Mechanisms of Neonatal Intestinal Failure-Associated Liver Disease: Exploring the Gut-Liver Axis

by

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Abstract

Neonates with intestinal failure (IF), who must rely on parenteral nutrition (PN) for growth and maintenance of health, are at risk for intestinal failure-associated liver disease (IFALD). In neonates, short bowel syndrome (SBS) as a result of intestinal resection is the most common cause of IF. SBS patients without a remnant ileum and ileocecal valve (ICV) are considered to have the worst prognosis. The inclusion of fish oil (FO) in the PN lipid emulsion has recently been used as both a prevention and treatment strategy for IFALD. However, the relationship between IFALD and intestinal anatomy in SBS has not been formally studied. Similarly, the molecular mechanisms that promote improvement of the disease with the use of FO-containing PN lipids are poorly understood.

The overall objective of this thesis was to define the mechanisms by which remnant SBS anatomy and PN lipid composition impact the development of IFALD. Outcome measures included bile flow and biochemical indicators of liver disease, intestinal structure and permeability of the jejunum, bacterial translocation and characterization of the gut microbiota, bile acid identification and quantification, and gene expression of proteins involved in epithelial barrier function, the host immune response, and bile acid metabolism.

To examine the effects of remnant anatomy on IFALD, we studied neonatal piglets that were randomized to a 75% intestinal resection including resection of the ileum and ICV (JC), 75% intestinal resection leaving intact ileum and ICV (JI), non-resected but PN-fed sham (Sham), or sow-fed control (SF); JC, JI and Sham piglets were 100% PN-fed for 14 days. As expected, PN-feeding resulted in cholestasis, as indicated by reduced bile flow and increase in markers of liver disease. However, we observed no differences between the PN-fed piglets, regardless of remnant anatomy. Further, in this neonatal model, we did not detect any differences

between any of the groups, whether SF or PN-fed, in jejunal permeability as measured directly through permeability studies, or as indicated through the expression of tight junction proteins. Bacterial translocation to the lymph nodes was greater in SBS (JC and JI) piglets compared to SF, but not different between SBS and Sham groups. Overall, sepsis had a greater impact on the development of IFALD than did remnant anatomy.

To determine whether PN lipid composition affects the gut microbiota and host immune responses, we compared PN-fed neonatal piglets given equivalent lipid doses of either a pure soybean oil (SO) or a mixed lipid (ML) containing FO for 14 days. We also studied a group of SF piglets. We characterized the gut microbiota with 16S rRNA gene sequencing, and we quantified the relative gene expression of tight junction proteins, mucins, antimicrobial peptides and inflammatory cytokines. Indeed, there were differences in the gut microbiota between all three groups. The microbiota of the ML group was more similar to that of the SF group than the SO group was to the SF group, indicating reduced disturbance with ML. Differences in gene expression relevant for epithelial barrier function (EBF) and the mucosal immune response were identified between SO and ML.

Finally, to investigate the molecular mechanisms that contribute to IFALD and to determine the impact of FO in PN lipid, we measured the expression of genes involved in bile acid metabolism and transport and determined bile acid composition of excreted bile, in neonatal piglets that were either SF or PN-fed for 14 days with either SO, ML, or pure FO as the PN lipid. While bile acid excretion was reduced with PN, FO-containing PN lipid resulted in greater expression of bile acid transport genes, and greater excretion of hepatotoxic, hydrophobic bile acids.

These findings suggest that remnant anatomy per se contributes minimally to the early emergence of IFALD. Rather, remnant anatomy likely has a greater impact on intestinal adaptation, and thus on the duration of PN. Furthermore, sepsis during PN is a key contributing factor to early IFALD. Further, these results provide empirical evidence that PN lipid modulation alters the gut microbiota, bile acid metabolism, and bile acid composition. Factors related to intestinal adaptation, the role of sepsis in IFALD, and the mechanisms by which PN lipid alters the gut microbiota should be further explored.

Preface

This thesis is an original work by Celeste Lavallee. The research project, of which this thesis is a part, received ethics approval from the University of Alberta Animal Care and Use Committee, project name "Omega-3 Fatty Acids in Parenteral Lipid for the Management of Parenteral Nutrition Associated Liver Disease" study ID AUP00000153, April 2010.

The research conducted for this thesis forms part of a multi-institutional research collaboration, led by Dr. Justine M. Turner and Dr. Benjamin P. Willing at the University of Alberta, and Dr. Paul W. Wales at the University of Toronto. The literature review in Chapter 1 and concluding analysis in Chapter 5 are my original work.

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Dedication

I dedicate this thesis to all the babies who have ever faced the challenges of intestinal failure, and to their families who have faced those challenges alongside them.

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Abbreviations

ACTB	beta actin
AMOVA	analysis of molecular variance
ANOVA	analysis of variance
CFU	colony forming units
CLDN	claudin
CRP	C-reactive protein
DHA	docosahexaenoic acid
EDTA	ethylenediaminetetraacetic acid
EPA	eicosapentaenoic acid
EN	enteral nutrition
EBF	epithelial barrier function
FGF19	fibroblast growth factor 19
FO	fish oil
GAPDH	glyceraldehyde-phosphate-dehydrogenase
GGT	gamma-glutamyltranspeptidase
GI	gastrointestinal
GLP-2	glucagon-like peptide 2
HPRT1	hypoxanthine phosphoribosyltransferase-1
ICV	ileocecal valve
IFN-γ	interferon gamma
IF	intestinal failure
IFALD	intestinal failure-associated liver disease
IL	interleukin
Isc	short-circuit current
IQR	interquartile range
JC	jejunocolic
JI	jejunoileal
LC-PUFA	long-chain omega-3 polyunsaturated fatty acids
LPS	lipopolysaccharide
MDA	malondialdehyde
ML	mixed lipid
Muc	mucin
MW	molecular weight
NEC	necrotizing enterocolitis
NMDS	non-metric multidimensional scaling
OCLN	occludin
OTUs	operational taxonomic units
P_{app}	apparent permeability
PCR	polymerase chain reaction
PN	parenteral nutrition
PNALD	parenteral nutrition-associated liver disease
PEG	polyethylene glycol
PUFA	polyunsaturated fatty acid
PD	potential difference
qPCR	quantitative PCR
SBS	short bowel syndrome
SD	standard deviation
SF	sow-fed
SO	soybean oil
SRTC	Swine Research and Technology Centre
TER	transepithelial electrical resistance
TLR	toll-like receptor
TNF-α	tumor necrosis factor alpha
ZO	zonula occludens

Proteins (*Genes*) in Bile Acid Metabolism

BSEP (*ABCB11*)	bile salt export pump (ATP-binding cassette, subfamily B member 11)
CYP7A1 (*CYP7A1*)	cholesterol 7 alpha-hydroxylase (cytochrome P450, family 7, subfamily A, polypeptide 1)
FXR (*NR1H4*)	farnesoid X receptor (nuclear receptor subfamily 1, group H, member 4)
MRP2 (*ABCC2*)	multidrug resistance protein 2 (ATP-binding cassette subfamily C member 2)
MRP3 (*ABCC3*)	multidrug resistance protein 3 (ATP-binding cassette subfamily C member 3)
OSTA (*SLC51A*)	organic solute transporter alpha (solute carrier family 51 alpha subunit)
SHP (*NR0B2*)	small heterodimer partner (nuclear receptor subfamily 0, group B, member 2)

Bile Acids

g-	glycine- or glycol-
t-	taurine- or tauro-

CA	cholic acid
CDCA	chenodeoxycholic acid
HCA	hyocholic acid
HDCA	hyodeoxycholic acid
LCA	lithocolic acid

CHAPTER 1: Introduction

Intestinal failure-associated liver disease (IFALD) occurs in about 66% of children with intestinal failure (IF). It is a leading cause of death in these children, with historically a mortality rate as high as 30%;[1] yet, we know very little about what causes the disease. Thus, it is imperative that we develop a better understanding of this significant clinical problem to improve the lives of children with IFALD.

1.1 INTESTINAL FAILURE

Intestinal failure (IF) can be defined as the impaired absorption of nutrients to a degree that health and growth in children,[2] or maintenance of health in adults,ch[3] cannot be supported. Vascular thrombosis, inflammatory bowel disease, radiation enteritis, intestinal obstruction, and intestinal resection are causes of IF.[4] Patients with IF who cannot tolerate feeding through the gut must rely on parenteral nutrition (PN) for the intravenous provision of nutrients. Although PN is a life-saving necessity, it is not without consequence as long-term dependence on PN increases the risk of catheter-related sepsis and IFALD.

1.1.1 Short Bowel Syndrome as a Leading Cause of IF

Short bowel syndrome (SBS) results from surgical resection of portions of the intestine due to congenital or acquired disease. While necrotizing enterocolitis (NEC) is the most common cause of resection in neonates, midgut volvulus, gastroschisis, and Hirschsprung's disease are also important causes of neonatal SBS.[2] In children, SBS is the most common cause of IF,[2] and can eventually result in IFALD. To date, IFALD has been the leading cause of death in infants with SBS.[5]

1.1.1.1 SBS Surgical Anatomy

There are three main anatomical subtypes of SBS.[5] Type 1 is a jejunoileal (JI) anatomy, wherein a portion of the mid-intestine is removed, leaving a remnant jejunum, ileum; and intact

ileocecal valve (ICV).[6] Overall, patients with a JI anatomy have the best prognosis.[5, 6] Type 2 is a jejunocolic (JC) anatomy, wherein a portion of the jejunum, the ileum, the ICV, and potentially a portion of the colon, are resected.[6] Type 3 is a high output jejunostomy in which a portion of the jejunum, the remaining intestine, the ICV and the colon are removed; a stoma is created in the abdomen and is attached to the remnant jejunum.[6]

1.1.1.2 Importance of Remnant Ileum and Ileocecal Valve

The ileum is a secretory source of glucagon-like peptide 2 (GLP-2),[7] and our group,[8] as well as others,[9] have shown that the presence of the ileum is very important for adaptation. It is the part of the intestine where water and vitamin B12 are absorbed.[6] Hence, resection of the ileum leads to diarrhea, dehydration, and malabsorption of fat and vitamins.[6] In addition, the ileum plays an important role in the enterohepatic circulation of bile acids, being the location where bile acids are reabsorbed.[10] Thus, absence of the ileum is expected to disrupt bile acid circulation, with the potential for reduced bile flow along with the build-up of toxic bile acids in the liver.

The ICV functions to slow transit time and prevent colonic content and flora from refluxing back into the ileum.[11] Absence of the ICV is a proposed risk factor for IFALD. Mayr et al.[12] found that neonates with an intact ICV had a better prognosis than those without. According to a 2015 multicenter retrospective cohort study conducted by the Pediatric Intestinal Failure Consortium, the ICV was resected in 48% of children with intestinal failure.[13] These children were less likely to achieve enteral autonomy than those with a preserved ICV, and thus were more likely to need prolonged PN support. Indeed, this finding is supported by others.[14-16] Finally, there is evidence that liver fibrosis is more advanced in patients with pediatric onset IF without an ICV than those with an ICV.[17] However, it cannot be ascertained from these studies

whether it is the absence of the ICV itself, or the loss of the terminal ileum that affects outcomes in these patients.

1.2 INTESTINAL FAILURE-ASSOCIATED LIVER DISEASE

1.2.1 Pathophysiology of IFALD

IFALD ranges in spectrum from mild cholestasis and steatosis, to fibrosis, cirrhosis and end stage liver disease.[1, 18-20] While steatosis is predominant in adults, cholestasis occurs more frequently in infants.[18, 21] Cholestasis is defined by decreased bile flow, but is indicated by various markers in serum. Elevated serum bilirubin, γ-glutamyltranspeptidase (GGT), alkaline phosphatase, aspartate aminotransferase, and alanine aminotransferase are biochemical indicators of the onset of IFALD, as are serum bile acids.[21] Serum unconjugated bilirubin is the most specific indicator of cholestasis, while GGT is the most sensitive marker.[22]

1.2.2 Aetiology of IFALD

The mechanisms of IFALD are still unclear and evidence suggests that aetiology of the disease is multifactorial, which complicates our understanding of the disease and its origins. Conditions known to increase the risk of IFALD include surgical SBS, lack of EN, and recurrent sepsis.[1, 23, 24] Sepsis is a common complication of PN therapy,[25] especially in SBS,[26, 27] as a result of central venous catheter infections or bacterial translocation. Risk factors for IFALD that relate to the neonate have been identified and include an underdeveloped GI tract in preterm infants, and low birth weight.[1, 23, 24] Finally, there are risk factors for IFALD that appear to be specific to the PN therapy itself. These include longer duration of PN; specific amino acid toxicity (i.e. methionine) or deficiency (i.e. taurine and cysteine); excess energy intake from carbohydrate or lipid; and soy-based lipid infusion of more than 1 g/kg/day.[23, 24] Historically, liver disease that occurred in the setting of IF and PN was referred to as parenteral nutrition-associated liver

disease (PNALD). However, the term IFALD recognizes the multifactorial nature of the disease, and is the more common term used today.[28]

Neonates with IF are especially at risk for a variety of reasons. The GI tract,[29] including the liver,[1] as well as the immune function[30] of preterm neonates are not fully developed. Even in neonates born at term, full development of GI function occurs with the exposure of oral or enteral nutrition (EN; i.e. via a tube) into the gut, and eventual weaning from breast milk or formula to solid foods.[29] A neonate's microbiota also develops over time, as an ecological process that is influenced by these same milestones, before it resembles that of an adult.[31] A key feature of this development is an increase in the number of diverse populations of commensal bacteria that colonize the gut. This colonization is especially disrupted in preterm neonates.[32] Moreover, these infants are often exposed to antibiotics and invasive procedures in the neonatal nursery which further disrupt their gut microbiota. Commencing EN promotes the development of the GI immune system and the colonization of the gut microbiota.[30] Thus, neonates who require PN without EN are not exposed to the factors that promote maturation of the GI tract, GI immune function, and the gut microbiota, putting them at increased risk for the complications of PN, including IFALD.

1.2.3 The Role of Parenteral Lipid in IFALD

Modulating the PN lipid composition was postulated as a potential treatment for IFALD,[1, 28, 33, 34] and is now used as a strategy for prevention[35] and treatment[36, 37] of the disease. The conventional or standard PN lipid provided to preterm neonates and those with IF is soybean oil (SO), which is now recognized to promote IFALD.[38] A mixed lipid (ML) emulsion has recently become available in Canada and contains a combination of soybean-oil, medium chain triglycerides, olive oil and fish oil (FO). A pure FO is also available. PN lipid emulsions that

contain fish oil (FO) have been recently recognized as providing a prevention[35] and treatment[36, 37] benefit in IFALD. Reversal of cholestasis, indicated by decreased bilirubin levels, has been seen in pediatric patients treated with FO-containing PN lipid.[36, 37, 39-41] In animal studies, our group and others have shown increased bile flow with the use of FO-containing PN lipid.[42-44] Yet, despite certain improved outcomes with the use of FO, the risk of end stage liver disease with fibrosis remains for pediatric patients.[17, 45-47] Thus, the mechanisms for improved bile flow and for improvements in the severity of IFALD with the use of FO-containing PN lipid require further investigation.

1.3 UNDERSTANDING LIVER PHYSIOLOGY TO BETTER UNDERSTAND IFALD

Given the cholestatic nature of IFALD, a review of liver physiology, bile flow, and enterohepatic circulation is needed to better understand the aetiology of IFALD. Bile acids aid in the digestion and absorption of fat and other nutrients, and are formed, in the liver, from cholesterol.[10] The classic, or neutral pathway for bile acid synthesis produces most of the bile acids in the overall bile acid pool,[48] and is regulated by the rate-limiting enzyme cholesterol 7 alpha-hydroxylase (CYP7A1).[10] There is also an alternative, acidic pathway for bile acid synthesis.[10]

Farnesoid X receptor (FXR) is bile acid receptor that is involved in the regulation of bile acid synthesis and transport.[49, 50] Bile acids from either the existing bile acid pool, or from de novo synthesis, bind to and activate FXR, which then induces the expression of the small heterodimer partner (SHP).[50] Through a negative feedback loop, SHP then represses, which results in inhibited bile acid synthesis.[50] Thus, in the presence of bile acids, FXR activity is upregulated[49] while CYP7A1 activity is downregulated via FXR.[49, 50]

Hepatic canalicular transport proteins are major determinants of bile flow.[51] Bile acids and organic solutes are secreted via the bile salt export pump (BSEP) and the multidrug resistance protein 2 (MRP2).[51] FXR is able to regulate bile acid transport by transcriptionally inducing BSEP and MRP2.[52] However, in cholestasis, organic solute transporter alpha (OSTA)[53] and multidrug resistance protein 3 (MRP3)[54] are upregulated. These hepatic basolateral bile acid transporters allow the efflux of bile acids into blood.

Although bile acids are FXR agonists, the numerous bile acid species upregulate FXR to varying degrees.[52] Bile acids can also differentially impact CYP7A1 activity.[55, 56] Importantly, CYP7A1 enzyme activity is suppressed by a hydrophobic bile acid pool.[57] Moreover, bile acid transporters are expressed differently depending on the substrate that binds to them, including specific bile acid species.[35, 53, 58]

1.3.1 Enterohepatic Circulation

Enterohepatic circulation (Figure 1-1) begins with synthesis of primary bile acids in the liver. Primary bile acids are conjugated with taurine or glycine to increase their solubility and thus facilitate their transport out of the liver, via BSEP and MRP2, into bile, and to minimize passive reabsorption in the intestine.[10, 59] EN stimulates the release of bile into the duodenum.[10] Primary bile acids are then deconjugated and dehydroxylated into secondary bile acids by bacteria in the ileum.[10] Approximately 95% of bile acids[10] are actively transported across the terminal ileum, and returned to the liver via the portal vein.[59] The remaining 5% enter the colon where they are further biotransformed by bacteria, which increases their hydrophobicity and thus potential passive absorption through the colon.[59] Importantly, an increasingly hydrophobic bile acid pool is linked to hepatotoxicity.[59]

The major physiological factor that regulates bile flow is the size of this circulating bile acid pool,[60] and de novo synthesis ensures a constant total bile acid pool. However, we expect PN[61] and absence of the ileum[62] in SBS to disrupt normal enterohepatic circulation. Disruption of this cycle may alter the composition of the bile acid pool by increasing its hydrophobicity, thus suppressing CYP7A1, and bile acid synthesis.

