certified animal pathologist (P. N. Nation).

9.2.3 Intestinal permeability

Using a modified Üssing chamber (Harvard Apparatus, Holliston, MA) procedure, as previously described,[235, 292] intestinal paracellular transport of radiolabeled mannitol and polyethylene glycol (PEG) was determined from jejunal segments taken 20cm distal to the ligament of Treitz, placed in Krebs's buffer for immediate transport and mounting on the Üssing chambers within one hour. Each segment was mounted on a segment holder and that is connected to the modified transport chamber.[293] This classing Üssing chamber tissues are mounted onto and compressed between two chamber halves. This modified system allows the investigation of multiple tissue types.[293] The apparent permeability coefficients (P_{app}, cm/s) were calculated at steady state by $P_{app} = dQ/dt \times [1/(A \times C_0)]$, where dQ/dt is the appearance rate of radiolabeled marker in the receiver chamber, A is the exposed surface area of the intestine, and C_0 is the initial concentration in the donor chamber.[235] Transepithelial electrical resistance (TEER) was calculated by the spontaneous transepithelial potential difference (PD) and the short-circuit current (I_{SC}) to reduce the PD across the tissue, as previously described.[235, 292]

9.2.4 Reverse-transcriptase Semi-quantitative Real-time PCR

Expression of genes related to growth factors, their receptors and tight junction proteins was assessed using total RNA isolated (RNeasy with QiaShredder; Qiagen, Toronto, ON, Canada) from the segments of jejunum taken 5cm from the jejunocolic anastomosis, and underwent reverse transcription (All-In-One Reverse Transcriptase MasterMix; Applied Biological Materials, Richmond, BC, Canada). Real-time semiquantitative PCR was performed using TaqMan Fast Advanced Master Mix (ThermoFisher Scientific, Mississauga, ON, Canada) using the primers (Life Technology, Burlington, ON, Canada) listed in **Table 9-1**. Relative mRNA expression was quantified using the delta-delta cycle threshold method[295] with 18S rRNA as the control, which has been previously validated as a stable porcine intestinal tract reference gene.[235, 298]

9.2.5 Statistical Analysis

Normally distributed data are expressed as mean and standard deviation; non-parametric data are expressed as median and interquartile range. A One-way ANOVA was performed with a Post hoc Tukey test for all comparisons of parametric data and a Kruskal-Wallis and Mann-Whitney U test for non-parametric data comparisons. Statistical tests were performed using SPSS (version 28; SPSS Inc, and IBM Company, Chicago, IL, USA). Differences were considered significant at $P<0.05$.

9.3 Results

9.3.1 Animal Outcomes

In total, 43 piglets were entered into the study and were randomized to the 4 groups:(control (CON, n=;10); GLP-2 analogue (TED, n=10); enteral IGF-1 (IGF, n=12); and IGF-1 in combination with GLP-2 (IGF/TED, n=11). Forty piglets reached the endpoint; 2 piglets did not complete the study due to protocol compliance errors (1 IGF/TED and 1 TED) and 1 because of an obstructive bowel adhesion (IGF). At baseline, piglets were not different in age (days) [CON: 3.8 (0.63), TED: 4.1 (0.78), IGF: 3.7 (1.2), IGF/TED: 3.6 (1.1); p=0.7], weight (kg) [CON: 2.4 (0.12), TED: 2.4 (0.16), IGF: 2.4 (0.18), IGF/TED: 2.3 (0.10); p=0.06], total small bowel length (SBL) (cm) [CON570 (46), TED: 583 (35), IGF: 601 (45), IGF/TED: 581 (61); p=0.5], or post-resection SBL (cm) [CON: 142 (11), TED: 145 (9), IGF: 150 (11), IGF/TED: 145 (15); p=0.5]. There was no difference in weight at the end of the study (kg) [CON: 3.6 (0.20), TED: 3.5 (0.35), IGF: 3.6 (0.29), IGF/TED: 3.3 (0.19); p=0.07].

9.3.2 Structural and Functional Adaptation

A significant impact of treatment on linear small bowel growth was found at termination, with increased SBL in both the TED and IGF/TED groups as compared to CON and IGF alone (p=0.001) (**Figure 9-1**.). No effect of IGF alone was found as compared to CON, and TED increased SBL (cm) to the same extent as IGF/TED treatment. Small bowel mass corrected for total body weight (g/kg) was also increased by treatment with TED and IGF/TED as compared to CON and IGF (p<0.001). There was no difference between any of the groups for jejunum scraping weight (p=0.1), villus height (p=0.2)and crypt depth (p=0.3) (**Figure 9-1.**). Finally, there was no impact of treatment on either measure of mucosal to serosal (M-to-S)

permeability mannitol (p=0.7) and Peg (p=0.5)] (**Table 9-2.**). Validity of permeability measurements was confirmed by measurement of transepithelial electrical resistance (TEER) in a subset of animals where PD >2mV confirmed tissue viability.

9.3.3 Gene expression

There was a significant impact of treatment on *GCG* expression with increased expression in the IGF treatment group as compared to CON, TED and IGF/TED (p=0.029) (**Figure 9-2.**). There was no impact of any of the treatments on the other transcripts for other intestinal growth factors [*IGF* (p=0.1), *EGF* (p=0.3)], their receptors [*GLP2R* (p=0.1), *GLP1R* (p=0.4), *IGF1R* (p=0.09), *EGFR* (p=0.1),] or tight junction proteins [*CLD1* (p=0.6), *OCLN* (p=0.2)].

9.4 Discussion

In this preclinical piglet model of SBS with 75% distal bowel resection and jejunocolic anastomosis, we investigated the efficacy of IGF-1 treatment alone and in combination with GLP-2 analogue treatment using teduglutide. Confirmatory of our previous studies,[212, 276] the GLP-2 analogue increased structural adaptation, measured via increased linear gut growth and increased small bowel weight, both with and without IGF-1. Treatment with IGF-1 alone had no significant impact on any measures of structural adaptation. Neither TED nor IGF-1 combined or alone had a significant impact on intestinal permeability, measured using both mannitol and PEG. While TED impacted structural adaptation, it had no effect on expression of intestinal growth factors or their receptors, or of tight junction proteins. However, IGF-1 significantly increased *GCG* expression over all other groups, notable as this is the gene encoding proglucagon, the precursor for GLP-2 (and GLP-1) release by the intestinal endocrine L cell.