1.3.2 Bile Acid Metabolism and Transport

Further, defects in the molecular mechanisms regulating enterohepatic circulation, including the expression of genes that regulate bile acid synthesis and transport, have been shown to occur in other cholestatic liver diseases,[10] and may also be implicated in IFALD. These mechanisms involve canalicular and basolateral bile acid transport proteins.[63] Interestingly, inflammatory cytokines, produced in response to infection, have been implicated in cholestasis as they inhibit the expression of transporters involved in bile acid uptake into the liver and transport into bile.[64] As mentioned, in cholestasis, the expressions of hepatic basolateral bile acid transporters OSTA and MRP3 are upregulated to allow efflux of bile acids into circulation.[53, 54]

1.3.3 Gut Microbiota and Bile Acid Metabolism

The terms microbiota and microbiome are often used interchangeably, but by definition, the microbiota consists of all commensal and pathogenic bacteria that inhabit the body, while the microbiome consists of the genomes of these bacteria.[65] The gut microbiota refers to the bacterial communities within the GI tract. In a healthy adult, commensal bacteria will dominate, while potentially pathogenic bacteria will exist but at levels insufficient to cause infection. For a preterm neonate whose gut is still maturing and whose immune defences are also reduced, there is a greater likelihood that potentially pathogenic bacteria can proliferate and displace commensal bacteria. The microbiota of a preterm neonate is reduced in bacterial diversity and

the shift to dominant pathogenic organisms can therefore readily occur.[32] Such variations in the type or quantity of bacteria from what are typically present in the microbiota are referred to as microbial dysbiosis.[66] The quantity of bacteria might well stay the same but a shift in the type, proportion, or diversity of bacterial populations within the microbiota can lead to severe microbial dysbiosis and illness. Such microbial dysbiosis has been noted in children with SBS.[67, 68] Lapthorne et al.[69] found colonic microbial dysbiosis in EN-fed piglets with JI anatomy. At 6 weeks post-resection, the dysbiosis was more pronounced than at 2 weeks post-resection, and was associated with colonic inflammation, as indicated by increases in interleukin (IL)-1 β, IL-8, IL-18 and tumor necrosis factor α (TNF-α).

Just as bacteria alter the composition of bile acids,[59] bile acids in turn have direct and indirect effects on gut bacteria.[70] Bile acids can alter the bacterial composition of the gut microbiota by inhibiting the growth of bile acid-intolerant bacteria, while promoting the growth of bile-acid tolerant bacteria.[70] Additionally, while the size of the bile acid pool regulates the gut microbiota, microbial dysbiosis can alter the size of the bile acid pool, as was demonstrated by Sayin et al.[71] who showed the bile acid pool in conventional mice was reduced by 71% compared to the bile acid pool in germ-free mice. Given the potential for microbial dysbiosis in the immature neonatal GI tract, such interactions between bile acids and bacteria have potential for significant impact on bile flow and bile acid metabolism in the neonate.

1.4 EFFECTS OF PARENTERAL NUTRITION ON THE GUT-LIVER AXIS

It is well known that gut atrophy, with decreased villus height, occurs as a result of PN.[72, 73] How this impact on gut structure is related to IFALD has yet to be explored. Aspects of GI health that could have potential relevance to the development of IFALD are epithelial barrier function (EBF), immune function, enterohepatic circulation of bile acids and the gut microbiota.

1.4.1 Parenteral Nutrition and Epithelial Barrier Function

The epithelial barrier functions as a defence mechanism that prevents the translocation of pathogenic bacteria across the epithelium and into the systemic circulation of the host.[74] The mucous layer that overlies the epithelium represents the first defence from pathogenic bacteria in the gut.[75] Overlying the epithelial cells of the villi, the mucous membrane works to keep bacteria from attaching to intestinal cells by providing a mobile physical barrier. Perturbations in the mucous layer have been noted in PN; Iiboshi et al.[76] observed a decrease in the mucous gel layer of rats after 4 and 7 days of PN. The decrease in the mucous layer was associated with decreased EBF noted by increased permeability of the intestine, though the mechanisms were not defined.

An increase in mucolytic bacteria is a plausible explanation for a decrease in EBF as these bacteria are able to use the mucus in the mucous layer as an energy substrate, and in doing so will degrade the first protective layer offered by the epithelial barrier. Indeed, several investigators have discovered an increase in the proportion of mucolytic bacteria with PN administration.[77-82] Specifically, higher levels of *Clostridium perfringens* [77] and *Clostridium difficile* [78] were observed in of PN-fed piglets; an increase in the proportion of mucolytic Bacteroidetes was found in PN-fed rats;[79] and a shift to Proteobacteria and Bacteroidetes was seen in PN-fed mice.[80, 81] In addition to increased levels of Bacteroidetes and Tenericutes in PN-fed mice, Wan et al.[82] also noted a lower level of intestinal alkaline phosphatase, as well as a lower relative density of mucin 2 (*Muc2*) and *Muc2* mRNA expression.[82] Decreased production of these mucin glycoproteins, which form part of the epithelial barrier, along with increased presence of mucolytic bacteria, decreases the integrity of the epithelial barrier and increases the risk of bacterial translocation in the host. Further, intestinal alkaline phosphatase deficiency has

been associated with increased gut permeability, microbial dysbiosis, and bacterial translocation in neonatal rat pups.[83]

Tight junction proteins play a key role in EBF by joining together the epithelial cells to form tight junctions, thereby decreasing intestinal permeability.[74] The lower the permeability and the greater the resistance of the gut, the less likely bacteria will be able to passively translocate across the epithelium. The EBF of preterm neonates is not fully developed, as demonstrated by high gut permeability at birth.[84] Although gut permeability decreases with age, the decrease is more significant after 4 weeks of feeding of human milk compared to commercial formula.[84] Furthermore, Buchman et al.[85] discovered an increase in gut permeability in humans administered PN for 14 days. Thus, it seems plausible that preterm neonates with a long-term dependence on PN, and without the opportunity for exposure to human milk, would have impaired EBF and an increased risk of infection from opportunistic pathogenic bacteria able to cross the epithelium. Rouwet et al.[86] investigated the gut permeability of 59 preterm neonates who were PN-fed for the first 7 days of life. They concluded that gut permeability increased and EBF decreased over those 7 days. Another study of preterm neonates found that although gut permeability was higher than in term neonates, the difference only existed during the first 2 days of life.[87] In that study, however, neonates were fed human milk or formula, which may explain different results than were noted with PN administration.

Further, increased gut permeability[79, 88, 89] and decreased epithelial barrier resistance [80] have been noted in PN-fed animals. Sun et al.[90] found that loss of EBF, indicated by increased gut permeability and decreased barrier resistance, was associated with PN administration in a mouse model of PN. The relative mRNA expressions of the tight junction proteins zonula

occludens (ZO)-1, ZO-2, occludin (OCLN), claudin (CLDN)-2 and CLDN-15 were all significantly lower in PN-fed mice than in chow-fed controls, also indicating diminished EBF.

1.4.2 Parenteral Nutrition and Gastrointestinal Immune Function

Although GI immune function develops prenatally, it does not fully mature until after birth.[30] Immune function for a preterm neonate is even less mature. Impaired immune function may be exacerbated with PN, and contribute to a greater risk of sepsis. The previously mentioned mouse model studied by Sun et al.[90] showed a significant decrease in intraepithelial lymphocyte-derived IL-10 expression with PN administration. The decreases in mRNA expression of tight junction proteins were attenuated when the PN-fed mice were given exogenous IL-10, a cytokine that down-regulates the inflammatory response, suggesting that altered EBF with PN may be the result of intestinal inflammation. This is supported by mouse studies of PN administration that found increases in various cytokines[80] and decreased expression of antimicrobial peptides,[81] lysozyme,[81, 82] and lysozyme mRNA.[82] Specifically, Miyasaka et al.[80] reported that PN increased levels of TNF-α, interferon gamma (IFN-γ), IL-1β, IL-2, and IL-6; they saw no significant change in IL-10 or IL-17. Although the group did not see a change in IL-10, the mice in their study were only provided PN for 5 days, rather than 7 days as in the study by Sun et al.;[90] the length of the study could impact the results. Conversely, Hodin et al.[79] found increased expression of antimicrobial peptide mRNA, lysozyme, and lysozyme mRNA in rats administered PN. A distinct difference between the studies was that PN was administered for 14 days in the study by Hodin et al.,[79] but only 5 days[80-82] and 7 days[90] in the other studies, which could account for the differing results.

1.4.3 Parenteral Nutrition and the Gut Microbiome

Microbial dysbiosis in neonates and infants with SBS has been associated with prolonged dependence on PN.[67] The longer a neonate remains on PN, the more likely complications such as liver disease will develop, ultimately leading to poorer outcomes for the neonate. The role of PN adversely impacting normal bacterial colonization in the pathogenesis of IFALD has not been well studied. In a study of children with IF, Korpela et al.[91] found liver steatosis was associated with intestinal microbial dysbiosis. Microbial diversity of the gut was reduced in children with liver steatosis, while the composition of the microbiota differed depending on whether the patient was currently fed via PN. Proteobacteria were most commonly found in the gut microbiota of patients currently receiving PN.

Several groups have reported microbial dysbiosis with PN administration in animals. In studies that compared PN-fed and EN-fed piglets, the microbiota differed between the two groups in type[77, 78] and concentration[78]. Other animal studies have also discovered differing bacterial populations in PN-fed and EN-fed animals, with a shift from Firmicutes to Bacteroidetes,[79-82] Proteobacteria,[80, 81] and Tenericutes.[82] While PN administration resulted in decreased diversity in the microbiota of PN-fed piglets,[78] another study found greater diversity in the microbiota of PN-fed mice.[81] An important difference between these studies is that the piglets were colostrum-deprived newborns whose GI tracts and gut microbiota had not yet matured, while the adult mice had previously been exposed to EN and thus had fully matured GI tracts and microbial colonization at baseline.

An association has also been found between PN administration in animals and bacterial translocation to the mesenteric lymph nodes,[82, 90, 92] as well as to the spleen and liver.[90, 92] It seems feasible that this translocation is due to decreased EBF, which is also seen with PN administration. Bacterial translocation was indeed associated with a reduction in the mRNA

expression of tight junction proteins[90] and epithelial barrier glycoproteins.[82] Interestingly, just as the reduction in mRNA expression of tight junction proteins was attenuated when exogenous anti-inflammatory cytokine IL-10 was provided, so too was bacterial translocation, suggesting that microbial dysbiosis is also associated with a proinflammatory immune response.[90] This is further supported by the findings of several groups who found microbial dysbiosis along with changes in the immune response.[79-82] As discussed above, microbial dysbiosis that results in increased mucolytic bacteria can lead to decreased integrity of the mucous layer as the bacteria utilize mucus as an energy substrate. Although the literature is lacking in studies considering both the presence of mucolytic bacteria and bacterial translocation with PN administration, a recent study[82] did report such an increase.

Until recently it was unknown whether it was the lack of nutrients in the gut or the PN per se that caused microbial dysbiosis. Wan et al.[82] compared diets provided to mice as either PN without EN, or as PN with partial EN, in which EN provided from 10% to 60% of energy intake. They found that when at least 20% of energy intake was provided as EN, bacterial translocation significantly decreased while the proportions and variety of most bacteria remained similar to chow fed mice. They also found intestinal tissue levels of lysozyme and the expression of lysozyme mRNA increased with partial EN of 20% of energy intake. These results suggest that it is the lack of nutrients that causes microbial dysbiosis, as even a small amount of EN ameliorates the microbial dysbiosis and immune function imbalances.

1.4.4 Parenteral Nutrition and Bile Acid Metabolism

It is well established that PN is a risk factor for cholestasis.[93, 94] Several animal studies have confirmed the relationship between PN and decreased bile flow in both adolescent[95] and

neonatal[35, 42-44, 61, 96-98] models. However, few have investigated potential molecular mechanisms that may be involved in decreased bile flow.

The bile acid profile was more hydrophobic in adolescent rabbits PN-fed for 14 days compared to control, while bile flow and bile acid secretion decreased.[95] In adult mice PN-fed for 8 days the bile acid profile differed in serum but not in liver.[99] Serum bile acids and CYP7A1 expression were increased in these mice, while bile flow and the expression of SHP and BSEP were decreased.[99] Clearly, normal bile acid metabolism is aberrant in PN.

Our laboratory previously studied the effects of PN on bile acid metabolism in a neonatal setting.[97, 100] Neonatal piglets were treated with PN along with either saline or GLP-2 for 17 days. While bile flow was decreased with PN, the decrease was not as marked with GLP-2 treatment compared to saline.[97] Liver bile acid content, the bile acid profile in bile, and the expression of hepatic genes involved in bile acid homeostasis and transport all differed with PN and between saline and GLP-2 treatments.[100] These results evoke the question whether remnant anatomy or PN lipid composition would similarly have an effect on bile acid metabolism.

1.5 EFFECTS OF PARENTERAL LIPID COMPOSITION ON THE GUT-LIVER AXIS

1.5.1 Parenteral Lipid Composition and IFALD

As discussed earlier, modulating the lipid composition of PN has emerged as a treatment and prevention strategy for IFALD. However, the exact mechanisms are poorly understood. Various compositional differences between SO and FO-containing lipid emulsions may explain reasons for improved outcomes with PN lipid modulation. These differences include vitamin E, phytosterol content, and fatty acid composition.

The high PUFA content in PN lipids can undergo peroxidation, which may contribute to liver damage, and thus IFALD.[101] Vitamin E is an antioxidant that is added to FO-containing

lipids to prevent this peroxidation.[102] The increased vitamin E content in FO-containing lipids compared to SO may be one reason for improved outcomes with FO. Studies of the effects of vitamin E in PN lipid in IFALD have found differing results.[96, 103]

Stigmasterol is a phytosterol in SO that has been shown to promote cholestasis via FXR antagonism.[104, 105] Thus, because FO does not contain phytosterols, it is plausible that improved FO-containing lipids are associated with improved bile flow because FXR activity is not repressed. However, adding phytosterols to FO did not result in the early onset of IFALD.[103]

Eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are omega-3 long chain polyunsaturated fatty acids (LC-PUFA). EPA and DHA have the potential to moderate inflammation[106] and the immune response,[107] while also providing antimicrobial defences[108-110] as they are incorporated into the epithelial cell membranes and the mucous layer.[111] Alternatively, omega-6 polyunsaturated fatty acids (PUFA) have the potential to be pro-inflammatory.[23] There are distinct differences in the fatty acid composition of PN lipids: SO contains high levels of omega-6 fatty acids but is devoid of EPA and DHA; ML provides omega-6 fatty acids along with monounsaturated fatty acids, and omega-3 fatty acids including EPA and DHA; and pure FO contains relatively low levels of omega-6 fatty acids, but very high levels of EPA and DHA.

An optimal PN lipid emulsion has the potential to prevent the onset of IFALD in preterm neonates. Increasing vitamin E content, reducing the phytosterol content, decreasing the omega-6 PUFA content, or increasing the LC-PUFA content of the PN lipid are all potential strategies for reducing the risk of IFALD. Thus, further investigations of the effects and potential mechanisms of PN lipid modulation are warranted.

1.5.2 Parenteral Lipid Composition and Epithelial Barrier Function

Evidence is lacking as to the effect of the lipid composition of PN on EBF. An in vitro study of human epithelial cells incubated with various PUFAs indicate that EPA and DHA may improve EBF through the increased expression of tight junction proteins, improving resistance, and decreasing permeability.[112] Future research should investigate whether modulation of the PN lipid formula can improve EBF in vivo.

1.5.3 Parenteral Lipid Composition and Gastrointestinal Immune Function

A study of healthy human volunteers found no difference in immune function with the infusion of a FO,[113] but the results do not directly translate to patients on PN. More pertinent studies in cancer and surgical patients have reported a modulated immune response with the inclusion of FO-based lipid emulsions.[114, 115] Specifically, provision of a ML resulted in lower levels of TNF-α and IL-6 in patients with surgical resection of gastric tumors,[114] and lower levels of TNF-α, IL-1, IL-6, IL-8, and IFN-γ in surgical patients,[115] compared to the provision of a SO. Cao et al.[116] reported that immune function in PN-fed rats was modulated with the inclusion of a ML. The applicability of these results to neonates is limited as these studies were all conducted in adult patients or adult rats.

Turner et al.[42] compared the inflammatory response of neonatal piglets receiving PN as either a SO or ML for 14 days. Although all post-treatment markers of inflammation were within the normal range, C-reactive protein (CRP) was significantly higher in the SO group than the ML group, and TNF-α was higher in both groups than at baseline.

1.5.4 Parenteral Lipid Composition and the Gut Microbiome

To date, only two studies have investigated whether the lipid composition of PN emulsions are associated with the gut microbiota.[117, 118] Harris et al.[117] studied a mouse model of PN associated liver injury to assess a potential link between the composition of PN lipid

emulsions and the gut microbiota. Adult mice were divided into ten groups that ranged in size from 5 to 14. Mice with liver injury were provided PN that contained either no lipid, SO or FO for 7 days, without EN. Various controls, with or without liver injury, were provided either chow or PN with a SO. Significant differences were seen in the fecal microbial communities of the groups provided either no lipid or FO compared to those provided chow or a SO. Although the group investigated bacterial translocation to the liver and did not find any, it is important to note that these were adult mice whose microbiotas were established prior to treatment. This would not be the case in neonates and, as noted earlier, could impact the severity of microbial dysbiosis and therefore have different implications as a result of lipid modulation.

A more clinically relevant study by Arboleya et al.[118] evaluated the impact of different PN lipid emulsions on the development of the gut microbiota of human preterm neonates. The authors analyzed the size and diversity of the bacterial communities in fecal samples of neonates provided a ML (n=8) or a standard SO (n=17). Samples were taken on days 1, 15 and 30, which allowed the authors to monitor changes in bacterial communities over time. Of seven bacterial communities that were analyzed, *Klebsiella* was the only one that was significantly different over the course of the study, as it was higher in the ML group on day 30. Otherwise, the composition of gut bacterial communities did not differ between groups; although a non-significant trend of increasing *Enterobacteriaceae* was seen in the ML group. In this study, PN accounted for at least 80% of the neonates' energy intakes; therefore, some neonates may have also received up to 20% of their energy through EN. The authors did not indicate whether differences were noted in those who received PN alone. Wan et al.[82] later reported that the gut microbiota of mice provided PN was maintained similar to that of chow-fed mice when a minimum of 20% of energy intake was provided through EN. Hence, changes in, or lack thereof, the fecal bacterial communities of

these neonates could be attributed to the inclusion of EN. It is also possible that the sample size was not large enough to detect differences in this clinical study. In addition, neonates with liver disease or problems associated with the bile duct were excluded from the study. As liver disease is one of the most common and serious complications in neonates and infants receiving PN[1, 34] this significantly limits the relevance of the study. Research in this population should be a priority to further our understanding of the role of microbial dysbiosis in IFALD, the most common life-threatening complication in these infants.

Another important factor to consider is the use of antibiotics, as microbial dysbiosis and decreased microbial diversity has been noted in preterm infants treated with antibiotics.[119, 120] Harris et al.[117] showed just that effect in the mouse model; the gut bacterial communities were quite different in PN-fed mice with liver injury that were treated with antibiotics compared to those that weren't. In animal studies, antibiotics have been shown to improve both gut barrier [121] and immune[122] function. Arboleya et al.[118] did not report on the neonates' individual diagnoses or any antibiotics provided as treatment for illness and comorbidities. These confounding factors could drastically affect the results of the investigation.

Finally, because these studies assessed bacterial content of fecal samples, they cannot report on where along the intestinal tract the microbiota may have changed. Further, bacterial translocation to the liver was assessed only in the animal study[117] and was not evaluated for tissues such as the lymph nodes or the spleen. It is possible that more severe microbial dysbiosis or translocation may have occurred along the intestinal tract, but these outcomes were simply not measured.