The piglets used in the current study represent type 2 anatomy of SBS, the most common anatomy of pediatric SBS, given most causes of neonatal SBS are associated with congenital loss or resection of the ileum and ICV.[2] In contrast, most animal models investigate type 3 SBS anatomy, which has some remnant ileum still present and a jejunoileal anastomosis, and affords patients the most optimal outcomes.[8] The JC model was used here as these piglets and also patients[356] have been shown to have reduced endogenous GLP-2 production given type 2 anatomy.[2] As this anatomy has limited endogenous production of GLP-2, we believe it is ideal to study GLP-2 replacement therapies or other trophic therapies with potential to work in concert with or independent of GLP-2.

It has been hypothesized that GLP-2 exerts its trophic effects through a paracrine or neural pathway because the GLP-2 receptor has been localized to gut endocrine cells present in the small bowel and colon, yet these cells do not include the epithelial cells that undergo proliferation in response to GLP-2.[365, 366] The GLP-2 receptor is expressed in the subepithelial myofibroblasts that underlie the epithelium, as well as in scattered enteroendocrine cells and neurons of the enteric nervous system.[365] Therefore, it is postulated that GLP-2 exerts its intestinotrophic effects via release of downstream mediators from the GLP-2 receptor.[365] Some hormones of interest include IGF, EGF and vasoactive intestinal polypeptide.[365] We and others[367] have postulated that IGF-1 may mediate the downstream effects of GLP-2 because studies in IGF-1 as well as in intestinal epithelial IGF-1 receptor[368] knockout mice have shown they were unresponsive to the growth effects of GLP-2 in both the small and large intestine.[229] With this, GLP-2 increased expression of IGF-1 and IGF-1 secretion in intestinal cultures, and further increased expression of IGF-1 in mouse small intestine.[229] Additionally, co-

administration of GLP-2 and IGF-1 together has been shown to induce greater trophic effects than those of GLP-2 alone.[369] However, of note, these were not SBS models, potentially explaining the differing results seen in healthy or sham rodents versus SBS piglets.[229, 234, 235]

Indeed, in our SBS model, we did not find that IGF-1 had any independent or synergistic action with GLP-2 on intestinal adaptation. We did find that IGF-1 alone but not in combination with TED increased expression of *GCG* compared to all treatment groups. This gene encodes proglucagon, the parent hormone for proglucagon-derived peptides, including GLP-1 and GLP-2.[370] Our study does not rule out that IGF-1 has a key role in GLP-2 release nor in downstream signaling, but does not suggest that this signaling is increased by adding more exogenous IGF-1. Furthermore, in the absence of exogenous GLP-2 treatment, there may be a role for IGF-1 signaling to increase endogenous GLP-2 release, plausible given the increase in *GCG* was observed only in the absence of GLP-2 provision. To clarify it would have been helpful if we had also studied our SBS model with the jejunoileal (JI) anatomy that has remnant ileum, to determine if exogenous IGF-1 treatment with upregulation of *GCG* could lead to an increase in circulating GLP-2 over that of untreated piglets.

The concentration of IGF-1 in colostrum is 5 times higher than human milk supporting an important role in early gut development that has led us and others to postulate a potential role in SBS treatment. In 7 day old piglets with surgical SBS with a 75% resection, it was shown, similar to our findings, that giving formula with added IGF-1 rich colostrum did not increase intestinal adaptation over placebo formula.[371] These piglets had a JI anastomosis and the bovine IGF-1 was dosed at 0.2 μg/mL or 25 μg/kg/d, comparing to our dose of 3.3 μg/ml or 200

μg/kg/d.[371] Interestingly, IGF-1 is 25 times higher in colostrum at birth of piglets than one week later in term sow milk, with IGF-1 levels peaking around day 4.[371-373]

Conversely, findings for IGF-1 treatment in animal models without SBS have been more positive. Adding recombinant human IGF-1 to formula for neonatal piglets without intestinal resection in the first 4 days of life did increase small intestinal weight, villus height, protein and DNA content.[374] These differing results from the previous study in SBS piglets,[371] as these were gut intact piglets that had never suckled and were treated for a shorter period of time.[374] The recombinant IGF-1 was dosed at 3.5 mg/kg/d.[374] In mice on TPN with mucosal atrophy given recombinant IGF-1 in TPN at a dose of 2.5 mg/kg/d, IGF-1 administration was able to prevent mucosal atrophy from TPN, along with had an increase in IGFBP-5.[233] In preterm piglets given sc injection of recombinant IGF-1 for 8 days at a dose of 2.25 mg/kg/d, there was increased systemic IGF-1 levels and increased intestinal weight and protein synthesis.[375] Finally, in premature infants without intestinal surgery given standard formula or formula supplemented with IGF-1 at a concentration twice that of human colostrum, gut permeability was measured via lactulose/mannitol excretion.[376] Bovine IGF-1 was dosed at 10 μg/100 mL of formula.[376] There was significantly decrease in gut permeability at 14 days of treatment for those infants given the formula supplemented with IGF-1.[376] The results from these later studies, without SBS, do not align with our findings where in an SBS late preterm model we saw neither intestinal adaptation or altered permeability with enteral IGF-1 supplementation.

In healthy rodents, GLP-2 has been shown to decrease intestinal permeability and increase the expression of several tight junction proteins through an IGF-1R dependent mechanism.[234] In our previous study, similar to this study, GLP-2 treatment alone did not

impact intestinal permeability,[235] consistent with the present Üssing findings and no changes in tight junction protein expression with GLP-2 treatment. In contrast, GLP-2 in combination with EGF decreased intestinal permeability in both a JI and JC model while, In the sham piglets, EGF alone decreased intestinal peremeability.[235] While we saw no significant impact of IGF-1 alone or in combination with GLP-2 on permeability in this study, we did not have a sham model and only investigated the JC model. Whether the presence of ileum would have changed these results will require future investigation. These results could also be hampered by the fact we could only perform Üssing analyses on a subset of the animals, due to the high cost and length of time to perform, along with Üssing requiring viable tissue harvested and then transported to be performed immediately. Unfortunately, due to a mechanical problem we also were not able to assess electrical activity TEER in all piglets in this study to ensure every sample analyzed had viable tissue.