1.5.5 Parenteral Lipid Composition and Bile Acid Metabolism

Vlaardingerbroek et al.[35] are the only authors to date to study the impact of PN lipid composition on bile acid metabolism. Piglets delivered by caesarean section 7 days preterm were PN-fed for 14 days. Piglets were provided a PN lipid of either SO, ML, or FO. Indeed, the bile acid concentrations in plasma and in liver differed with PN lipid modulation, as did the bile acid profiles in plasma and the expression of bile acid synthesis and transport genes in the liver. PN was associated with increased plasma bile acids, which were highest with SO. PN was also associated with decreased gene expression of FXR, CYP7A1 and BSEP. SO had the highest expression OSTA. Finally, the plasma bile acid pool in both SO and FO were the most hydrophobic, and thus hepatotoxic. The lipid dose provided in this study was 5 g/kg/d, whereas a standard lipid dose in piglets is 10 g/kg/d. Given that lipid restriction has been shown to reduce the risk of IFALD,[44] it is possible that molecular mechanisms would differ when lipid is provided at a standard dose.

1.6 THE NEONATAL PIGLET MODEL FOR UNDERSTANDING THE PATHOGENESIS OF IFALD

Use of animal models allows for invasive procedures, such as cannulation of the bile duct to measure bile flow, which requires anaesthesia over a long period of time and a terminal laparotomy. Further, animal models allow for the collection of tissues that would only be feasible post-mortem in a human neonate. In this regard, relatively large samples of intestinal scrapings can be collected from the neonatal piglet to allow for bacterial sequencing of various sites along the GI tract. Additionally, tissues from the liver and other organs can be sampled from an animal model without subjecting a human infant to disruptive and aggressive procedures.

For an animal to be a suitable model for human neonates, it must display similar characteristics in terms of the systems or diseases being studied. The intestinal anatomy,

ontogeny, physiology and metabolism, as well as body composition, of newborn piglets are similar to premature infants.[123] The rapid growth rate of piglets,[123, 124] which is 5 times that of human infants, allows for quick detection of changes in the various body systems. However, energy needs[125] are also greater for piglets; thus, a lipid dose of 10g/kg/d in a piglet is translatable to a dose of 2g/kg/d in a human infant, and often appears to clinicians as an extremely high-dose treatment.

Neonatal piglets are a validated model of PN-fed human infants,[73, 125] as well as neonatal SBS[126] and IFALD.[61, 127] Piglets offer an advantage in allowing the assessment of mechanisms that underlie long-term PN under controlled conditions, without an ethical requirement to provide EN, whereas the clinical priority in caring for a human neonate is to advance EN feeds as quickly as is safe and feasible for the infant. Molecular mechanisms of bile acid metabolism are similar between piglets and human infants, but differences in bile acid composition between piglets and humans limits the ability to ascertain the role that specific bile acid species play.[128, 129]

Nejdfors et al.[130] concluded that the rate and extent of GI permeability in pig correlated with that in human. The microbiota of the GI tracts of pig and human are similar in that Firmicutes and Bacteroidetes are the dominant phyla, though differences in dominant genera should be noted.[131] Specific to the neonate, *Bacteroides* and *Escherichia/Shigella* are similarly predominant in the early stages of life, although differences exist in the relative abundance and presence of Bifidobacteria, *Lactobacillus*, and *Streptoccus*.[131]

1.7 KNOWLEDGE GAPS

1.7.1 The Role of Remnant SBS Anatomy in IFALD

Currently there is a marked paucity of evidence-based treatments for IFALD.[1, 28, 34] Despite the incidence of IFALD being greatest for preterm neonates and infants with long-term dependence on PN,[33, 34] most of what we know about the impact of PN on the gut comes from studies in animals and adult humans. Further, in human infants, diseases such as NEC and intestinal atresia commonly involve the ileum; thus, the JC anatomy is the most common clinically encountered anatomy.[5, 17, 132] Despite this, animal studies have traditionally used models of the JI anatomy.[69, 133-137] Moreover, many studies of SBS are confounded by the provision of EN.[69, 134, 136, 138-140] As little as 20% of energy provided through EN preserves EBF while impacting immune function and the gut microbiota.[82] Thus, investigations of the mechanisms of neonatal IFALD, including SBS anatomies relevant to the neonate, and in the setting of 100% PN are warranted.

To date, a single study by Mutanen et al.[141] has investigated the impact of remnant anatomy on markers of IFALD. Specifically, the study examined fibroblast growth factor 19 (FGF19) in relation to histological liver injury. The study was conducted in young children with IF (2.2 to 12.6 years of age), the majority of whom had weaned off PN a median of 3.9 years prior to the study. The authors concluded that absence of the ileum resulted in decreased levels of FGF19 that corresponded to hepatic inflammation and fibrosis.

Studies assessing remnant anatomy on EBF, immune function, and bile flow are lacking. A study in rats analysed the expression of bile acid transporters in the ileum along with bile acid pool size given intestinal resections varying in length and location.[139] As the rats were chow-fed adults, the results are likely not relevant to the PN-fed neonate.

There is a long-held assumption that absence of the ICV increases bacterial translocation. Yet, the limited evidence that is available, albeit conducted in adult rats, suggests that bacterial

translocation to the lymph nodes and portal blood is lower in gut-resected rats without of an ICV compared to those with the ICV in situ, in the setting of either EN[142] or PN.[143] Such evidence is lacking in the neonatal population. Even though remnant anatomy per se is not modifiable, understanding the mechanisms related to remnant anatomy that promote IFALD will allow for the development of pertinent, evidence-based treatments.

1.7.2 The Effect of Parenteral Lipid on the Gut Microbiome and Host Responses

Although we have in vivo evidence that decreased EBF[86] and microbial dysbiosis[118] in preterm infants are associated with PN, we do not know whether these factors contribute to IFALD. Animal studies have begun to elucidate the relationships between PN and EBF, immune function, and the gut microbiota,[79, 82] but more evidence is needed to understand the etiology of IFALD. Modulation of the PN lipid was shown to improve EBF in vitro,[112] but in vivo evidence is lacking in the literature. There is also evidence in adult populations that immune function may be improved with the inclusion of FO in the PN,[114, 115] but research in neonatal and infant populations is quite limited. Lastly, evidence is beginning to emerge that PN lipid composition does affect the gut microbiota,[117, 118] but further exploration is needed to determine the ratio of fatty acids that is needed to promote optimal composition of the microbiota in relation to decreasing the risk of IFALD in preterm neonates. Further, if the relationship between EBF, immune function, the gut microbiota and IFALD is better understood, then probiotic and antimicrobial treatments also become plausible.

1.7.3 The Impact of Parenteral Lipid on Bile Acid Metabolism and Transport

Until recently, the sole treatment for IFALD was a complete transition to EN, with the discontinuation of PN. There is evidence that ML and FO parenteral lipids improve IFALD[35-37] in comparison to conventional SO.[38] But the molecular mechanisms implicated in the

development of IFALD, and the improvement of the disease with the use of FO-containing PN lipid, are poorly understood. The first evidence that PN lipid modulation alters bile acid metabolism was discovered in preterm piglets provided low-dose PN lipid.[35] However, although PN lipid restriction is used in clinical practice to prevent and treat IFALD,[36, 41, 144] clinical guidelines from the American Society for Parenteral and Enteral Nutrition rate the evidence for use of the practice as very low, and give only a weak recommendation for its use.[1] As such, the molecular mechanisms in bile acid metabolism warrant further elucidation when lipid is provided at a non-restricted dose.

Clearly, many gaps remain in our knowledge of the pathogenesis of IFALD. A better understanding of the mechanisms of IFALD, and potential treatments, will allow us to develop clinical practice guidelines that will improve the outcomes for preterm neonates with IF.

1.8 RESEARCH OBJECTIVES AND HYPOTHESES

IFALD is a life-threatening complication, especially in neonates and infants who depend on PN. There remains much to understand about the mechanisms involved in IFALD, leaving us with few solid recommendations for preventing the disease. It is important that we develop a better understanding of the pathogenesis of IFALD to develop strategies for its prevention, especially for the highest risk population: the neonate with IF.

In our proposed pathogenesis of the disease we suggest that PN leads to IFALD because of an interplay between decreased EBF, effects on GI immune function, alterations in bile acid metabolism, and changes in the gut microbiota (Figure 1-2). We also suggest that an optimal PN lipid composition can moderate these effects of PN and hence prevent the onset of IFALD. Sepsis is a risk factor for IFALD,[1] and may be a direct result of contamination in the PN

emulsion. Sepsis may directly impact bile acid metabolism and be a cause or consequence of PN-related alterations in EBF, immune function, or the microbiota.

1.8.1 Objectives

The purpose of this thesis is to define the mechanisms through which SBS surgical anatomy and parenteral lipid impact the development of IFALD. The specific objectives are:

1. To determine the impact of remnant SBS anatomy on the onset and progression of IFALD in 100% PN-fed neonatal piglets.
2. To determine the impact of PN lipid formulation on the gut microbiome and host-responses in 100% PN-fed neonatal piglets.
3. To investigate mechanisms for improved bile flow with the use of FO-containing PN lipid formulations in 100% PN-fed neonatal piglets.

1.8.2 Hypotheses

1. PN-fed SBS piglets without ileum and ICV valve will have early onset and more rapid progression of IFALD; demonstrated by lower bile flow compared to PN-fed SBS piglets with intact ileum and ICV.
2. FO-containing PN lipid will reduce the host immune response and maintain a microbiota associated with reduced inflammation in the GI tract of PN-fed neonatal piglets.
3. FO-containing PN lipid, as compared to SO lipid, will result in increased gene expression of FXR, resulting in decreased expression of CYP7A1 and increased gene expression of canalicular bile transporters BSEP and MRP2.

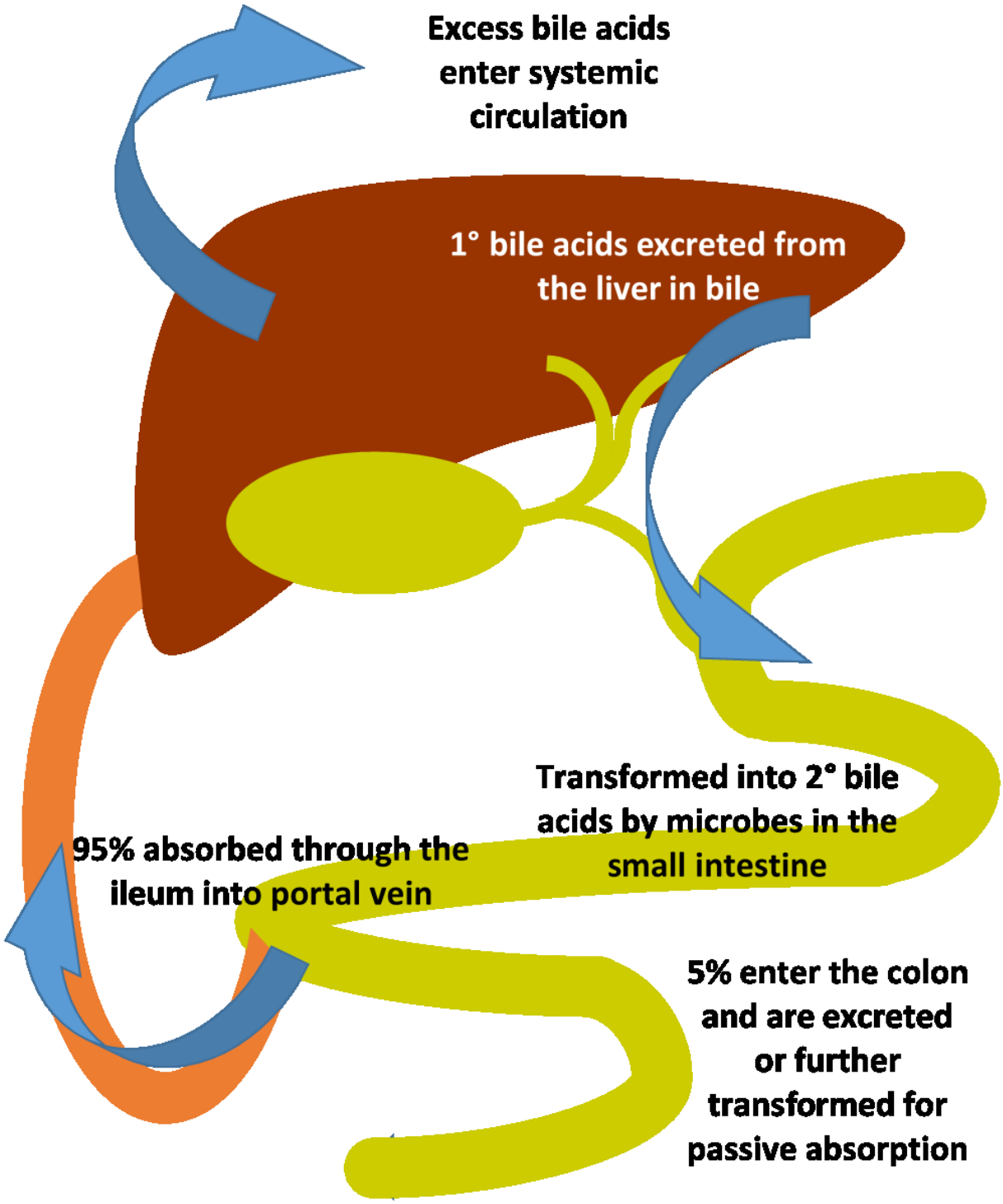

Figure 1-1: Enterohepatic circulation of bile acids.

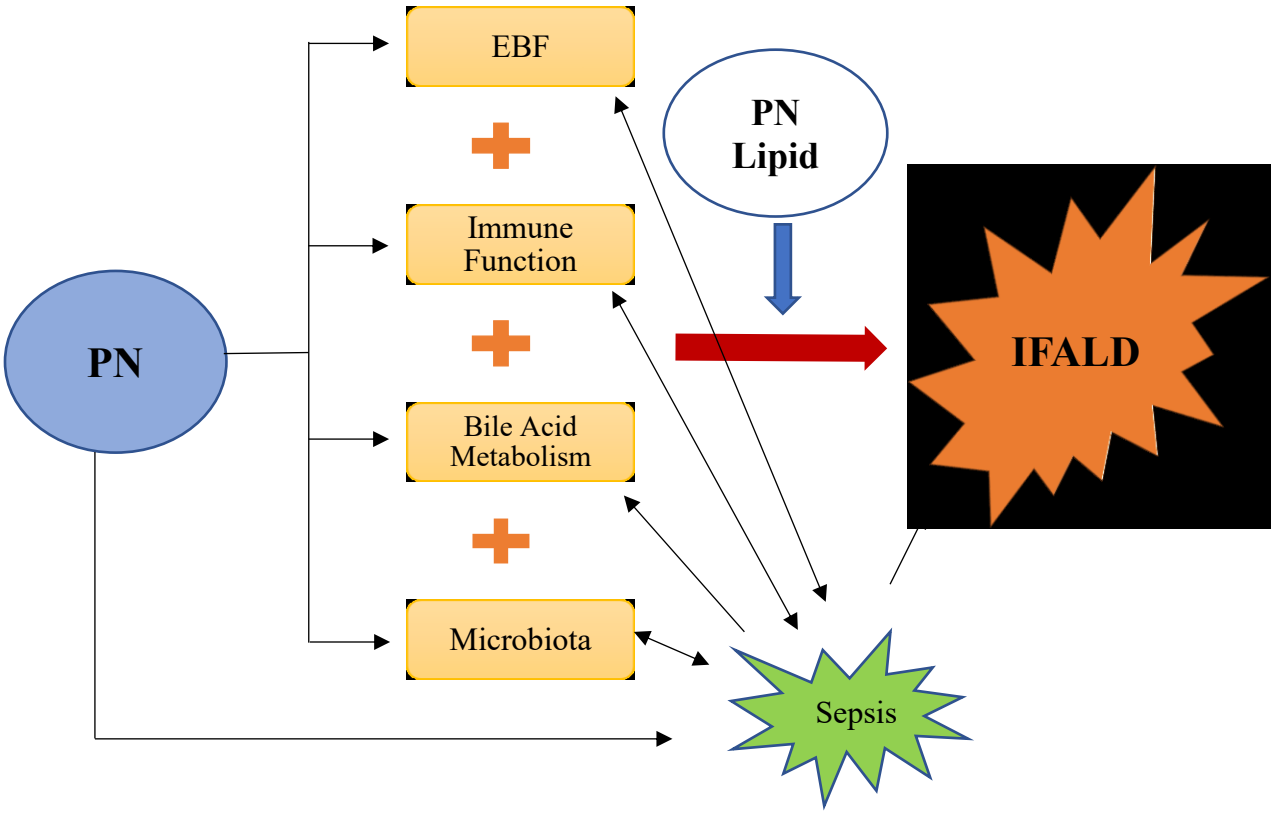

Figure 1-2: Proposed pathogenesis of IFALD.

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CHAPTER 2: Surgical Anatomy does not Impact the Progression of Intestinal Failure-Associated Liver Disease in Neonatal Piglets

Adapted from:

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2.1 ABSTRACT

Background: Intestinal failure-associated liver disease (IFALD) causes significant morbidity in neonates with SBS who are dependent on PN. Resected ileum, with loss of the ICV, is the most common anatomy in SBS, yet its impact on IFALD has not been adequately studied

Methods: Neonatal piglets were randomized to 75% intestinal resection with jejunocolic anastomosis and no ileum (JC, n=12); 75% resection with jejunoileal anastomosis and intact ileum (JI, n=13); sham without resection (Sham, n=14); or sow-fed control (SF, n=8). Surgical and Sham piglets received 100% PN for 14 days before bile flow was measured and blood chemistry, liver pathology, jejunal permeability, and bacterial translocation assessed.

Results: Bile flow was lower for PN-fed (SBS and Sham) compared to SF (P = .002), but not different between the PN-fed groups. Total bilirubin (P = .03) and liver pathology (P < .001) were greater in PN-fed than SF, but not different between PN-fed groups). Serum bile acids were increased in Sham (P = .01), but not different between SBS groups. PN-fed piglets with sepsis had lower bile flow (P = .001) and increased bilirubin (P = .04) than those without. Neither jejunal permeability nor bacterial translocation were different between JC, JI or Sham groups.

Conclusion: Contrary to our hypothesis, the remnant anatomy, including absence of the ileum and ICV, does not appear to worsen the progression of IFALD. However, the role of sepsis in IFALD should be further explored, in addition to other mechanisms, including PN factors, host immune responses and intestinal bacterial dysbiosis.

2.2 INTRODUCTION

Intestinal failure-associated liver disease (IFALD) occurs in infants and children with intestinal failure that are reliant on long-term PN.[1] IFALD is a leading cause of death in infants

with SBS.[2] Although IFALD is a devastating condition, its mechanisms are not well understood. Infants with severe IFALD often need a liver transplant, but many die while awaiting transplant.[3] Recently, it has been identified that soy-based parenteral lipid has a role in IFALD pathogenesis.[4] However, end stage liver disease with fibrosis has also been noted in patients receiving omega-3 parenteral lipids,[5] and hence the need for transplantation remains. Thus, it is crucial that we better understand the mechanisms of IFALD so we can design further treatments to reduce the incidence and severity of the disease.