This study has some limitations. Firstly, it was of short duration and it is possible that treatment-related differences would be seen with a greater length of study or with increased animal numbers. However, longer study periods increase both animal morbidity and risk of mortality, as well as research costs. We also acknowledge that while we observed increased small bowel length and weight, we did not find changes in villus height, arguably the gold standard for assessing structural adaptation. However, there was an average increase in villus height with TED treatment over control of 22% and for TED over IGF of 19%. We anticipate that while numerically small, such increases in average individual villus height could have clinical significance when multiplied over the length of the intestine. We did not examine changes in levels of the IGF binding proteins (IGFBP), that act as reservoirs for both paracrine and

circulating IGF-1 to prolong its half-life.[377] These IGFBPs could increase or decrease cIGF-1 signalling and, therefore, the downstream intestinotrophic effects.[377] IGFBP-4 has been shown to bind IGF-1 and have a negative effect on basal intestinal growth, but exerts a positive effects on the intestinotrophic actions of GLP-2.[377]

Finally, it is plausible that our study was underdosed. The dose of recombinant human IGF-1 studied in preterm piglets and the effective range of doses throughout the literature studied in piglets, ranges from 50-3500 μg/kg,[378-384], with some of these doses clearly being supraphysiological.[385] We aimed to study a dose within the range of sow colostrum, but did not intend to study more supraphysiological doses.[230, 386, 387] However, we acknowledge our dose was in the mid-range of sow colostrum and regardless that some of the literature indicates supraphysiological doses may exert greatest trophic effect.[385] However if such a strategy would be a safe or cost effective approach in humans is arguable, particularly when GLP-2 treatment that is already very expensive.[374, 385]

9.5 Conclusion

While GLP-2 analogues have drastically impacted the clinical care and outlook for patients with SBS, allowing weaning from TPN and in some cases enteral autonomy, other trophic factors that are important for gut maturation could offer the potential alone or in combination with GLP-2 to further modulate adaptation. This is a reasonable goal given current studies in pediatrics suggest at best 12% of children will achieve autonomy[201] with GLP-2 therapy in the short term and up to 32% in the longer term.[204] Our study did not demonstrate a significant benefit of IGF-1, alone or in combination with GLP-2, for structural or functional

adaptation. Confirmatory of our prior studies and the literature, the GLP-2 analogue teduglutide significantly increased mucosal hyperplasia and linear gut growth. Regardless, this study does not negate the physiological role of IGF-1 in GLP-2 signaling. The impact of exogenous IGF-1 therapy on the expression of *GCG* encoding the proglucagon parent prohormone is noteworthy.

This study and our prior study of EGF in combination with GLP-2 highlight the complexity of the relationship between different trophic factors in gut development and adaptation, and how different endpoints can be impacted by different trophic factors alone or in combination, depending on remnant intestinal anatomy. We believe there is benefit in continuing to study trophic factors in combination, to both improve the clinical utility of GLP-2 therapy and ideally to reduce the high costs of GLP-2 alone. However, this current study does not show benefit of the addition of IGF-1 to GLP-2 analogue treatment for adaptation in neonatal piglets with SBS without ileum.

Table 9-1. Primers used for PCR

Gene name	**Assay ID**
GCG	Ss03384069_u1
GLP2R	Ss04322851_m1
GLP1R	Ss04953518_g1
IGF1	Ss03394499_m1
IGF1R	Ss03394281_m1
EGF	Ss03391285_m1
EGFR	Ss03393423_u1
OCLN	Ss03377507_u1
CLDN1	Ss03375708_u1
18S	Hs99999901_s1

Table 9-2. Functional Adaptation via Üssing Chamber

	CON (n=5)	TED (n=6)	IGF (n=5)	IGF/TED (n=5)	P Value
Mannitol (Papp, cm x 10^{-6})	13.8 (12.1)	6.1 (12.7)	5.8 (10.0)	12.9 (12.4)	0.7
PEG (Papp, cm x 10^{-6})	1.2 (2.1)	2.1 (2.7)	1.9 (2.0)	3.9 (4.4)	0.5

Jejunal permeability. Jejunal mucosal-to-serosal (M-to-S) permeability of mannitol and PEG. Papp, apparent permeability. PEG, polyethylene glycol. Data are shown as median (interquartile range) and were analyzed via Kruskall-Wallis test.

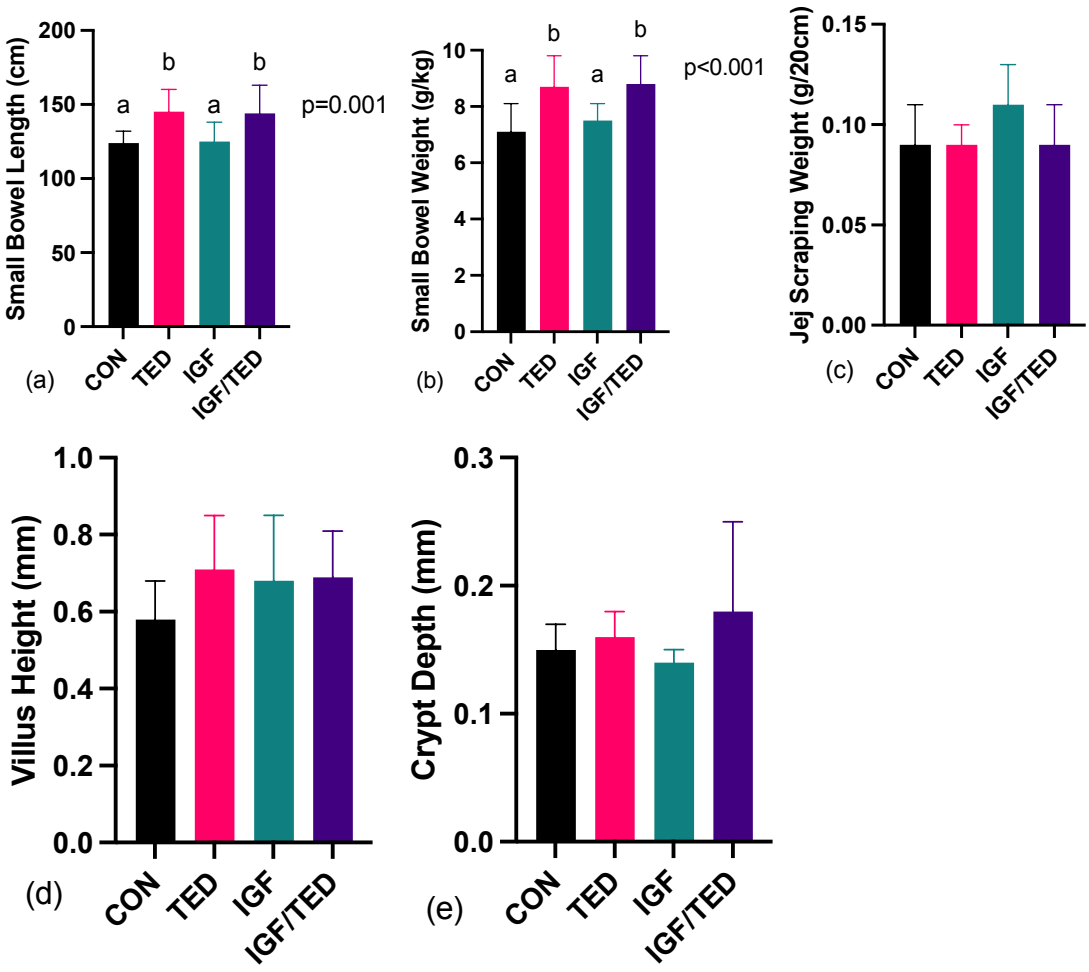


Figure 9-1. Structural Adaptation.

Small bowel length (a), small bowel weight adjusted for body weight (b), jejunum scraping weight (c), villus height (d) and crypt depth (e). Data is mean (standard deviation). Data is analyzed via one-way ANOVA, analysis of variance with post hoc tukey.

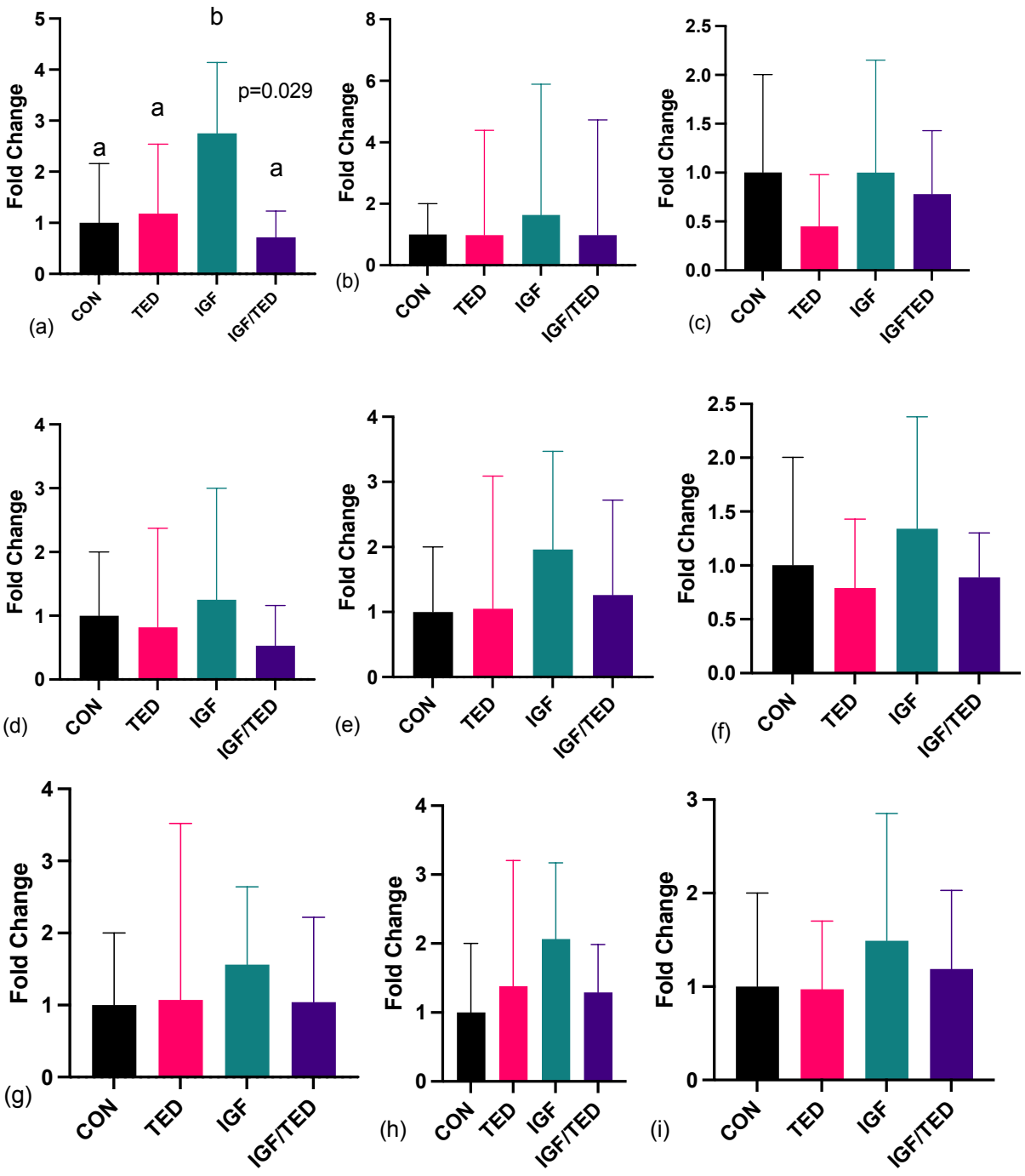


Figure 9-2. Intestinal Gene Expression of Growth Factors, their Receptors and Tight Junction Proteins.