In SBS associated with severe intestinal failure it is common to have resection of most or all of the ileum,[2, 6, 7] and absence of the ICV is believed to be a risk factor for IFALD.[8] In a cross-sectional study of patients with pediatric-onset intestinal failure, either weaned from or continuing to receive PN, liver fibrosis was observed to be more advanced in patients without an ICV, and lack of the ICV was determined to be the strongest predictor of fibrosis.[7] Yet despite observations that the provision of PN and remnant anatomy in SBS may increase the risk of IFALD, studies of IFALD mechanisms often use gut-resected, enterally-fed animal models with the ileum and ICV in situ.[9-13] Evidence is beginning to emerge on the differential effects of surgical anatomy on the development of IFALD,[14] but much is yet to be learned. Investigations of the mechanisms of IFALD should also include the most relevant surgical anatomies, including absence of the ileum and ICV. While remnant anatomy per se, may not be modifiable in the clinical setting, it may well be related to an increased risk of bacterial dysbiosis and translocation that could potentially be a modifiable factor in the progression of IFLAD, in addition to the PN formulation.

The purpose of this study was to determine the impact of remnant SBS anatomy, with or without ileum and ICV, on the progression of IFALD in a well-established neonatal piglet model

of SBS provided 100% PN. Liver disease was hypothesized to be most severe in SBS piglets without ileum or ICV, followed by SBS piglets with intact ileum and ICV, and least severe in gut-intact sham piglets. A sow-fed control was included and expected to show no signs of liver disease. A secondary hypothesis was that bacterial translocation would be associated with sepsis and that, again, this would be greatest in piglets without ileum, followed by those with ileum, then gut-intact shams.

2.3 METHODS

2.3.1 Animal Surgeries and Care

This study was conducted as per the guidelines given by Canadian Council on Animal Care and with approval of the University of Alberta Animal Care and Use Committee (AUP#00000153), in a bio-secure swine research facility. Male and female Duroc x (Large White x Landrace) piglets from the University of Alberta Swine Research and Technology Centre (SRTC) were randomized by litter where possible to: 75% distal intestinal resection with jejunocolic anastomosis and no ileum or ICV (JC); 75% proximal resection with jejunoileal anastomosis and intact ileum and ICV (JI); PN-fed sham without resection (Sham); or sow-fed control (SF).

At 2-5 days of age, SBS and Sham piglets underwent surgery for jugular catheter placement and intestinal resection as per their treatment allocation, after which they received 100% PN for 14 days. They were housed in individual cages, attached to a swivel system to allow PN infusion and freedom of movement. The room was temperature-controlled with a 12-hour light/dark cycle. SF piglets remained with their sow, and all piglets were humanely euthanized 14 days later at 16-19 days old. PN was commenced following the surgery, using a sterile PN solution prepared in our laboratory as reported elsewhere.[15] Nutrient targets were

previously validated in a neonatal piglet model of parenteral nutrition to meet the needs of piglets that grow at a rate 5 times that of human infants.[16] As such, lipid was provided at a dose of 10g/kg/d, which is comparable to a dose of 2g/kg/d in a human neonate; dextrose was provided at 29g/kg/d, and protein at 16g/kg/d. The lipid formulation used was Intralipid® (Fresenius Kabi, Bad Homburg, Germany), which is a soybean oil emulsion expected to be associated with development of IFALD in this model, including decreased bile flow and increased bilirubin, serum bile acids, and GGT.[17, 18]

Post-surgery, to reduce the risk of sepsis through the venous catheter, piglets were administered ampicillin (10mg/kg twice a day; Sandoz, Boucherville, QB, Canada), and trimethoprim (20mg/d) with sulfadoxine (100mg/d; Merck Animal Health, Kirkland, QB, Canada) on days 1-4 and 8-12. Piglets were monitored for signs of sepsis, including lethargy, vomiting, and fever; at first signs, blood was drawn for culture analysis. Piglets with suspected sepsis were treated with either extra days of ampicillin and trimethoprim with sulfadoxine or, if they failed to improve, with the addition of enrofloxacin (5mg/kg/d; Bayer Animal Health Mississauga, ON, Canada) or gentamicin (3mg/kg/d; Sandoz, Boucherville, QB, Canada). A positive blood culture confirmed sepsis.

2.3.2 Bile Flow and Liver Chemistry

To measure bile flow from the liver, a terminal laparotomy was performed on day 14 and the common bile duct cannulated as previously described.[15] Blood was collected at the same time for the measurement of bilirubin, serum bile acids, and GGT.

2.3.3 Liver Pathology

A section of liver was preserved in 10% buffered formaldehyde (Histoprep; Fisher Scientific, Ottawa, ON, Canada). Five-micrometer sections were then stained for histological

analysis. A liver histology grading system[19] was modified and used to quantitatively compare liver pathology. Nine parameters, including necrosis, cholestasis and apoptosis, were scored as either 0 - normal, 1 - mild to moderate, or 2 – severe, for a maximum possible total of 18, as previously reported.[18]

2.3.4 Jejunal Structure

At 40cm distal to the ligament of Treitz, a 1cm cross section of jejunum was collected and preserved in 10% buffered formaldehyde (Histoprep; Fisher Scientific, Ottawa, ON, Canada). Sections were then embedded in paraffin, trimmed, placed in cassettes, and stained to measure jejunal villus height and crypt depth as described elsewhere.[18] Measurements were completed by a veterinary pathologist blinded to the treatment groups.

2.3.5 Jejunal Permeability

Apparent permeability (P_{app}) across *ex vivo* jejunum was measured using a modified Ussing chamber methodology.[20] Following dissection of the intestine, the first 20cm of jejunum distal to the ligament of Treitz was placed in ice-cold Krebs buffer supplemented with sodium L-glutamate (4.9mM), disodium fumarate (5.4mM), sodium pyruvate (4.9mM) and D-glucose (11.5mM), bubbled continuously with O_2/CO_2 (95%/5%). Jejunal segments were then mounted in modified Ussing Chambers (Harvard Apparatus Inc, Holliston, MA), and bathed in oxygenated Krebs buffer at 37°C, as previously described.[21] The exposed surface area was 1.15cm^2. After equilibrating the tissues for 30 min, radiolabelled markers [^{14}C]mannitol and [^{3}H]polyethylene glycol (PEG) (Perkin Elmer) were added to donor chambers at time 0. Samples were taken from the receiver chamber every 20 min over 180 min. P_{app} was calculated at steady state in cm/s. Potential difference (PD), short-circuit current (Isc), and transepithelial electrical resistance (TER) were analysed in a subset of piglets (JC=8, JI=4, Sham=12). All tissues were

viable and had baseline PD values >2mV. TER was calculated using the Isc and PD, as described previously.[21]

Gene expression of tight junction proteins ZO-1, OCLN, and CLDN-1 in jejunal tissue were measured as another indicator of differences in permeability. The GeneJET RNA Purification kit (Thermo Scientific) was used to extract total RNA using Lysis buffer supplemented with β-mercaptoethanol. Manufacturers instructions were followed with an additional on-column DNAse (QIAGEN) treatment for 15 min. RNA was quantified on a Nanodrop ND-1000 (NanoDrop Technologies, Wilmington, DE, USA), and 1µg RNA was used to synthesize cDNA with the qScript^{TM} Flex cDNA Synthesis Kit (Quanta Biosciences) using a mixed primer strategy. Quantitative PCR (qPCR) was performed using the DyNAmo Flash SYBR Green qPCR Kit (Thermo Scientific) with Sus scrofa primers (Table 2-1) on a StepOnePlus Real-Time PCR System (Applied Biosystems). A 10µl reaction volume was used under the following conditions: 95°C for 3 min followed by 40 cycles at 95°C for 10 s and annealing temperature for 30 s. Glyceraldehyde-phosphate-dehydrogenase (GAPDH) was chosen as an endogenous standard for normalization. Relative gene expression was calculated with the delta delta CT method.

2.3.6 Bacterial Translocation

Bacterial translocation from the gut to the lymph nodes as well as into portal blood, and its association with sepsis was a secondary outcome of interest beyond IFALD. Therefore, bacterial densities in mesenteric lymph nodes and portal blood were determined. Lymph nodes were collected from the mesentery at and distal to the cecum, and placed in 1.5 ml bead-beating tubes filled with 250µl glass beads and 1ml ice-cold phosphate buffered saline with cysteine. The tubes were weighed before and after adding the lymph nodes to obtain a bacterial density of

colony forming units (CFU)/gram of lymph node tissue. The tubes were beat 3 x 1 min, and cooled on ice between each beating. Blood was collected from the portal vein in a blood tube with ethylenediaminetetraacetic acid (EDTA) immediately prior to euthanasia. Under anaerobic conditions, 100µl of the lymph node mixture and 100µl of portal blood were plated in duplicate on fastidious anaerobic agar and incubated for 24 hrs at 37°C before being counted. Counts for the 2 mesenteric lymph node samples for each piglet were averaged and standardized to CFU/g for lymph nodes. Counts were determined as CFU/ml for portal blood.

Bacteria found in the lymph nodes of a subset of piglets were identified by sequencing the 16S rRNA gene, to indicate the potential origin of the bacteria. Individual colonies were picked, boiled for 10 min, and stored at -20°C for colony polymerase chain reaction (PCR). Hypervariable regions of the *16S rRNA* gene were amplified using 8F: 5′-AGAGTTTGATCCTGGCTCAG-3′) and 926R: 5′-CCGTCAATTCCTTTRAGTTT-3′ primer pairs in 50µl reaction volumes. The conditions for the PCR were: initial denaturation at 94°C for 5 min; 40 cycles of 94°C for 10s, 58°C for 30s and 72°C for 60s; then a final extension at 72°C for 7 min. Amplicons were sequenced using Sanger DNA sequencing. RDP Release 11, Update 5 (September 30, 2016; https://rdp.cme.msu.edu/) was used for sequence classification.

2.3.7 Statistical Analysis

Data are expressed as mean ± standard deviation for parametric data, and median ± interquartile range for non-parametric data; $P \leq .05$ was considered significant. Statistical software "R", version 3.2.,3[22] along with "car"[23] and "agricolae"[24] packages, was used for statistical analysis. Analysis of variance (ANOVA) and Welch's ANOVA were performed for parametric data with homogeneous or non-homogeneous variance respectively. Kruskal-Wallis ANOVA was performed for non-parametric data. Post hoc testing for parametric data was conducted using

Tukey's Honestly Significant Difference or pairwise comparisons for unequal variances with the Holm-Bonferroni Method for multiple inferences. Mann-Whitney-Wilcoxon tests were conducted for post hoc tests of non-parametric data. Student's t-tests, the Welch-Satterthwaite variance approximation, or Mann-Whitney-Wilcoxon tests were used to compare septic and non-septic piglets for parametric data with homogeneous variance, parametric data with non-homogeneous variance, and non-parametric data respectively.

2.4 RESULTS

2.4.1 Animal Outcomes

A total of 47 piglets (12 JC, 13 JI, 14 Sham, 8 SF) were used in the study. Seven (7) piglets did not reach the study endpoint due to mortality: 2 JC and 1 JI from sepsis with *Escherichia coli*, 1 JI due to aspiration post-surgery, 1 JI due to advanced liver failure as determined by a veterinary pathologist, 1 JI from sepsis with *Lactobacillus plantarum*, and 1 Sham from sepsis with *Enterococcus faecium*. Thus, data from 10 JC, 9 JI, 13 Sham, and 8 SF piglets were collected for analysis.

Of those piglets used in the study, confirmed sepsis was more prevalent in Sham (7/14=50%) than JC (4/12=33%) or JI (4/13=31%) piglets. Although another 1 JC and 2 JI piglets were clinically suspected of having sepsis, blood culture results were negative. Blood cultures from septic piglets were positive for *Candida parapsilosis* (n=3), *Enterobacter cloacae* (n=1), *Enterococcus hirae* (n=1), *Enterococcus faecium* (n=3), *Escherichia coli* (n=5), *Lactobacillus plantarum* (n=4), and *Staphylococcus aureus* (n=1), either alone or in combination.

2.4.2 Bile Flow and Liver Chemistry

Bile flow was measured in 9 JC, 9 JI, 12 Sham, and 7 SF piglets as the bile duct could not be cannulated in 3 animals (1 JC, 1 Sham, 1 SF). While bile flow was lower in PN-fed compared to SF piglets (P = .002), it was not different between JC, JI or Sham groups (Figure 2-1). In PN-fed piglets, bile flow was lower in those with sepsis than those without (1.3±0.6 vs 4.8±3.7µg/g liver/10min respectively; P = .001).

Total bilirubin is a common clinical biomarker used to indicate the presence of IFALD, and a cut-off of 10µmol/L (just above the peak value in the SF control group) was chosen to define the presence of IFALD. Bilirubin could not be obtained in 1 JC piglet. IFALD was present in 78% of JC (7/9), 67% of JI (6/9), and 85% of Sham (11/13) piglets. Bilirubin was higher in PN-fed animals compared to SF (P = .03), but it was not different between treatment groups (Figure 2-2A). Bilirubin levels were higher in PN-fed piglets with sepsis than those without (23.4±6.0 vs 13.25±10.3µmol/L; P = .04).

Serum bile acids were higher in Sham than any other group (P = .01), but not different between JC, JI, and SF groups (Figure 2-2B). In PN-fed groups, bile acids tended to be higher in septic animals, but this did not reach statistical significance (septic animals=20.7±16.5µmol/L, non-septic animals=10.5±20.7µmol/L; P = .06).

PN-fed piglets had significantly higher GGT levels than SF (P < .0001). Although GGT was elevated in JC, JI, and Sham piglets, it was similar between all 3 of these groups (Figure 2-2C). GGT was not different between septic and non-septic PN-fed piglets (399.0±259.0 vs 237.5±347.25 IU/L; P = .39).

2.4.3 Liver Pathology

Liver histology was available for 9 JC, 8 JI, 11 Sham, and 8 SF piglets. An elevated liver pathology score indicates greater liver pathology. Livers in all PN-fed piglets had higher

pathology scores than SF (JC=4.3±2.2, JI=5.0±3.1, Sham=5.1±3.0, SF=0.0±0.0; $P < .001$), but the scores were not different between PN-fed groups (Figure 2-3). Liver pathology scores were not different between PN-fed septic and non-septic piglets (5.6±3.3 vs 4.6±2.5; $P = .41$).

2.4.4 Jejunal Structure

Villus height was shorter in all PN-fed groups than SF (JC=371±93µm, JI=405±77µm, Sham=395±89µm; SF=666±89µm; $P < .0001$), but did not differ amongst SBS and Sham groups (Figure 2-4). Interestingly, septic piglets had longer villi than non-septic PN-fed piglets (4.4±0.8 vs 3.7±0.8µm; $P = .03$). Crypt depth was similar between all groups (JC=154±28µm, JI=162±22µm, Sham=149±27µm, SF=164±21µm; $P = .53$), and did not differ between septic and non-septic PN-fed piglets (P=0.38).

2.4.5 Jejunal Permeability

The jejunal mucosal-to-serosal P_{app} of mannitol and PEG were not different between groups (Figure 2-5). The P_{app} of mannitol was significantly higher in septic compared to non-septic PN-fed piglets (6.3±2.5 vs 4.2±2.5 x 10^{-6} cm/s; $P = .02$). However, there was no difference in the P_{app} of PEG between septic and non-septic PN-fed piglets (6.3±1.5 vs 4.2±2.3 x 10^{-6} cm/s; $P = .35$). There were no differences in electrical parameters between any of the groups analysed; PD (JC=5.4 ±1.3 mV, JI=4.5±1.1 mV, Sham=5.2±1.4 mV; $P = .51$), Isc (JC=86.5±30.1 uA/cm^2, JI=106.3±41.2 uA/cm^2, Sham=114.4±23.0 uA/cm^2; $P = .13$), or TER (JC=77.6±21.0 ohms/cm^2, JI=58.7±34.0 ohms/cm^2, Sham=53.3±20.3 ohms/cm^2; $P = .09$).

Gene expression of tight junction proteins ZO-1, OCLN, and CLDN-1 were not different amongst any of the groups (Figure 2-6). Comparing septic to non-septic PN-fed piglets, gene expression was not different for ZO-1 (1.6±1.2 vs 1.5±1.0 fold change from sow-fed; $P = .56$),

OCLN (1.4±0.6 vs 1.5±0.6 fold change from sow-fed; P = .56), or CLDN-1 (1.5±0.9 vs 1.4±1.1 fold change from sow-fed; P = .78).

2.4.6 Bacterial Translocation

Any bacterial growth deemed too numerous to count was set at an upper limit of detection of Log_{10} 8.0 CFU/g; bacterial counts of zero were set to a lower limit of detection of Log_{10} 2.0 CFU/g or CFU/ml. Bacterial density in the lymph nodes was lower in SF than JC and JI piglets, but not different from Sham piglets (P = .03); it was similar amongst all SBS and Sham groups (Figure 2-7A). Septic piglets had greater bacterial density in the lymph nodes than non-septic PN-fed piglets (4.8±0.7 vs 3.4±1.1 Log_{10} CFU/g; P < .01). Bacterial density in portal blood was not different between groups (P=0.27) (Figure 2-7B), nor between septic and non-septic PN-fed piglets (P = .34).

To get an indication of the potential source of these bacteria, a subset of bacterial isolates was characterized by Sanger sequencing of the 16S rRNA gene. A total of 10 sequences of sufficient quality to identify bacteria isolated from lymph nodes were obtained. Except for one isolate identical to *Staphylococcus epidermidis*, the best sequence matches for the isolates were sequences from gut origin including *Clostridium perfringens, Clostridium innoccuum* (2), *Escherichia coli, Enterococcus faecalis,* unclassified *Enterococcus*, unclassified *Bacteroides*, unclassified *Enterobacter*, and unclassified *Enterobacteriaceae*.

2.5 DISCUSSION

Surgical anatomy has long been assumed to be associated with IFALD severity; however, in this preclinical, translational model of neonatal SBS with intestinal failure, we did not find that to be the case. In the setting of 100% PN, surgical resection appears of less importance than the PN solution or the occurrence of sepsis for the development of IFALD.

Decreased bile flow is a key indicator of cholestatic liver disease and we hypothesized that bile flow would be lowest in JC piglets, then increase progressively in JI, then Sham, and finally SF piglets. No difference was seen in bile flow of PN-fed piglets with differing intestinal anatomies. Hyperbilirubinemia is the most common clinical biomarker used to indicate the presence of IFALD, but bilirubin levels were not significantly different between JC and JI piglets. All other indicators of liver disease, such as bile flow, serum bile acids, GGT, and liver pathology scores were not different between these SBS anatomies. This contrasts with our previous report of worse liver pathology in JC compared to JI piglets.[18] However, those piglets were provided both PN and enteral nutrition and therefore the models are not directly comparable. Certainly, based on prior findings of limited adaptation in the JC group in that study, we believe it is plausible that SBS anatomy has a greater influence on intestinal adaptation, and hence on the severity or duration of intestinal failure, than on severity of liver disease. Absence of the ICV has long been associated with increased duration of PN.[25, 26] Contreras-Ramirez et al.[27] found that adaptation was seen in only 64% of patients lacking the ICV compared to 87.5% with the ICV, and that it took longer to achieve this adaptation. The reduced intestinal adaptation seen in SBS patients without an ICV may lead to longer dependence on PN and thus a greater risk of IFALD. The 14-day trial period in the current study may not have been of sufficient duration to allow for such differences in adaptation; thus, a longer trial period should be considered for future studies.

The reason for greater incidence of sepsis in Shams is unclear. First, it must be noted that not all bacteria are culturable, hence, blood culture results may be negative in piglets despite signs of sepsis. In this regard, it is notable that presumed sepsis was equivalent across all three groups. Second, it is also possible that since Sham piglets have a fully intact intestine, there is a

greater surface area through which bacteria could potentially translocate across the epithelium; this hypothesis should be tested in future studies.

As expected, villus height was shorter in PN-fed piglets. The reason for longer villi in septic piglets is unknown. Longer villi were noted in antibiotic treated compared to non-treated neonatal piglets provided PN for 3 days, followed by 3 days of enteral nutrition.[28] Although all piglets in the current study were provided antibiotics, those who showed signs of sepsis were given additional antibiotics.

Although we hypothesized that jejunal permeability would be highest in JC, followed by JI, then Sham and SF groups, no difference was found. Outliers in the JC and Sham groups resulted in high standard deviations in jejunal P_{app}; however, no explanation could be found as to why these piglets had increased permeability and thus no indication for excluding them from the analysis. These results are consistent with those from a study of mice in which no difference in permeability was seen between bowel-resected and sham animals 14 days post-surgery.[12] The mice in that study were provided a liquid diet, while the piglets in the current study did not receive any enteral nutrition, but post-operative functional adaptation may explain why P_{app} was not different in the current study. Similar to the current findings regarding gene expression of tight junction proteins, this laboratory previously reported no difference in the expression of OCLN between piglets provided a soybean-oil based PN-emulsion and SF piglets.[29] Sun et al.[30] found increased permeability and increased expression of ZO-1 and OCLN in PN-fed mice. That study was conducted in adult mice provided 7 days of PN and does not report which part of the small intestine was analyzed. It is plausible that variation in adaptive responses across animal species, animal stages of development and regions of the intestine account for these differences found in permeability and gene expression of tight junction proteins. In the current study of

neonatal piglets with SBS we utilised jejunal permeability, a region that was comparable between all groups.

Again, contrary to what was expected, loss of the ileum and ICV did not increase bacterial translocation from the gut to the lymph nodes. Rather, bacterial density in lymph nodes was greater in septic animals. Increased bacterial translocation to the lymph nodes and portal blood has previously been observed in resected rodents compared to non-resected,[31] although those rodents were fed enterally. A prior study in adult Wistar rats also found increased bacterial translocation in gut-resected animals, whether enterally- or PN-fed.[32] Interestingly, that study also found that PN-fed rats without the ICV had reduced bacterial translocation compared to those with the ICV. This, along with the increased rate of sepsis in the Sham group of the current study may suggest neither the ileum nor ICV have any protective roles in bacterial translocation.

Except for *Staphylococcaceae*, bacteria sequenced from the lymph nodes were those typically of gut origin; however, this does not preclude them from being from the metabolic cage environment. Characterization of lymph node bacteria was conducted in a subset of bacterial isolates; including a larger sample size would allow for deeper analysis. In addition, while bacteria were cultured on a media that aims to support growth of fastidious anaerobes as well as facultative anaerobes, it is possible that not all bacteria present in the lymph nodes were detected.

The results of this study suggest that surgical SBS anatomy is not a critical factor in the early development of IFALD; as such, other mechanisms must be explored. If IFALD is secondary to prolonged PN due to a lack of intestinal adaptation after resection of the ileum, treatments to improve intestinal adaptation may prove to be most effective at reducing the incidence of IFALD.[33] Although bacterial translocation was not affected by SBS anatomy in this study, the bacterial densities and communities within the remnant intestine have yet to be

quantified and characterized. Bacterial overgrowth and dysbiosis both have the potential to alter bile acid composition and metabolism, which may play critical roles in the development of IFALD. Factors that influence intestinal adaptation, reduce episodes of sepsis, or alter host immune responses should all be considered as potential mechanisms in the development and severity of life-threatening IFALD.

Table 2-1: Sus Scrofa primers used for qPCR to analyze host gene expression in piglets.

Gene	Primer (5'-3')	Annealing Temp. (°C)	Product Length
pZO-1_F	GAGTTTGATAGTGGCGTT	62	298 bp
pZO-1_R	GTGGGAGGATGCTGTTGT		
pOccludin_F	ATCAACAAAGGCAACTCT	62	157 bp
pOccludin_R	GCAGCAGCCATGTACTCT		
pCldn1_F	TGATGAGGTGCAGAAGATGC	60	174 bp
pCldn1_R	CCAGTGAAGAGAGCCTGACC		
pGAPDH_F	GTTTGTGATGGGCGTGAAC	63	147 bp
pGAPDH_R	ATGGACCGTGGTCATGAGT		

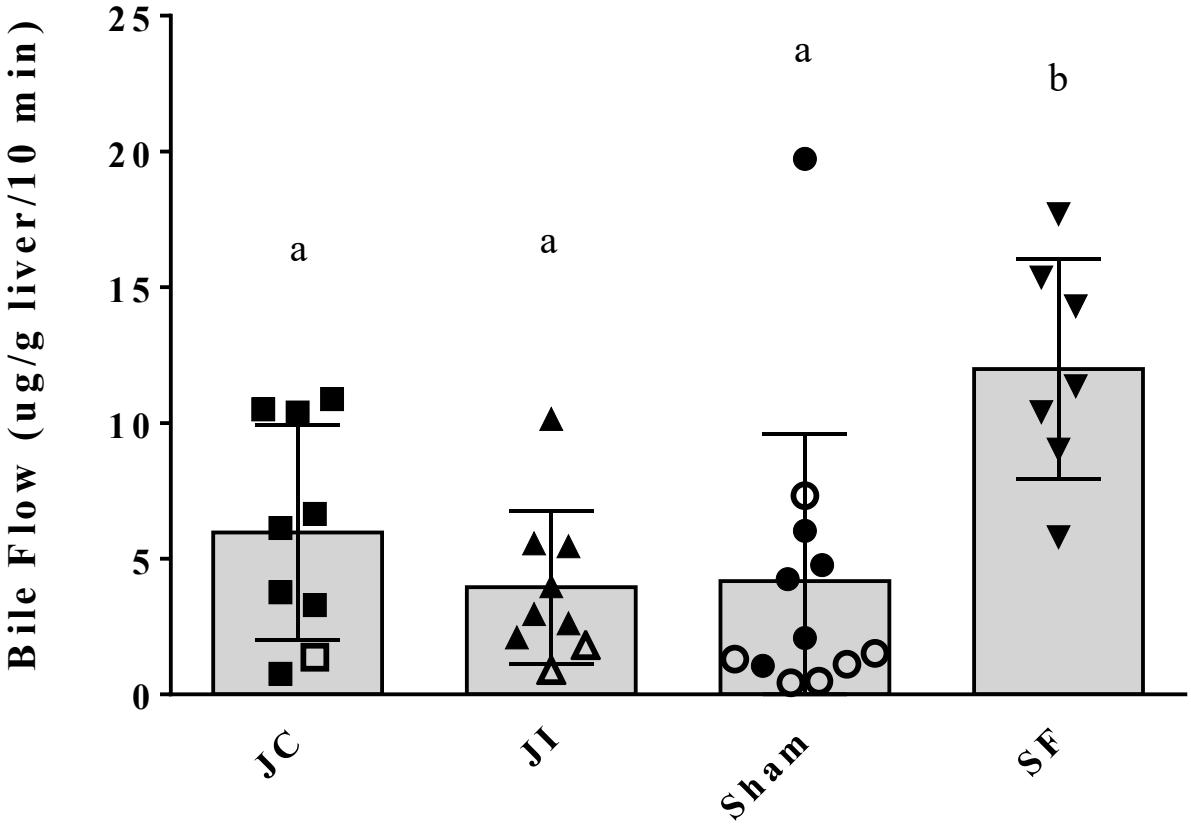

Figure 2-1: Bile flow in short bowel and gut-intact piglets.

Bile flow was lower in JC, JI and Sham groups compared to SF (P = .002), but not different between treatment groups. Mean ± SD; analysis of variance; a,b: between-group differences are noted by different letters. Septic piglets are denoted by open symbols.

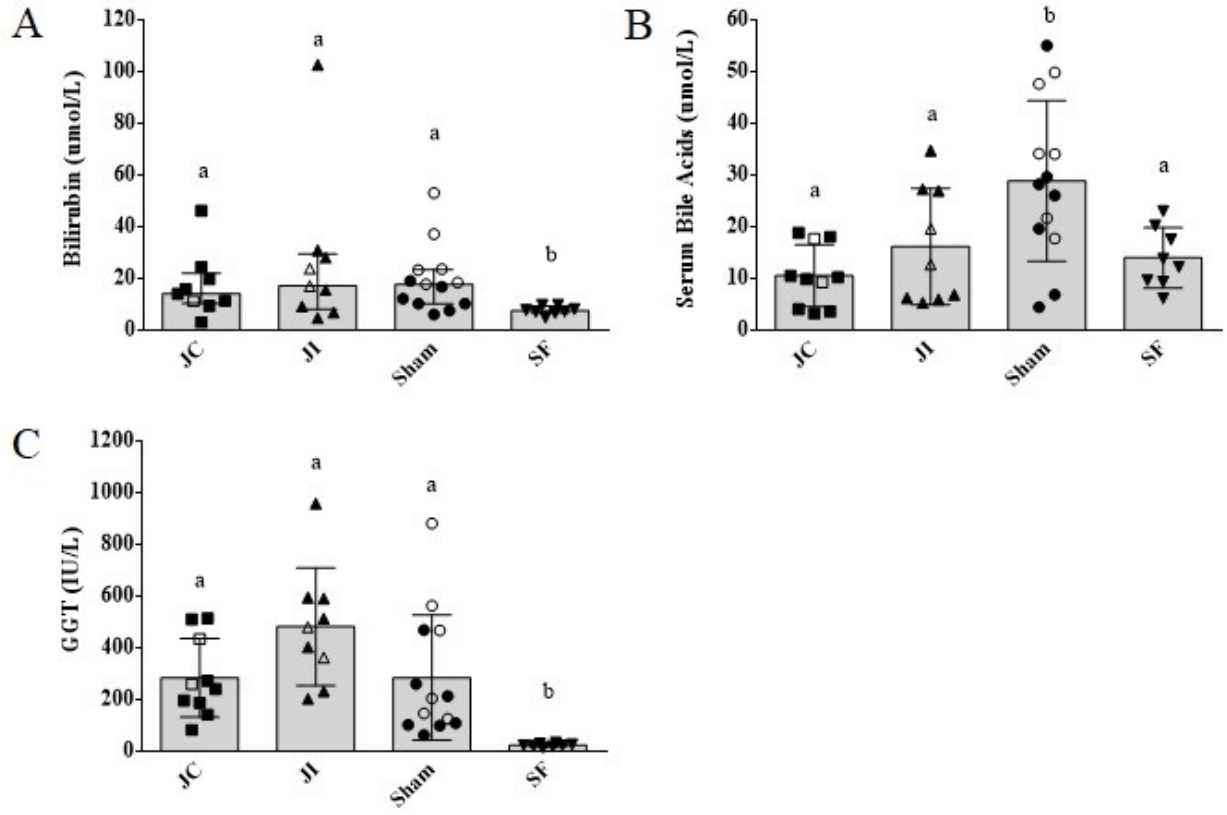

Figure 2-2: Liver chemistry in short bowel and gut-intact piglets.

A) Bilirubin was higher in JC, JI and Sham groups compared to SF (P = .03), but not different between treatment groups; median ± IQR; Kruskal Wallis analysis of variance. B) Serum bile acids were higher in Sham than all other groups (P = .01), but not different between JC, JI, and SF groups; mean ± SD; Welch's analysis of variance. C) GGT was higher in JC, JI and Sham groups compared to SF (P < .0001), but not different between treatment groups. Mean ± SD; Welch's analysis of variance. a,b: between-group differences are noted by different letters. Septic piglets are denoted by open symbols.

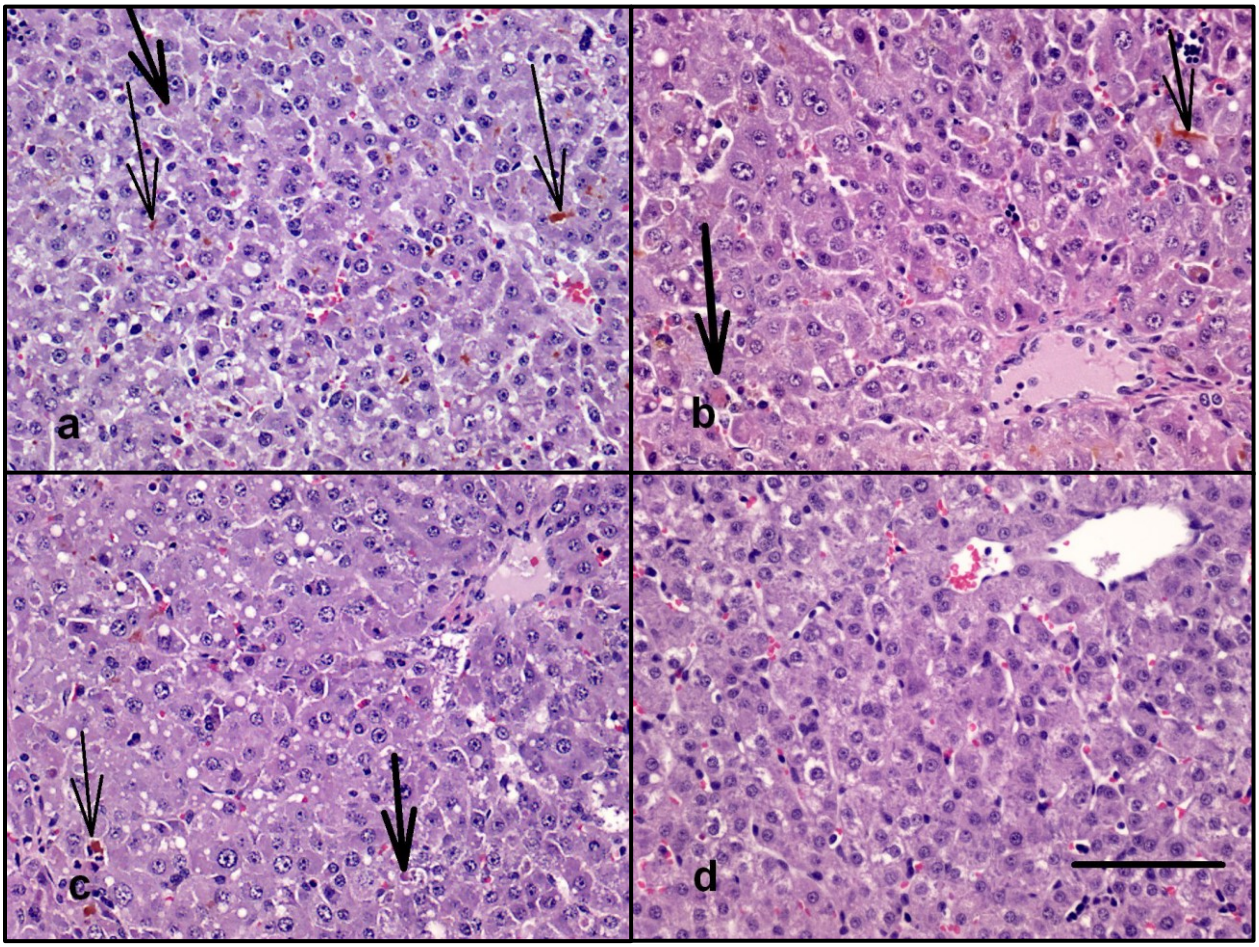

Figure 2-3: Histopathology of liver (200x magnification) from representative short bowel and gut-intact piglets.

a: JC, b: JI; c: Sham, d: SF. Thin arrows show bile in canaliculi. Thick arrows show individual necrotic hepatocytes. Bar = 0.1 mm.

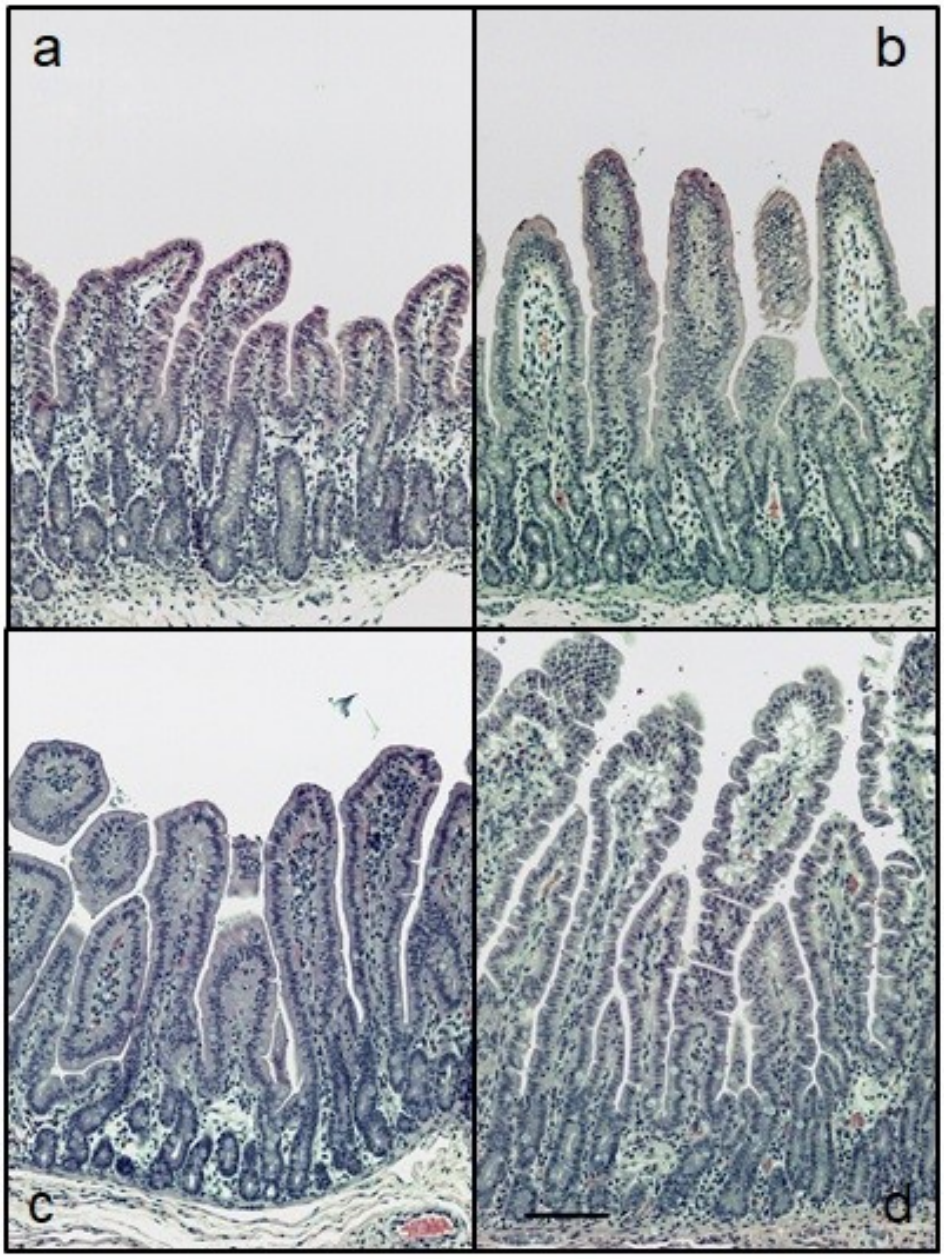

Figure 2-4: Jejunal histology (200x magnification) from representative short bowel and gut-intact piglets.

a: JC, b: JI; c: Sham, d: SF. Bar = 0.1 mm.