Gene expression of *GCG* (a), *GLP2R* (b), *GLP1R* (c), *IGF1* (d), *IGF1R* (e), *EGF* (f) *EGFR* (g), *OCLN* (h), *CLDN1* (i). Data are adjusted to fold change of control. Data are shown as mean and standard deviation, and were analyzed via Kruskal-Wallis test.

Chapter 10. Trophic Factors, Sepsis and the Microbiome in Short Bowel Syndrome: Conclusions and Future Directions

Short bowel syndrome is the leading cause of intestinal failure in infants, where individuals are unable to absorb sufficient nutrients for survival and growth.[2] SBS traditionally had a 25-50% mortality rate in infants dying from complications of parenteral nutrition.[2] Over the last 25 years this has drastically changed due to multidisciplinary IF teams, changes in PN, home parenteral nutrition, and antibacterial locks.[357] Now the focus for SBS research has shifted towards ways to improve quality of life and decrease sepsis in these patients.[357]

In order to improve quality of life, weaning from PN, also called enteral autonomy, should be the goal of every IF treatment program. In SBS, this is achieved through gut adaptation. Gut adaptation is a compensatory process that involves both structural and functional changes in the intestine. This includes lengthening of the gut, increasing villus height and bowel dilation. As well as, slowing of motility and increased nutrient transporter expression. All these changes ultimately enhance nutrient absorption, allowing weaning off PN. The process of adaptation takes months to years. Children receiving PN often receive it 7 nights a week, negatively impacting their own and their caregivers sleep and quality of life.[357]

Every time a child with SBS becomes septic, this negatively impacts their enteral tolerance and hence their gut adaptation, ultimately the only way they can wean off PN.[4] Sepsis in SBS children comes from central line associated bloodstream infections, from line contamination and potentially gut bacterial translocation. Bacterial translocation occurs across an unhealthy mucosa and has been shown in both infants and animal models with SBS.[44, 147] Ultimately, CLABSI risk can only be eliminated if the patient is able to achieve enteral autonomy and get off of PN, but the risk can be reduced with antibacterial locks. Antibacterial locks, like 70% ethanol have been used, but product availability and catheter patency issues have led to

the development of new options like Kitelock™, a 4%-tetrasodium EDTA solution.[312] Kitelock™ use in children has been shown to significantly decrease CLABSI.[312]

Many patients with SBS suffer intestinal microbiome dysbiosis as a result of prematurity, prolonged hospitalization, recurrent antibiotics and altered intestinal anatomy. In infants it has been shown that microbial dysbiosis in the small bowel can negatively impact adaptation and weaning from PN.[155, 388] Probiotics have been suggested as a way to modify the microbiome in SBS but there have been few studies to date with mixed results. Probiotics have shown a positive impact on nutritional status and growth.[163]

Recently, glucagon-like peptide-2 analogues have gained interest as trophic therapies aiming to improve adaptation in the management of SBS.[79] Teduglutide is a GLP-2 analogue approved for adults and pediatrics ($\geq$1 year of age) in the US and Canada. A 24 week study in children who entered the study with little to no ability to reduce PN had a reduction in PN by at least 20% in 68% of patients.[201, 357] Apraglutide is a newer GLP-2 analogue that has a longer half-life than teduglutide.[389] Our studies into teduglutide and apraglutide in a neonatal piglet model have shown both analogues can enhance intestinal growth and adaptation, with apraglutide having superior benefit on mucosal hyperplasia.[390] Although this study was unable to show a functional benefit,[390] our previous study with exogenous GLP-2 in SBS piglets allowed fewer days on PN,[184] which is an aim of human pediatric studies.[200, 201] Insulin-like growth factor-1 is hypothesized to mediate the trophic response to GLP-2 treatments.[229] Our collaborators have found in mice that GLP-2 increases IGF-1 expression and that IGF-1 is required to mediate the response to GLP-2.[229]

In this thesis we aimed to study novel treatments that ultimately if translated to human infants would improve quality of life and reduce sepsis. We used our neonatal piglet model of SBS that we believe to be the most valid translational model for human infants currently widely available.

In chapter 6, we studied the impact of 4%-T EDTA use retrospectively after this refinement to our animal protocols was initiated in our laboratory. We analyzed this in both our TPN and SBS piglet animal models. There was a significant difference in culture confirmed sepsis with the use of the locking solution for both TPN and SBS piglets. As well, although we did not reach statistical significance for line replacements or occlusions, there were no catheter replacements needed for occlusions in either surgical group of piglets with the use of the locking solution. While not statistically significant, largely due to the small animal numbers, this meant there was a 58% cost reduction for TPN piglets and a 16% cost reduction for SBS piglets. This greater reduction in cost with the use of T-EDTA over a longer period is important, as typically we see sepsis starting after day 7. This reduction in line replacement and culture costs shows the significant impact on our laboratory to reach longer experimental endpoints and to reduce antibiotic use, a confounder in microbiome experiments. Sepsis is a clear confounder of key outcomes we seek to study, including intestinal failure associated liver disease, inflammation and the microbiome. The use of this locking solution has allowed us to not use antibiotics in studies up to 10 days long, which allows us to investigate the microbiome in SBS on a longer timeline.

With 70% ethanol having concerns with catheter patency along with availability and cost, a novel solution such as T-EDTA we have studied in our piglets is timely and warranted for

children with SBS. This translational piglet model provides further data that this both antibacterial and anticoagulant locking solution could work as well, if not better for IF patients. In the future, with a larger sample size and more contemporary antibiotic regimen it would be vital to do another retrospective study to confirm the impact of T-EDTA within our laboratory. A more evidence based approach would be to conduct a randomized trial, with treatment groups getting our previous control, saline with heparin, and some piglets getting T-EDTA. However, to conduct such a study would bring up an ethical question, when we already have shown a marked decrease in CLABSI with the use of T-EDTA in our laboratory: is it justified to have animals given control? One could consider the use of T-EDTA a valid refinement of our approach to animal use that should be continued to support animal welfare. Ideally, as our sample size increases with our experience using T-EDTA we will be able to achieve statistical significance with a retrospective study to determine the impact on catheter occlusions and line replacements with the use of T-EDTA.