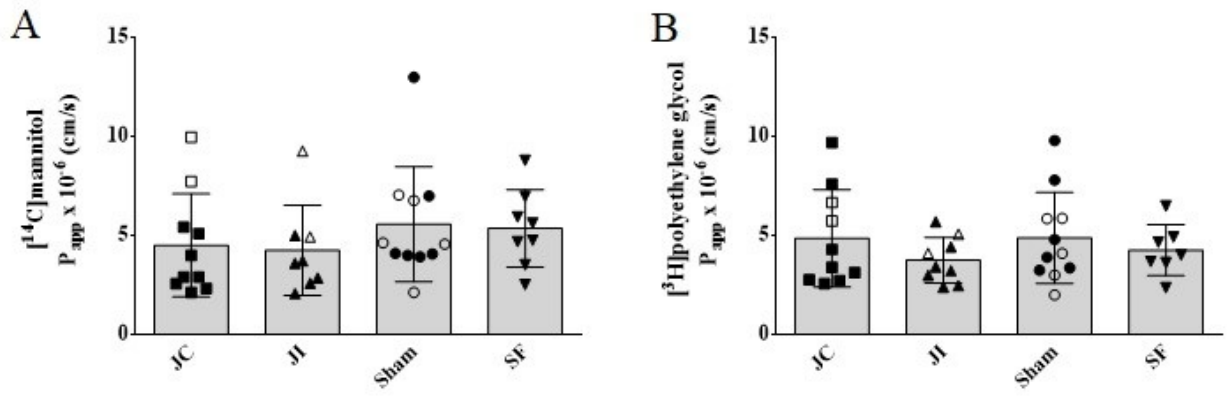

Figure 2-5: Jejunal permeability in short bowel and gut-intact piglets.

Mucosal-to-serosal P_{app} was not different between groups to A) [^{14}C]mannitol (P = .68) or B) [^{3}H]polyethylene glycol (P = .55). Mean ± SD; analysis of variance. Septic piglets are denoted by open symbols.

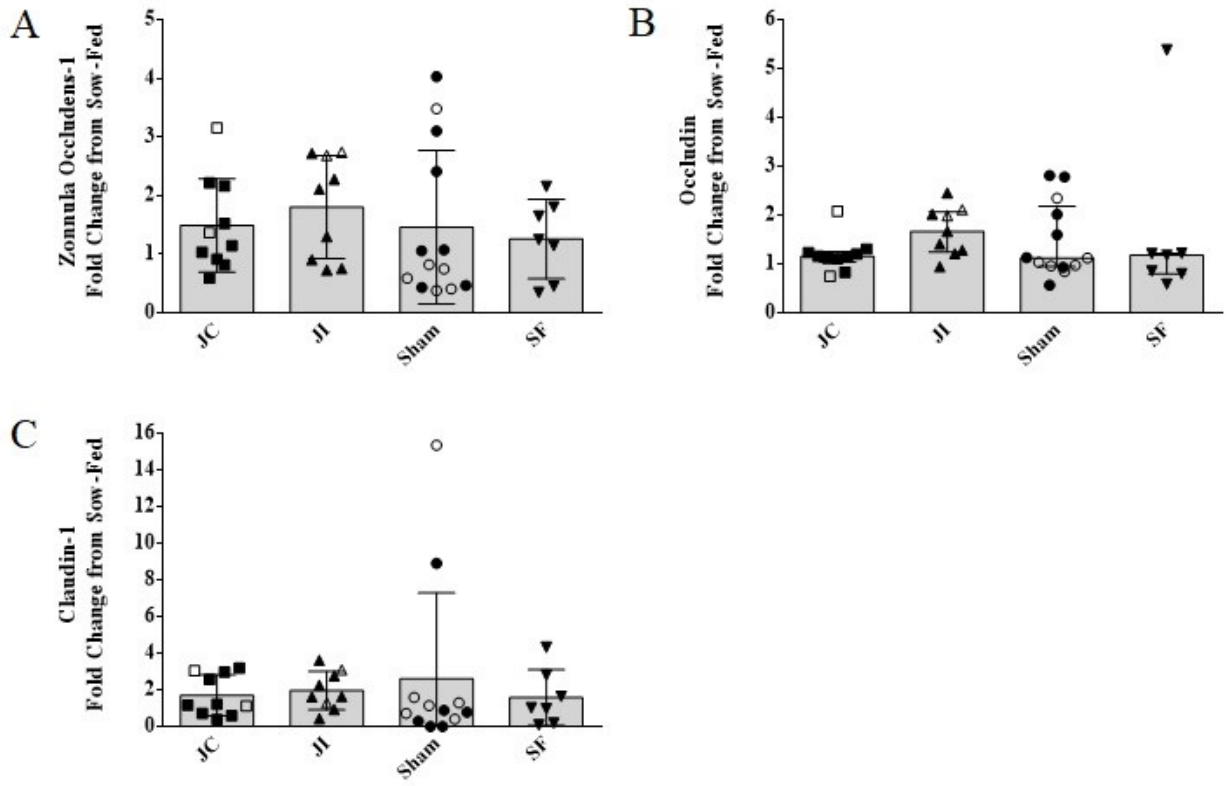

Figure 2-6: Tight junction protein gene expression in short bowel and gut-intact piglets. Gene expression of tight junction proteins was not different between groups for A) ZO-1 (*P* = .74), mean ± SD, analysis of variance; B) OCLN (*P* = .18), median ± IQR; Kruskal Wallis analysis of variance; or C) CLDN-1 (*P* = .86), mean ± SD, Welch's analysis of variance. Septic piglets are denoted by open symbols.

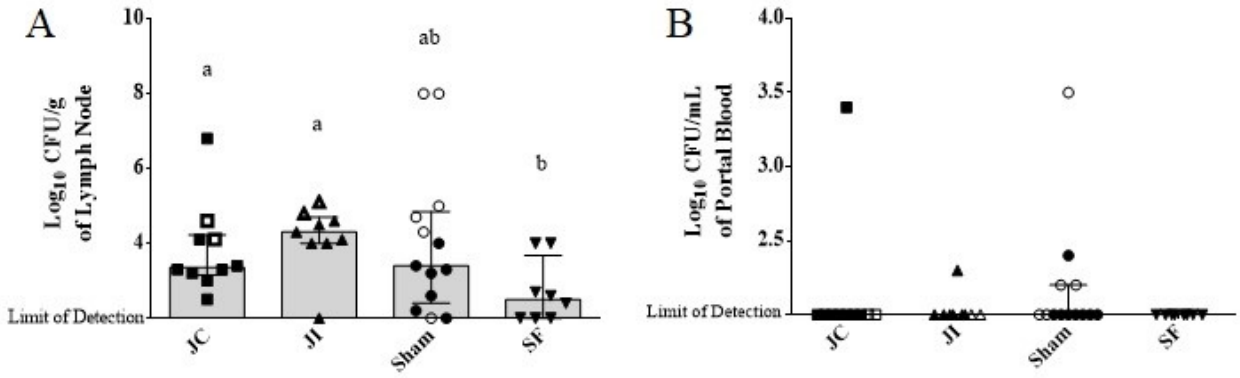

Figure 2-7: Bacterial densities in lymph nodes and portal blood of short bowel and gut-intact piglets.

A) Bacterial density in lymph nodes was lower in SF than JC and JI piglets (P = .03), but not different between SF and Sham or between JC, JI and Sham. B) Bacterial density in portal blood was not different between groups (P = .27). Median ± IQR; Kruskal Wallis analysis of variance. Septic piglets are denoted by open symbols.

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CHAPTER 3: Polyunsaturated Fatty Acid Composition of Parenteral Nutrition Impacts Intestinal Microbiota and Host Responses in Neonatal Piglets

Adapted from:

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3.1 ABSTRACT

Background: Intestinal failure-associated liver disease (IFALD) is a life-threatening complication of PN therapy for IF. PN causes intestinal microbial dysbiosis and impaired EBF. The role of this dysbiosis and impaired EBF in the pathogenesis of IFALD needs to be clarified. Specifically, the composition of the parenteral lipid emulsion may modulate both outcomes. Accordingly, we compared the effects of a lipid emulsion containing long-chain omega-3 polyunsaturated fatty acids (LC-PUFA; SMOFlipid®) to a predominantly omega-6 PUFA emulsion (Intralipid®) on microbial composition and host response at the mucosal surface.

Materials and Methods: Neonatal piglets were provided isocaloric, isonitrogenous PN for 14 days and compared to sow-fed controls (SF). Equivalent lipid doses (10g/kg/d) of either SMOFlipid® (ML: n=10) or Intralipid® (SO: n=9) were given. Ileal segments and mucosal scrapings were used to characterize microbial composition by 16S rRNA gene sequencing and quantitative gene expression of tight junction proteins, mucins, antimicrobial peptides and inflammatory cytokines.

Results: Microbial composition of PN piglets differed from SF, while ML and SO differed from each other (AMOVA; $p<0.05$); ML were more similar to SF as indicated by UniFrac distance ($p<0.05$). SO showed a specific and dramatic increase in *Parabacteroides* ($p<0.05$), while ML showed an increase in *Enterobacteriaceae* ($p<0.05$). Gene expression of mucins, CLDN-1, beta defensin 2 and IL-8 were higher in PN; overall increases were significantly less in ML compared to SO ($p<0.05$).

Conclusion: The formulation of parenteral lipid is associated with differences in gut microbiota and host response of PN-fed neonatal piglets. Inclusion of omega-3 LC-PUFA

appears to improve host-microbial interactions at the mucosal surface, although mechanisms are yet to be defined.

3.2 INTRODUCTION

Parenteral nutrition (PN) provides carbohydrate, protein, lipid, and micronutrients intravenously to neonates that cannot tolerate enteral feeding. It is a means to prevent malnutrition until EN can be supported, and is essential for survival, growth and development of neonates with intestinal failure. However, PN has adverse effects that cause significant morbidity and increased mortality. IFALD is one major complication of prolonged PN and includes steatosis, cholestasis, and cirrhosis leading to liver failure and death.[1-3] Understanding the etiology of these diseases and developing strategies to improve the outcomes for infants on PN are essential.

PN has been shown to result in changes in the microbial community at the mucosal surface whether the formulation is fat-free[4] or provides omega-6 fatty acids.[5-7] PN is associated with mucosal inflammation and a loss of epithelial barrier function,[8, 9] which can lead to bacterial translocation, bacteremia, and sepsis.[10] Increased bacterial translocation is an important contributor to non-alcoholic fatty liver disease[11] and recent evidence indicates this is also the case for infants on PN.[12] Improved outcomes with the inclusion of antibiotics, and the fact that intestinal injury is reduced in the absence of microbial recognition receptor toll-like receptor (TLR) 4[13], suggest that microbes contribute to the disease.

In prior studies we observed improved liver outcomes[14, 15] and reduced inflammatory markers[15] with the provision of a mixed lipid (ML; SMOFLipid®) emulsion containing soybean oil, medium chain triglycerides, olive oil and fish oil as compared to the standard soybean oil (SO; Intralipid®) emulsion in PN-fed piglets. SMOFlipid® provides eicosapentaenoic acid and

docosahexaenoic acid, the longer chain omega-3 polyunsaturated fatty acids (LC-PUFA); however, as we do not completely understand the mechanisms by which SMOFLipid® prevents IFALD,[15] we now consider the potential role of the microbiome being modulated by the inclusion of omega-3 fatty acids. Few studies to date have focused on how PN varying in lipid emulsion formulation, alters the intestinal mucosa and the associated microbial community at the mucosal surface. Previous studies have found both an increase[6] and a decrease[4] in antimicrobial peptides coinciding with dysbiosis of the gut microbiota; however, little is known about whether changes in antimicrobial production are a cause or consequence of microbial dysbiosis. Due to their anti-inflammatory[16] and antimicrobial[17-19] properties, we expect the provision of omega-3 LC-PUFA in the PN emulsion to reduce the host immune response and maintain a less inflammatory gut microbiome. To determine whether the lipid emulsion formulation of the PN alters host-microbial interactions at the mucosal surface, we compared microbial composition and innate defense mechanisms in the ileum of piglets receiving PN containing different lipid emulsions.

3.3 METHODS

3.3.1 Animals and Surgery

This study was performed according to the guidelines provided by Canadian Council on Animal Care and with approval of the University of Alberta Animal Care and Use Committee (AUP#00000153), and was conducted in a bio-secure swine research facility.

Male Landrace-Large White cross piglets were obtained from the University of Alberta SRTC. At day 1 of life, all piglets received a ceftiofur antibiotic (3.0mg/kg; Excenel RTU, Zoetis). To avoid litter effects, there were never more than 2 piglets from the same sow. After general anesthesia, piglets underwent jugular catheter insertion to allow for continuous delivery

of PN, without EN. To reduce the risk of central venous catheter sepsis, broad spectrum antibiotics ampicillin (10mg/kg twice a day; Sandoz, Boucherville, Quebec), and trimethoprim (20mg/d) with sulfadoxine (100mg/d; Merck Animal Health, Kirkland, Quebec), were administered on days 1 to 3 and 8 to 12. Piglets were monitored for signs of sepsis, including fever, vomiting, and lethargy. At the onset of these signs, blood cultures were drawn and the piglet was treated with enrofloxacin at 5mg/kg/d for the remainder of the study. Piglets that further deteriorated despite this regimen were treated with clindamycin (3mg/kg/d; Table 3-1).

Piglets were provided isocaloric, isonitrogenous PN for 14 days. The PN solution was prepared in our laboratory under sterile conditions and provided equivalent amino acids, dextrose, vitamins, and minerals. To create an all-in-one mixture, the lipid emulsion was added to the amino acid–based PN solution just prior to infusion. Nutrition targets for PN-fed piglets were previously validated against targets for sow-fed piglets and are based on a growth rate of piglets being five times that of human neonates.[20] Lipid dose (10g/kg/d) was the same between groups, but composition varied according to treatment: ML piglets received SMOFlipid® (n=10) while SO piglets received Intralipid® (n=9); a lipid dose of 10g/kg/d is equivalent to 2g/kg/d in a human infant. Nutrient delivery for PN-fed piglets is shown in Table 3-2.

Treatment piglets were housed similarly in individual cages. A swivel system allowed for freedom of movement while PN was infused. Cages were housed in a temperature-controlled room with a 12-hour light/dark cycle. After 14 days of treatment, piglets 16 to 21 days old were humanely euthanized and samples collected for analysis. Healthy, sow-fed (SF) piglets (n=8) served as controls and underwent terminal laparotomy at the same age as treatment piglets.

Whole tissue and mucosal scrapings were collected from 20 to 40 cm proximal to the ileocecal valve. They were then snap frozen in liquid nitrogen and stored at -80 °C until analysis.

Ileal sections fixed in 10% buffered formalin were embedded in paraffin, cut in 5 um sections and stained with haematoxylin and eosin. A pathologist blinded to treatment groupings evaluated sections and measured villus height and crypt depth in 10 consecutive villi in well-oriented sections.

3.3.2 Microbiome Analysis

Microbial composition was analyzed in ileal mucosal scrapings. Total DNA was extracted with the QIAamp DNA Mini Stool Kit (Qiagen, Inc. Mississauga, ON, Canada) following the manufacturer's instructions, with the addition of a bead beating step (FastPrep instrument, MP Biomedicals, Solon, OH). The hypervariable regions (V1 to V3) of the bacterial *16S rRNA* gene were amplified with nucleotide-barcoded primer pairs 27F: 5′-AGAGTTTGATCMTGGCTCAG-3′ and 519R: 5′- GWATTACCGCGGCKGCTG-3′. The forward primer contained Roche/454 Titanium adaptor A (CCATCTCATCCCTGCGTGTCTCCGACTCAG) and unique 10-bp barcodes, the reverse primer contained adaptor B (CCTATCCCCTGTGTGCCTTGGCAGTCTCAG). Triplicate DNA amplification was carried out in 20-µl reaction volumes containing 0.2µl Phusion high-fidelity DNA polymerase (Thermo Scientific), 4µl of 5 × HF buffer, 0.4µl 10mM dNTPs, 1µl of the extracted template DNA and 1µl each of forward and reverse primers (10ng/µl). The PCR for each method was carried out in an S1000 Thermal Cycler (Bio-Rad, Hercules, CA, USA) with the following parameters: initial denaturation at 98°C for 1 min, followed by 35 cycles of 98°C for 10 s, 59°C for 30s and 72°C for 30s, with a final extension at 72°C for 7 min. Following PCR amplification, triplicate DNA amplification products were mixed and run at 100 V for 1 hour by gel electrophoresis on a 1% agarose gel (SYBR Safe stain, Invitrogen). The bands corresponding to bacterial *16S rRNA* were excised and gel-purified (QIAquick gel extraction kit, Qiagen,

Valencia, CA). Each amplicon (100 ng) was pooled and pyrosequenced using a 454 Titanium platform (Roche, Branford, CT). All of the obtained sequences were submitted to NCBI Sequence Read Archive (accession number SRP07538). Sequence data that passed Roche's quality thresholds were processed according to the mothur 454 SOP [21] accessed on June 16, 2015. Barcodes were trimmed and quality sequences were obtained by removing sequences containing ambiguous bases, quality read length <200 bases. Sequences passing quality filter were aligned to the silva bacterial reference alignment. Sequences were clustered into operational taxonomic units (OTUs) using UClust, based on a dissimilarity cutoff of 0.03. Hypotheses testing were performed with normalized data in mothur. Subsamples of quality sequence reads, equal to the sample with the least reads (938), were taken to normalize the data and were subsequently represented as a percentage of total reads. The Inverse Simpson diversity index was used as a measure of alpha diversity. UniFrac weighted distance matrices were used to calculate similarity of ML and SO to SF.

3.3.3 Gene Expression

Total RNA was extracted from whole ileal tissue using the GeneJET RNA Purification kit (Thermo Scientific) according to manufactures instructions. The RNA was quantified using a Nanodrop ND-1000 (NanoDrop Technologies, Wilmington, DE, USA) and 1ug was used for cDNA synthesis using the Thermo Scientific Maxima First Strand cDNA kit. RNA integrity was determined on the Agilent RNA ScreenTape System (Agilent 2200, Germany) and samples with RIN scores less than 7 were not included. Real-time PCR was performed using a DyNAmo Flash SYBR Green qPCR kit (Fisher Scientific) with Sus Scrofa primers (Table 3-3). The PCRs were performed on the Viia7 Real-time PCR System (Applied Biosystems) with 10uL reaction volumes and the following 2-step cycling conditions: 95°C for 10 min, and 40 cycles at 95°C for

15 s and annealing temperature for 30 s. Glyceraldehyde-phosphate-dehydrogenase (GAPDH) and beta actin (ACTB) were found to be appropriate endogenous controls and were used for normalization. Relative expression was calculated using the ddCT method and corrected for primer efficiencies according to Pfaffl.[22] Appropriate target amplification was confirmed by sequencing PCR amplicons.

3.3.4 Intestinal Oxidative Stress

To determine whether increased oxidative stress may have contributed to altered microbial composition between treatments, MDA concentration was measured in ileal tissue. The TBARS Assay Kit (Cayman Chemical, No. 700870) was used to measure malondialdehyde (MDA) concentration in ileal tissues. Approximately 100 mg of tissue was ground with liquid nitrogen and added to 1 mL of sterile PBS with protease inhibitor (Pierce Biotechnology, PI78410). Samples were homogenized using bead beating for 1 minute. The homogenates were centrifuged at 1,600 x g for 10 minutes at 4°C. 100 µL of the supernatant was aliquoted into a labeled 1.5 mL microcentrifuge screw cap vial and then mixed with 100 µL of TCA Assay Reagent (10%) by swirling. 800 µL of Color reagent was added to each vial and vortexed. The vials were then placed upright in boiling water for one hour. Following boiling, vials were immediately placed in an ice bath to stop the reaction and then incubated on ice for 10 minutes. Vials were centrifuged for 10 minutes at 1,600 x g at 4°C and 200 µL (in duplicate) of the supernatant was transferred to the microplate. Absorbance was read at 535 nm and the values of MDA (µM) were calculated from a standard curve.

3.3.5 Statistical Analysis

Data are expressed as mean ± SEM and significance was defined as $p \leq 0.05$. Differences in villus height, gene expression, and oxidative stress were tested by one-way ANOVA with

Tukey's post hoc test. UniFrac distance of SO and ML to SF were analyzed by student's *t*-test. Relative abundance of specific bacterial genera and all taxonomic levels were normalized by log transformation and analyzed by ANOVA. AMOVA with Bonferroni correction for multiple comparisons was used to test differences in microbial communities.

3.4 RESULTS

Piglets were observed for signs of sepsis through the course of the study. Four of nine SO and three of ten ML piglets were given additional antibiotics due to suspicion of sepsis. Blood cultures revealed that none of the ML piglets had proven bacteremia, whereas two SO piglets were positive for either *Enterococcus faecalis* or *Klebsiella pneumoniae*.

3.4.1 Intestinal Structure

Assessment by a blinded pathologist noted that all of the ileal sections were microscopically normal, and that there were no ulcerations in any of the intestinal sections observed. Villus height (ML: 471±64μm; SO: 503±82μm; SF:470±32μm) and crypt depth (ML: 159±15μm; SO: 162±6μm; SF:140±8μm) measurements were not different between groups, however, all SF piglets showed more developed Peyer's Patches than PN piglets, as can be observed in representative section from each treatment in Figure 3-1.

3.4.2 PN Lipid Formulation Alters Ileal Microbial Composition

After quality filtering, an average of 2633±250 reads were obtained per sample and only samples with more than 938 quality reads were included from the analysis. Three samples were removed due to low reads, leaving 8 individuals per PN treatment group and 4 in the control group. The microbial community differed between all three groups (AMOVA<0.05 with Bonferroni correction for multiple comparisons) as visualized in the non-metric multidimensional scaling (NMDS) plot with weighted UniFrac distance metrics (Figure 3-2A).

While all treatment groups differed from each other, microbial composition was more similar to control in ML than SO piglets ($p<0.05$) as indicated by weighted UniFrac distances from the control group (Figure 3-2B). Furthermore, beta-diversity, as indicated by within-group weighted UniFrac distances, was lower (mean ± SEM; $p<0.01$) for ML (0.640 ± 0.074) as compared to SO (0.902 ± 0.039), indicating a more cohesive shift in the ML piglets. It should be noted that microbial differences between parenteral treatments and control reflect both the difference in nutrition delivery as well as antibiotic treatment; however, in general ML and SO piglets received the same antibiotic regimen. Differences between treatments at the bacterial family level are represented in Figure 3-3.

As a result of the large effects of both antibiotic treatment and lack of enteral nutrition, the main comparison was between ML and SO. The effects of lipid formulation described below were consistent irrespective of whether the piglets received additional antibiotics to treat potential sepsis (2/8 ML; 3/8 SO), therefore they were not excluded from the microbial analysis. SO was associated with an increased proportion of the genus *Parabacteroides* ($p<0.05$), and was the only genus representing the family *Porphyromonadaceae*. This increase was not consistently represented by a single OTU, however it was quite dramatic, representing an average of 16.2 ± 7.0% of reads, whereas *Parabacteroides* were rarely detected in ML piglets (never more than 0.05%) and represented 1 ± 0.7% of reads in SF piglets. Conversely, ML piglets had a much larger proportion of the family *Enterobacteriaceae* than SO piglets ($p<0.05$). Another interesting observation was an increased abundance of *Halomonas* in SO piglets. This genus represented up to 30% of the microbial community and was more than 8% in 3 of 8 SO, whereas this genus was only detected in 1 control and 1 ML at <2%. Alpha diversity as indicated by inverse Simpson diversity index was not different between SO (3.89±1.05) and ML piglets (2.32±0.51) ($P = .17$).

3.4.3 Innate Defense and Inflammatory Cytokine Gene Expression

Previous studies have shown that PN is associated with altered expression of innate defense molecules, increased intestinal permeability, and inflammation.[7, 9] To determine whether PN formulation altered these outcomes, gene expression of tight junction proteins, mucins, beta defensins, and inflammatory chemokine IL-8 were measured (Figure 3-4). The expression of mucin genes Muc1, Muc2, and Muc4 were all elevated in PN-fed piglets compared to control ($P<0.05$). Although mucin gene expression was elevated in both PN treatments compared to SF, the expression was higher in SO piglets as compared to ML ($p<0.05$). Tight junction protein CLDN 1 was most highly expressed in SO, intermediate in ML and lowest in SF piglets, with all groups being different from each other ($p<0.05$). Similarly, beta defensin 2 showed increased expression in PN treatments, with ML having lower expression than SO ($p<0.05$); beta defensin 1 did not show a significant change in expression between PN groups. IL-8 is a neutrophil recruiting chemokine produced by macrophages and epithelial cells in the intestinal tract. SO piglets had higher IL-8 expression compared to control and ML piglets suggesting a more inflammatory environment. Together these results indicate that there was a disturbance at the mucosal surface in PN piglets, and this difference was more pronounced in SO than ML piglets.

3.4.4 Altered Microbial Populations are Not Associated with Increased Oxidative Stress

To determine whether increased oxidative stress may have contributed to altered microbial composition between treatments, MDA concentration was measured in ileal tissue. Interestingly, oxidative stress was lowest in PN-fed animals (Figure 3-5). The difference was significant for ML ($P<0.05$) and showed the same trend in SO piglets ($P=0.06$), however, ML and SO piglets showed very similar levels of oxidative stress.

3.5 DISCUSSION

We characterized mucosal-associated microbe populations in total PN-fed neonatal piglets to determine the effect of PN lipid emulsions that vary in omega-3 PUFA content on intestinal dysbiosis. Neonatal piglets have been validated as a suitable model of PN-fed human infants [3], and we have validated piglets as a model for intestinal failure and for IFALD.[23] Non-discriminant analysis identified that the communities were indeed different from each other, and consistent with previous animal studies,[4-7, 24-26] the mucosal microbiota of PN-fed piglets differed from that of enterally-fed piglets.

A specific increase in *Parabacteroides* and *Halomonas* was associated with SO piglets, whereas ML piglets showed a specific increase in *Enterobacteriaceae.* Although there are differences between the gut microbial communities of infants and piglets, the data here suggest that the type of lipid provided in the PN alters the microbiome. The increase in *Enterobacteriaceae* observed in ML piglets is consistent with a recent report looking at the inclusion of SMOFlipid® in infants receiving PN.[27] Previous studies have shown increased *Enterobacteriaceae* in response to inflammation,[28] however, it does not seem to be the case in this study, because of the lower expression of inflammatory cytokines in ML as compared to SO piglets. It is important to note that *Enterobacteriaceae* are more prevalent in the neonatal period[29] and were found at up to 15% of the community in SF piglets. With reduced IL-8 expression in ML, it is expected that the enriched *Enterobacteriaceae* (largely represented by OTUs identified as *Escherichia coli*) were not pathogenic, however, this is not discernable with 16S rRNA sequencing. It is yet unclear why *Parabacteroides* and *Halomonas* increased in SO piglets. *Halomonas* is not typically an abundant member of the piglet microbiota, yet represented more than 8% of the community in 3 of the SO piglets. This genus has shown some capacity for

pathogenicity in humans;[30, 31] however, no evidence of such a relationship is demonstrated here. Previous studies have shown an increase in the relative proportion of mucous utilizing bacteria, such as *Clostridium perfringens*, in PN-fed piglets,[5] however, a reduction in the relative proportion of OTUs that were closely related to *C. perfringens* was observed in the current study. This reduction may be a result of antibiotic treatment in PN piglets. That antibiotics were required to attempt to prevent sepsis, and that sepsis did indeed occur in this model, are limitations of the study that we recognize; however, it is also a pragmatic reality that would be noted in studies of human infants on PN support and this reality likely contributes to microbial dysbiosis in both settings. Interestingly, the bacteria cultured in SO piglets are also common pathogens in human infants with sepsis in the setting of PN delivery via central venous catheters.[32]

Two other studies have recently reported on the effects of lipid composition on the gut microbiome. Significant differences were noted in the microbiome of mice[25] and preterm infants[27] provided a PN emulsion containing omega-3 LC-PUFAs compared to soybean oil. LC-PUFAs, in particular of the omega-3 series, have direct antimicrobial activity,[17-19] therefore it is not surprising that they alter microbial composition when fed enterally. Since nutrition was not provided enterally, our study provides evidence that the PN lipid emulsion formulation alters the microbiome through a yet to be defined mechanism that is likely dependent on modifications in host physiology. The change in intestinal microbes may be associated with altered bile flow, reduced inflammation, or changes in the mucosal secretions, which are utilized as a nutrient source by intestinal microbes. Tian et al.[26] hypothesized that the changes in the microbiota of infants receiving SMOFlipid® were associated with changes in oxidative stress. We therefore

measured MDA concentrations as an indicator of oxidative stress, but found no difference in the ileum of SO and ML piglets.

Although villus height and crypt depth were not different between groups, there were less developed Peyer's Patches in PN-fed than SF piglets, which is consistent with atrophy seen in Peyer's Patches of PN-fed rabbits.[33] It is unclear why PN-fed piglets have less developed Peyer's Patches than SF piglets, particularly given the increased expression of inflammatory chemokines in PN-fed piglets. This therefore merits further investigation.

Increased antimicrobial peptide gene expression was observed in the ileum of PN-fed as compared to SF piglets. This is consistent with a previous study that found the expression of lysozyme as well as gene expression of lysozyme and α-defensins 5 and 8 were increased with duration of PN.[6] Differences in gene expression of tight junction protein CLDN-1, between ML and SO piglets, indicates that tight junction integrity is likely impacted by lipid formulation. Future studies should explore the impact of PN lipid formulation on intestinal permeability and visualization of tight junction proteins to understand the role of altered gene expression on actual integrity. At this time, without such functional analysis, it is difficult to draw inferences from our data.

The firmly adherent mucus layer has been observed to be smaller in rats receiving PN for 14 days as compared to control, based on Periodic acid Schiff staining.[34] That was associated with increased susceptibility to pathogen penetration through the mucosal surface. Mucin gene expression analysis was not performed in that study and it should be noted that the authors also observed reduced mucus thickness in PN as compared to control. Recent studies have found reduced abundance of Muc2 in tissues and intestinal washes of parenterally-fed as compared to chow-fed mice,[24] as well as reduced Muc2 gene expression in parenterally-fed mice.[35] Those

results are counter to the results of the current study where increased expression of mucin genes, including Muc2, was observed. The differences in animal species, animal age, treatment duration and antibiotic treatments may contribute to the differences in results. The increased expression of mucin genes observed here may reflect an increased mucosal stimulation by bacterial products. Several studies have found that lipopolysaccharide (LPS) induces the expression of mucin genes by binding to TLR4[36, 37] and subsequent activation of the NF KB pathway.[38] Similar to LPS, flagellin and lipoteichoic acid, a component of the gram-positive cell wall, have been shown to induce mucin gene expression.[39-41] Increased expression of mucin genes through NF-κB activation is consistent with the greater level of IL-8 in intestinal tissues. A limitation of the current study is that intestinal sections were not sufficiently preserved to assess whether changes in gene expression were associated with mucus thickness.

It should be noted that factors other than fatty acid composition differed between ML and SO lipid emulsions. Differing phytosterol and vitamin E content between treatments may have also contributed to the differences. Work from our lab recently showed that vitamin E content had no impact on markers of inflammation nor oxidative stress in a similar model[42], suggesting that differences in Vitamin E content were not responsible, however further work is needed to confirm the specific role of fatty acid composition.

In conclusion, differing lipid emulsion compositions were associated with differences in the microbiome composition and host-microbial interactions at the mucosal surface in total PN-fed piglets. The provision of PN, per se, increased antimicrobial peptide gene expression compared to EN, regardless of the fatty acid formulation. It is unclear whether the shifts in microbial composition were a direct or indirect result of the PN lipid formulation. Further studies are needed to determine the cause-effect of the differences in the microbiome seen with PN and

whether the change in intestinal microbes is associated with altered bile flow, reduced inflammation, altered permeability or changes in mucosal secretions.

Table 3-1: Antibiotic treatment for PN-fed piglets treated for sepsis.

Treatment	Antibiotics Provided to Treat Suspected Sepsis	Tested Positive For
SO	ampicillin and trimethoprim-sulfadoxine day 13-14, and enrofloxacin days 10-14	*Enterococcus faecalis*
SO	ampicillin and trimethoprim-sulfadoxine days 13-14, and enrofloxacin day 14	
SO	ampicillin and trimethoprim-sulfadoxine day 7 and 13-14, enrofloxacin days 12-14 and clindamycin days 12-14	*Klebsiella pneumoniae*
SO	ampicillin and trimethoprim-sulfadoxine days 4-7 and 13-14, enrofloxacin days 9-14 and clindamycin days 12-14	
ML	ampicillin and trimethoprim-sulfadoxine days 4-7 and 13, enrofloxacin days 4-13	
ML	ampicillin and trimethoprim-sulfadoxine days 4 and 6-7	
ML	ampicillin and trimethoprim-sulfadoxine days 4-7 and 13, and enrofloxacin days 5-7	

Table 3-2: Nutrient delivery for PN-fed piglets.

Nutrient Content	ML Piglets	SO Piglets
Total energy, MJ/kg/d	1.1	1.1
Total lipid, g/kg/d	10	10
Soybean oil	3	10
Olive oil	2.5	0
Fish oil	1.5	0
Medium-chain triglycerides	3	0
Total protein, g/kg/d	16	16
Total dextrose, g/kg/d	29	29
Key fatty acid intakes, g/kg/d*		
Palmitic acid (C16:0)	0.9	1.1
Oleic acid (C18:1 ω-9)	2.7	2.4
Linoleic acid (C18:2 ω-6)	1.8	5.3
α-Linolenic acid (C18:3 ω-3)	0.2	0.8
Arachidonic acid (C20:4 ω-6)	0.05	0.02
Eicosapentaenoic acid (C20:5 ω-3)	0.3	0
Docosahexaenoic acid (C22:6 ω-3)	0.2	0
Phytosterols, mg/kg/d*	10	22
Vitamin E, mg/kg/d* α-tocopherol equivalents	6.5	2.8

*Amounts provided by manufacturer (Fresenius Kabi, Bad Homburg, Germany); approximate values only since exact values may vary from batch to batch, due to variations in natural raw materials.

Table 3-3: Sus Scrofa primers used for qPCR to analyze host gene expression in piglets.

Gene	Primer (5'-3')	Annealing Temp. (°C)	Product Length	Efficiency
pIL-8_F	TTCTGCAGCTCTCTGTGAGGC	59	92 bp	78
pIL-8_R	GGTGGAAAGGTGTGGAATGC			
pOccludin_F	ATCAACAAAGGCAACTCT	62	157 bp	99
pOccludin_R	GCAGCAGCCATGTACTCT			
pCldn1_F	TGATGAGGTGCAGAAGATGC	60	174 bp	108
pCldn1_R	CCAGTGAAGAGAGCCTGACC			
pMuc1_F	GGTACCCGGCTGGGGCATTG	60	146 bp	99
pMuc1_R	GGTAGGCATCCCGGGTCGGA			
pMuc4_F	GATGCCCTGGCCACAGAA	60	89 bp	96
pMuc4_R	TGATTCAAGGTAGCATTCATTTGC			
pBD-1_F	CTCCTCCTTGTATTCCTCCT	60	141 bp	112
pBD-1_R	GGTGCCGATCTGTTTCAT			
pBD-2_F	GACTGTCTGCCTCCTCTC	60	148 bp	110
pBD-2_R	GGTCCCTTCAATCCTGTTG			
pMuc2_F	CTGCTCCGGGTCCTGTGGGA	60	101 bp	103
pMuc2_R	CCCGCTGGCTGGTGCGATAC			
pACTB_F	AGAGCGCAAGTACTCCGTGT	60	68 bp	101
pACTB_R	ACATCTGCTGGAAGGTGGAC			
pGAPDH_F	GTTTGTGATGGGCGTGAAC	63	147 bp	107
pGAPDH_R	ATGGACCGTGGTCATGAGT			

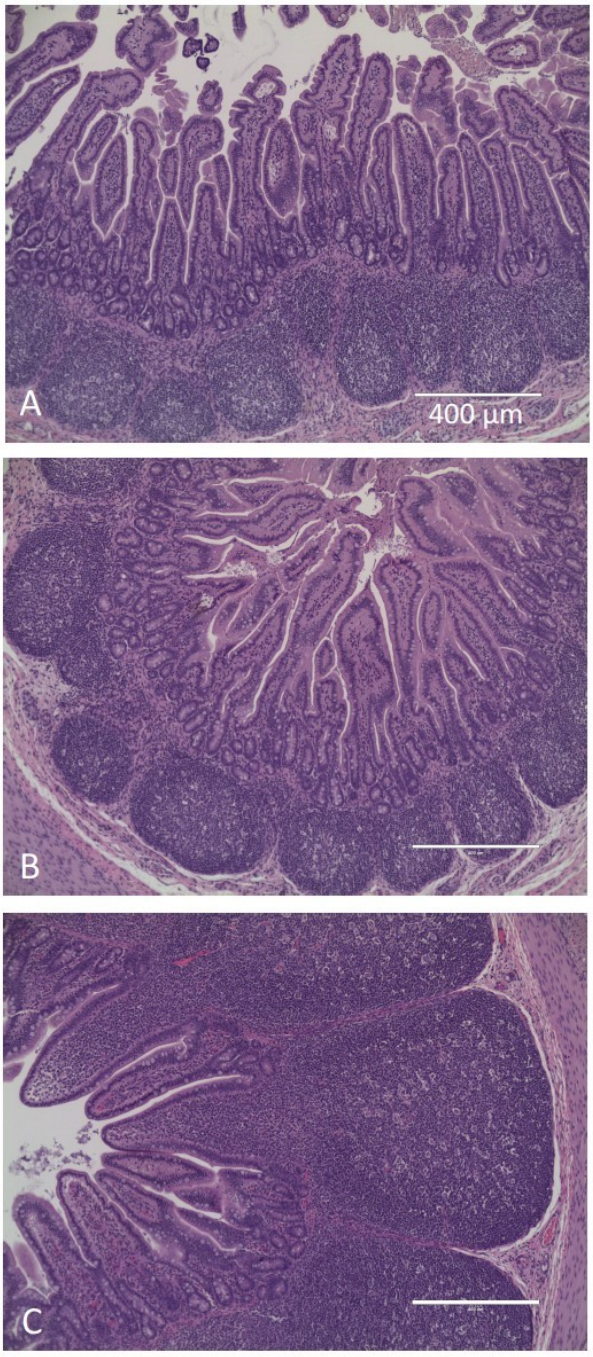

Figure 3-1: Cross-sections of the ileum at 40x magnification.