Chapter 7 investigated the impact of a combination probiotic compared to placebo and a standard of care antibiotic on structural and functional measures of the microbiome and adaptation in piglets with SBS. This study was over 10 days, and the standard of care was metronidazole, often used for patients with SBS to treat small bowel bacterial overgrowth, a presumed consequence of dysbiosis seen in SBS, such as decreased bacterial diversity and increased potentially proinflammatory bacteria. There was a drastic decrease in SCFAs important for colonic energy salvage with use of the antibiotic. While there is a reluctance due to a lack of clinical data to use probiotics in pediatric SBS, SCFA production was highest with the use of probiotic over metronidazole and placebo. This could have important implications for

patients with SBS for colonic energy salvage, showing an impact of the probiotic not only on the structure of the microbiome but also the function. Probiotic use increased relative abundance of beneficial bacteria in both the mucosal and luminal microbiota, along with increased alpha diversity and changes in alpha and beta metrics compared to the antibiotic, metronidazole. Over placebo, both metronidazole and probiotic increased total jejunum weight, with a trend towards increased villus length.

This study showed the negative effects of use of metronidazole on the structure and function of the SBS gut microbiota. This has already led to a further study that is currently underway. We are investigating this same probiotic in SBS piglets using a similar study design, including study duration, but a larger sample size. With a sample size of 15 per group placebo versus probiotic, we believe we will have the power to demonstrate that structural adaptation is observed with probiotic use versus placebo. We were able to use the pilot data from this thesis research to perform a post-hoc power analysis and more appropriately power the follow on study. Furthermore, in this follow up study, we are looking at inflammation, measured via PCR of genes associated with inflammation, along with blood levels of C-reactive protein and cytokines for systemic inflammation and lipopolysaccharide binding protein for evidence of bacterial endotoxemia. Importantly, we have cultured blood at termination from all piglets, regardless of them showing clinical symptoms of sepsis, to better determine any possible impact on sepsis rates via CLABSI. We will specifically also culture for the probiotic to better determine its safety. Finally, to determine any differences in bacterial translocation, including of the probiotic, we will culture lymph nodes under anaerobic and aerobic conditions on agar specific to the probiotic. We have postulated these changes to the microbiome and mucosa

would lead to changes in intestinal barrier function, so are performing Üssing chamber work on the jejunum piglets from this study. One further important addition to this follow on study will be the use of metagenomics and metabolomics. While the first study had significant results showing increased microbial function, with SCFA analysis, sequencing the entire genome of the microbiome present gives us further information into the activity of the GIT microbiome. We will confirm if the probiotic is established in the GIT and better characterize its function through gene expression.

A future area that could merge both chapter 6 and 7, would be extending these type of microbiome studies to day 14 with the use of T-EDTA. Any strategies that reduce the risk of sepsis will allow for longer studies. A further refinement to make this possible could be instilling the locking solution into the line for 4 hours, the clinical standard, instead of 2 hours per day. This possibly would give the locking solution further time to act, both as an anticoagulant and antimicrobial and allow for longer study durations without confounders of prophylactic antibiotics or the cost of loss of piglets from sepsis during trials.

From a clinical standpoint, it will be interesting to see if this preclinical work on probiotic use in piglets with SBS translates to human care. Although, there are some other questions that could be addressed first in piglet studies. What about in a different anatomical model, with the ICV? What about the timing of administration after surgery? Our neonatal piglets started the probiotic two days after surgery, while we know that the surgery itself has a major impact on the changing microbiome. What about the use of probiotics in more established microbiomes of older children with SBS? A unique benefit of probiotics in pediatrics may be the potential to

have a lifelong impact on the microbiome. However, in adults once probiotic treatment is stopped the microbiome typically transitions back to its original or baseline composition.[391]

A future area I am particularly interested in exploring, is the use of a probiotic combination with the *Lactobacillus* species removed. While *Lactobacillus* species have many benefits in infants, especially in combination with *Bifidobacterium* for digesting HMOs, SBS infants are often not able to be fed human milk. As well, much of the literature shows cases of *Lactobacillus* not *Bifidobacterium* causing possible sepsis from probiotic case studies in SBS.[170] *Lactobacillus* species also come with the concern if they can produce D-lactate. Although, as used in the current study *Lactobacillus rhamnosus* is a L-lactate not a known D-lactate producer. Further to this, *Lactobacillus casei* along with *Bifidobacterium breve* and prebiotic galactoligosacchairde have been used to decrease D-lactate levels and remission of D-lactic acidosis in an SBS patient.[392] In our study, as seen in chapter 7, there was increased *Lactobacillus fermentum* for probiotic compared to placebo.[310] However, we did not measure lactate levels and microbial function beyond SCFA production was not assessed.

The microbiome of many children with SBS is often higher in *Lactobacillus* than non SBS children, so possibly the addition of *Lactobacillus* is not beneficial. There is data in piglets where a *Lactobacillus* containing probiotic might have had a harmful impact.[340] In SBS piglets, when *Lactobacillus rhamnosus GG* and even *Lactobacillus* as a synbiotic with short-chain fructooligossaccharides was given, there was significantly less adaptation than the prebiotic alone.[340] This study postulated that this was due to the *Lactobacillus* led to less intraluminal butyrate due to changes in the GIT microbiome composition and/or provided less support to the process of adaptation due to another mechanism.[340] It would be helpful to study head to

head individual probiotics to clarify these issues, although combination treatments may work differently and could be designed based on data from piglet studies. It is possible that the benefits of *Lactobacillus* species like *Lactobacillus rhamnosus* when added to combination probiotic therapies outweigh the potential negatives. Colonization of this niche might decrease excessive proliferation of acid-resistant D-lactate producing bacteria like *Lactobacillus acidophilus, Lactobacillus fermentum, Lactobacillus delbrueckii, Lactobacillus buchneri* and *Streptococcus bovis.*[392]

To better understand how probiotics may influence intestinal adaptation it is reasonable to look beyond their role in gut energy metabolism and consider their relationship to gut trophic factors. Few studies have looked into the effect of the microbiome on endogenous GLP-2 expression,[393, 394] Studies in mice show that when the microbiome is shifted towards a more anti-inflammatory state, this increases endogenous GLP-2 expression, which in turn improves gut barrier function.[394] When TPN was supplemented with butyrate in piglets with a JI anastomosis and a 80% intestinal resection, butyrate led to increased measures of adaptation like villus height and proliferating cell nuclear antigen expression.[144] Butyrate also lead to increased levels of plasma GLP-2.[144] This lead the authors to postulate that butyrate could mediate the effects on adaptation via GLP-2.[144] Another study has examined the impact of exogenous GLP-2 treatment on the intestinal microbiome of young rats.[395] GLP-2 resulted in an increase at the phylum and genus level of several beneficial bacteria and there was a reduction in some pathogenic bacterial genera.[395] We believe there is the real possibility that the microbiome might influence endogenous GLP-2 expression and that exogenous GLP-2 treatments in turn could influence the microbiome. This relationship may be a factor in

explaining the benefits of probiotics for adaptation, enhanced mucosal barrier function, and reduced intestinal inflammation in SBS; as well as raising the possibility of enhancing probiotic benefits in combination therapy with GLP-2 analogues. This is clearly a research gap that needs to be addressed.

Chapter 8 we investigated two different GLP-2 analogues and their impact on structural measures of adaptation and pharmacokinetics to determine optimal dosing in our piglet model. Teduglutide, the GLP-2 analogue that has started us into our new era of care for pediatric IF, increased small bowel length at termination compared to control. Apraglutide, the longer acting GLP-2 analogue increased small bowel length, but also small bowel weight and villus height compared to control. While many of these differences were not significant when comparing apraglutide to teduglutide, a superior intestinotrophic effect of apraglutide compared to teduglutide was demonstrated. Fecal fat was measured, but no significant differences were demonstrated. This study has important clinical implications, as is, apraglutide has additional benefits and decreased injections, from once daily with teduglutide to once weekly with apraglutide for pediatric use.

This study could have included additional measurements of functional adaptation. While fecal fat measurement is an important outcome, there are limitations in our model, including that feeds were only given at trophic amounts and so we often need a much larger sample size to achieve significance for such a minimal amount of fat malabsorption. A better approach would have been consideration of Üssing chamber studies.

Another possible study design to better confirm if longer acting GLP-2 analogues are superior would be to perform a weaning study, as has previously been done in this

laboratory.[184] This would involve over 2 weeks decreasing the PN volume, to see if PN can be discontinued with adequate weight gain, like in humans, weaning from PN being the ultimate goal of enteral autonomy. This would show that these piglets are able to absorb enough nutrition from their EN and are adapting. As volumes of EN increase fat balance studies could be undertaken that might be more meaningful than at trophic EN volumes. A goal of this study could be to mirror the human studies with a goal of a decrease in PN volume by ≥20% from baseline to the end of the study. This would also provide a novel comparison of teduglutide to apraglutide in terms of weaning potential, where such a comparison has currently not been published.

A limitation of this work is that the studies are performed in animals immediately following intestinal resection. The majority of patients that achieve spontaneous enteral autonomy will achieve this within the first two years after intestinal resection,[46, 226] how these different analogues work after this initial adaptation will be a novel area of future study. However, given the rapid growth of pigs such a study is not feasible with our current laboratory set up. However, it is important to clarify the benefits of these analogues on the adaptation process occurring after initial post-surgical adaptation. This could help determine how well teduglutide works after resection, it may be more effective used sooner after surgery, but this is currently uknown.[225]

As mentioned another important area of study will be looking at the effect of different GLP-2 analogues on the microbiome. Future studies could simply collect fecal samples at termination from these piglets for us to start addressing these questions. A question that will need to be answered would be is the exogenous GLP-2 analogue impacting the microbiome

directly, or is it changes in mucosa and transit time that are impacting the microbiome by changing the host environment. This could be answered based on the microbiome of the piglets on the different GLP-2 analogues compared to control SBS piglets to establish the difference in the microbiomes of the different analogues compared to each other and compared to the controls. This could then be compared to inflammation measured in the intestines and to transit time measures. Alone, this would not clarify if the analogue slowing transit time and promoting adaptation changes the microbiome or is it the GLP-2 analogue directly altering the microbiome. Using medications to slow or speed up the microbiome in both treatment groups could help clarify.

It seems plausible there is a relationship between the microbiome and gut derived trophic factors. The microbiome stimulates enteroendocrine cells, inducing the secretion of hormones like GLP-1 and GLP-2.[396, 397] Microbes produce SCFAs that can bind to FFA receptors and stimulate hormone production or release.[396] To further answer this metagenomics could be used to assess differences in the microbes genome and to use metabolomics to look at microbial derived metabolites that can interact with the L-cells that produce GLP-2.[398] One putative focus would be genes for butyrate production, this could include the pathway of dihydroxyacetone-phosphate to produce pyruvate, acetyl-CoA, then genes to convert butyryl-CoA like phosphate butyryltransferase (encoded by *ptb)* and butyrate kinase (encoded by *buk)* to produce butyrate.[399] Other genes involved in butyrate production include *entH, menI, tesA, tesB, ybgC, ybhC, yciA, yigI.*[399]

An area touched on in the study is the concept of why a longer acting GLP-2 analogue would exert different pharmacodynamic effects. Two important concepts come up when

looking at dosing, pharmacokinetics and pharmacodynamics. As discussed, there is drug absorption, distribution, metabolism and excretion for pharmacokinetics. But, for pharmacodynamics, this is the physiologic effects or actions of that drug.[191] How a drug exerts its effects is through receptor binding, post-receptor effects and chemical interactions.[191] Some important questions for GLP-2 analogues include Kd, the relationship between the drug binding the receptor and the concentration of the drug already at the receptor site.[191] The smaller the Kd value, the greater the affinity of the drug for the target (i.e. GLP-2 receptor).[191] Receptor occupancy, the more receptors that are occupied by a drug, typically the greater effect of that drug, but all receptors don't have to be occupied for many drugs to get their Emax.[191] With long-term exposure to a drug, can get downregulation of the receptor, i.e. a decreased number of GLP-2 receptors with chronic GLP-2 analogue use.[191] The question for very long acting analogues like apraglutide is whether greater affinity for the GLP-2 receptor is what is leading to the greater effect? Or is it more beneficial to give a shorter acting analogue more frequently to act like physiological GLP-2 released after a meal, with more frequent peaks and valleys in GLP-2 receptor binding.

In chapter 9, we investigated the impact of adding IGF-1, given as enteral therapy alone or in combination with subcutaneous GLP-2 to promote adaptation in SBS piglets. While we found that IGF-1 treatment increased mRNA expression for *GCG,* encoding proglucagon the precursor for GLP-2, we found no benefit of IGF-1 treatment on structural adaptation. Despite this negative finding, identifying combination therapies to improve the efficacy of GLP-2 analogue therapy in SBS should continue, given the costs of GLP-2 therapy and the to date only modest impact on enteral autonomy.

Interestingly, both for the chapter 9 study for IGF-1, along with in our previous study from this laboratory on EGF the recombinant trophic factors were produced from bacteria.[235] The EGF was produced from *Lactococcus lactis* and derived from the supernatant[235] while the IGF-1 was produced by *Escherichia coli*. Further studies could investigate the use of probiotic bacteria that have an increase in genes and/or metabolites that stimulate the production of trophic factors like IGF-1, EGF and GLP-2. The most promising would most likely be those bacteria that readily ferment fiber into the SCFA butyrate and could therefore promote adaptation. This would most likely be a Firmicutes that produces butyrate that is selected out for the ability to either produce butyrate from a small amount of substrate or to produce a larger response.[400] Alternatively, the use of probiotics that have been engineered with increased genes that have been shown to produce these trophic factors. For example, *E. coli* engineered with overexpression of *ychJ* have been shown to increase EGF secretion, while *bcsB* overexpression has increased continuous fermentation.[401] As genes associated with increased fermentation, specific SCFA production, trophic factor production etc. are discovered this could open up the potential for targeted probiotics that are engineered for different disease states.

Dreuille et al. recently found when looking at jejunum biopsies from adult SBS patients before and after treatment with teduglutide there was a significant increase after teduglutide treatment in *PAPPA2*, the gene encoding pappalysin-2 responsible for cleavage of IGFBPs and increase in IGF-1 bioavailability.[402] There was an inverse correlation between *PAPPA2* expression before teduglutide treatment and response to treatment, suggesting those with lower levels of *PAPPA2* expression at initiation have an upregulation of *PAPPA2* with treatment (hence potentially in IGF-1); they also had greater reduction in PN volume.[402] This study further

suggests a role for IGF-1 in mediating the effects of GLP-2 analogues like teduglutide. If in our study the piglets were already maximally responding to teduglutide, producing adequate endogenous IGF-1, we postulate that the addition of exogenous IGF-1 did not confer additional benefit. Conversely, when IGF-1 was given alone as a mediator of the downstream effects of GLP-2, it led to an increase in *GCG* expression without any further adaptive effects. What is the relationship with *PAPPA2* expression, does it increase as *GCG* expression increases? This newly recognised relationship between proglucagon, GLP-2, pappalysin-2, IGFBPs and IGF-1 could be investigated further in future studies. Such work will help us better understand the mechanistic relationship between IGF-1 and GLP-2 for intestinal adaptation. An important next step might be to compare findings in piglets with a jejunoileal anastomosis to a jejunocolic anastomosis. When IGF-1 is administered alone, it is possible this leads to an upregulation of *GCG* for the parent hormone proglucagon, but not to a high enough degree to impact adaptation in animals that lack enteroendocrine L cells that produce GLP-2. When combined with exogenous GLP-2, this saturation of GLP-2 receptor with the exogenous GLP-2 does not lead to the same need to upregulate proglucagon production for GLP-2. Looking at plasma GLP-2 levels in these models under IGF-1 treatment could help clarify. *PAPPA2* levels would increase in those piglets that respond to the GLP-2 analogues, along with increasing IGF-1 bioavailability. Due to the fact the JC piglets did not respond to IGF-1 but did have an increase in *GCG* along with did respond to GLP-2, one could postulate there was an increase in *PAPPA2* with GLP-2, along increased IGF-1 bioavailability to a level that exogenous IGF-1 could not further augment. As IGF-1 is a downstream mediator of GLP-2, it did not impact *PAPPA2* or induce adaptation without the exogenous GLP-2 due to decreased endogenous GLP-2 in the JC piglets. Finally, the addition of a

western blot would be beneficial to confirm if the increased expression we observed of *GCG* mRNA is being translated into the protein.

In summary, the work presented herein has demonstrated the possible impact of novel treatments for improving adaptation and outcomes for pediatric SBS and IF. While multidisciplinary teams, changes in PN, antibacterial locks and HPN have substantially changed the outlook for SBS patients, there is still much that can be done in terms of novel treatments to promote enteral autonomy and reduce sepsis. GLP-2 analogues and the microbiome have become the focus of current SBS research. GLP-2 analogues offer the potential to improve quality of life for SBS patients by decreasing nights on PN or achieving enteral autonomy.[357] For patients that can't achieve enteral autonomy, reduced nights on PN has potential to improve quality of life and decrease healthcare costs. This still comes with the risk of a septic episodes, currently a leading cause of death in SBS patients.[1] Catheter related sepsis is also the most important factor that influences the cost of HPN, leading to recurrent hospitalizations.[1] Hence, recurrent sepsis also negatively impacts quality of life.[1] Antibacterial locks, like Kitelock™, offer the potential to decrease CLABSI,[312] but small bowel bacterial overgrowth, and bacterial translocation remain as an issue due to the altered microbiome or dysbiosis of SBS patients. Probiotics could be a potential candidate to promote a "healthier" microbiome for these patients. We identified the benefits on CLABSI and cost with the use of Kitelock™ in both our TPN and SBS piglet model. We showed a benefit on not only structural adaptation with probiotics over the standard of care, broad spectrum oral antibiotic metronidazole, but on both structure and function of the gut microbiome in SBS piglets. We demonstrated the superior intestinotrophic effects of GLP-2 analogue apraglutide over teduglutide in promoting structural

adaptation in SBS piglets. Finally, we showed the lack of benefit but mechanistic role of IGF-1 alone or in combination with GLP-2 analogues for structural and functional adaptation.

Overall, by exploring these novel treatments in this neonatal animal model of SBS, this thesis achieves a critical first step in research and development in order to translate to improve the care and quality of life for infants and children with SBS.

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