Piglets received 14 days of parenteral nutrition containing A) Intralipid® (SO) or B) SMOFlipid® (ML), or were C) sow-fed (SF) control. SF piglets showed more developed Peyer's patches.

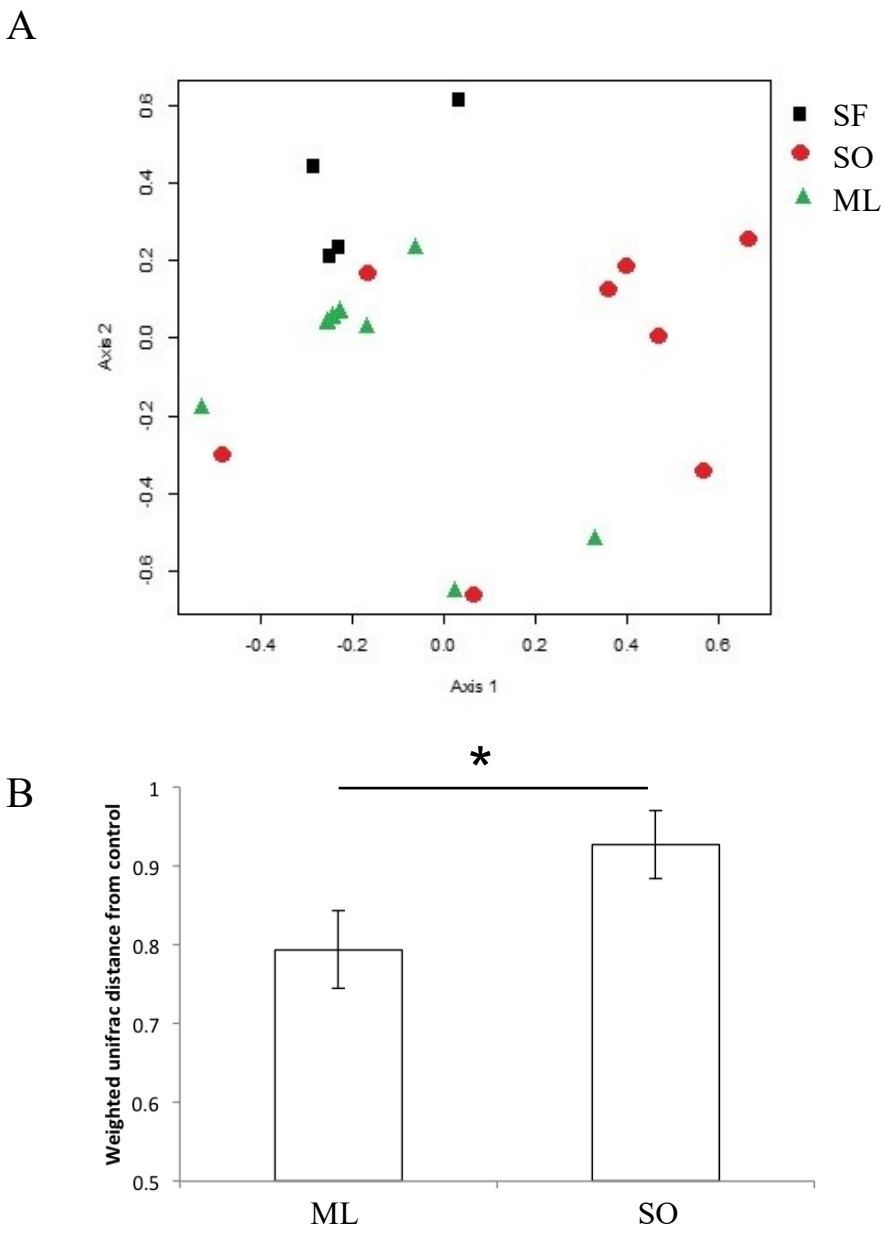

Figure 3-2: Microbial community differences in PN-fed piglets.

A) Weighted UniFrac distance in a non-metric multidimensional scaling plot showing the first two of three dimensions. Individuals administered additional antibiotics are denoted by an open circle. B) UniFrac distance between microbiota of treatment and control piglets; bars indicate the mean with SEM (*: $p<0.05$, Student's *t*-test).

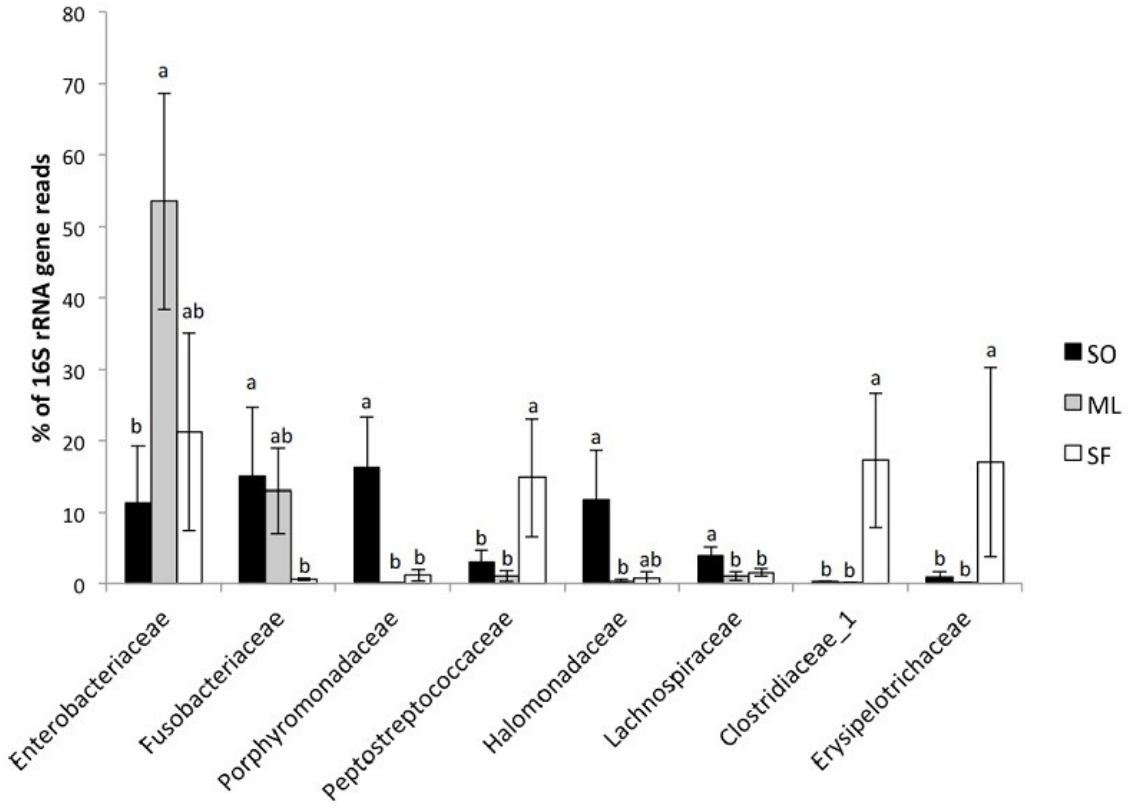

Figure 3-3: Bacterial families differed between groups in ileal scrapings of piglets that received PN.

Bars indicate the mean with SEM; a,b,c: means that do not share a letter are significantly different ($p<0.05$ by ANOVA).

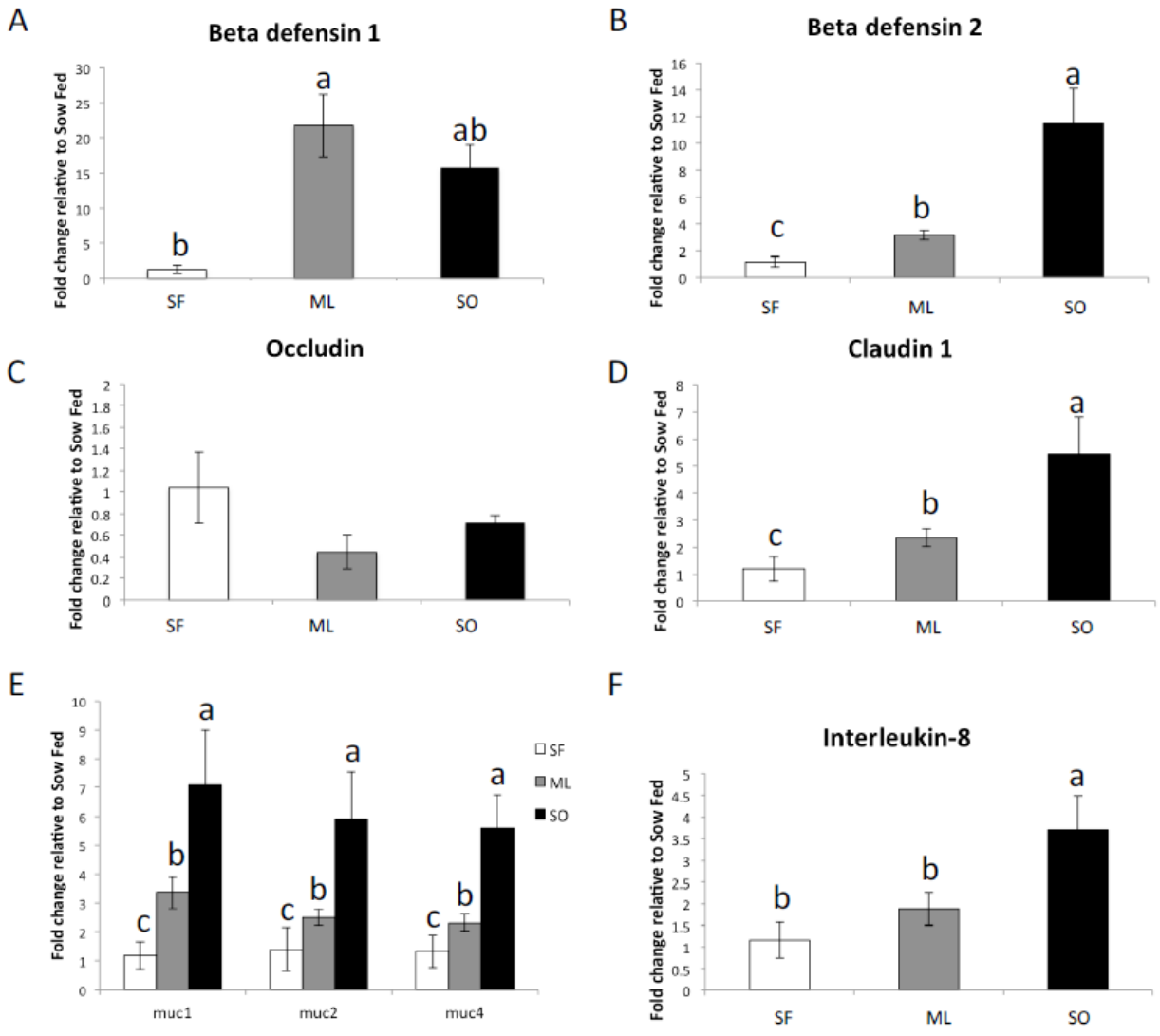

Figure 3-4: Gene expression by qPCR in the ileum of piglets that received PN.

A) beta defensin 1, B) beta defensin 2, C) OCLN, D) CLDN-1, E) mucin genes, and F) IL-8. Bars indicate the mean with SEM; a,b,c: means that do not share a letter are significantly different ($p<0.05$ by ANOVA).

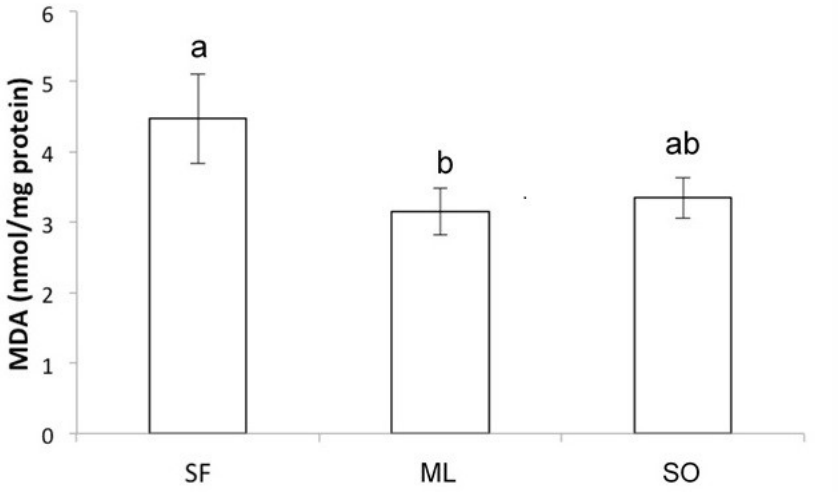

Figure 3-5: Oxidative stress measured by MDA assay in ileal scrapings from piglets that received PN.

Bars indicate the mean with SEM. a,b,c: means that do not share a letter are significantly different ($p<0.05$ by ANOVA).

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CHAPTER 4: Clinical Usage of Parenteral Lipid Emulsions and Impact on Bile Acid Metabolism and Composition, Studied in Neonatal Piglets

4.1 ABSTRACT

Background: Neonates with intestinal failure who are dependent on PN are at risk of IFALD. PN lipid composition relates to the risk of IFALD, but the mechanisms are poorly understood. We investigated the effects of SO, a ML emulsion containing FO, and a pure FO on gene expression, bile acid composition, and their relationship to bile flow as potential mechanisms for IFALD.

Methods: Neonatal piglets (3-6 days old) were allocated to receive one of SO, ML or FO throughout 14 days of PN feeding, and compared to SF controls. Relative expression of genes involved in bile acid synthesis and transport were determined through quantitative polymerase chain reaction. Bile secreted from the liver was collected and measured. Bile acid composition was determined using tandem mass spectrometry. Regression analysis was used to determine predictors of bile flow.

Results: PN reduced bile acid secretion ($P < .001$). FO-containing PN lipid was associated with greater expression of bile acid and organic solute transport genes ($P < .05$) and greater secretion of hydrophobic bile acids ($P < .001$). Farnesoid X receptor ($P = .02$), bile salt export pump ($P < .01$), multidrug resistant protein 2 ($P < .01$), and unconjugated hyocholic acid ($p = 0.02$) independently predicted bile flow.

Conclusions: PN lipid modulation altered bile acid metabolism and composition. These alterations may explain the hepatoprotective effects of FO-containing PN lipids, and supports their use in the prevention and treatment of IFALD.

4.2 INTRODUCTION

Intestinal failure impairs the absorption of nutrients to a degree that growth and health are compromised, leading to the need for PN. Long-term dependence on PN leads to IFALD, which

can result in the need for liver transplantation or death.[1] The aetiology of IFALD is multifactorial, with main risk factors being prematurity, lack of EN, recurrent sepsis, and factors related to the duration and composition of the parenteral nutrition.[1] Despite identifiable risk factors for IFALD, the molecular mechanisms of the disease are not well understood.

While neonates were traditionally at greatest risk of death from IFALD, recent advances in parenteral lipid formulations have markedly improved outcomes.[2] It is now recognized that use of SO promotes IFALD,[3] while lipid emulsions containing FO both prevent[4] and treat[5, 6] IFALD. Possible reasons for improved outcomes with FO-containing lipid emulsions can be related to compositional differences, including added vitamin E, and absence or reduced phytosterol content, as well as key differences in fatty acid composition, such as the presence of EPA and DHAin FO.

Using PN-fed neonatal piglets, a translational model for human neonates, we have previously shown that both pure FO (Omegaven®, Fresenius Kabi)[7] and a ML emulsion containing SO, medium chain triglycerides, olive oil and FO (SMOFlipid®, Fresenius Kabi)[8] increased bile flow to high-normal ranges compared to conventional SO (Intralipid®, Fresenius Kabi) (SF range = 6.8-12.2 microgram/g/10 min;[8] SO = 6.4 microgram/g/10 min;[8] ML = 13.0 microgram/g/10 min;[8] FO = 13.4 microgram/g/10 min[7]). The current study was undertaken to investigate the mechanisms for altered bile flow with these FO-containing lipid emulsions. We hypothesized that FO-containing PN lipid would result in reduced bile acid synthesis, increased canalicular bile acid transport and in a more hydrophilic bile acid pool.[9-11]

4.3 MATERIALS AND METHODS

4.3.1 Animals and Care

This study and procedures were approved by the University of Alberta Animal Care and Use Committee (AUP#00000153) and performed as per the Canadian Council on Animal Care guidelines, in a bio-secure swine research facility. The study utilized samples from piglets studied in our previous published findings of the effects of lipid emulsions on bile flow.[7, 8] Sample size and allocations are therefore based on the prior studies. The piglets were male, Large White cross Landrace, 3 to 6 days old. Study interventions and methods were as previously reported.[7, 8]. In brief, PN-fed piglets had a surgically placed central venous catheter and commenced PN, day 0, with subsequent daily care as was reported.[7, 8] Piglets were allocated to receive one of three PN lipid emulsions and doses: SO at 10 g/kg/d (n=8); ML at 10 g/kg/d (n=10), or low-dose pure FO at 5 g/kg/d (n=8). A lipid dose of 10 g/kg/d for a rapidly growing piglet is equivalent to 2 g/kg/d for a human neonate. Lipid doses were chosen based on standard of care and clinical practice. The standard of care for human babies is to provide SO at 1 to 4 g/kg/d.[5, 12] Studies have found ML to be safe when provided to pediatric patients[13, 14] at 2.5 g/kg/d[15]; furthermore, it is common clinical practice to provide both SO and FO for the prevention and treatment of IFALD at restricted doses of 1 g/kg/d.[5, 12, 16]

On study day 14, PN-fed piglets were anaesthetised (mean age 18.4 days) and underwent terminal laparotomy for measurement of basal bile flow.[7, 8] The liver was excised and randomly dissected it into smaller specimens. Bile and liver specimens were flash frozen in liquid nitrogen, then stored at –80°C until the analysis that is the subject of this report. The same data was obtained from SF control piglets (n=10; mean age 19.1 days).

4.3.2 Quantification of Gene Expression

We used the RNeasy Mini Kit (Qiagen, Germantown, MD, USA) to extract RNA from 30 mg of frozen liver disrupted and homogenized using Buffer RLT supplemented with β-mercaptoethanol. We quantified RNA by spectrophotometric assay and used 10µL of RNA to synthesize cDNA with a High Capacity cDNA Reverse Transcription Kit containing MultiScribe™ Reverse Transcriptase (Applied Biosystems, Foster City, CA, USA). cDNA was stored at -20°C. We performed qPCR using TaqMan® Sus scrofa primers (Life Technologies, Carlsbad, CA, USA; Table 1) with TaqMan® Universal Master Mix (Invitrogen, Burlington, ON, Canada) on a 7900HT Fast Real-time PCR System (Applied Biosystems). We used a 10µl reaction volume under the following conditions: 95°C for 10 min followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. We used hypoxanthine phosphoribosyltransferase-1 (HPRT1) as the endogenous standard for normalization and calculated relative gene expression using the delta delta CT method. We excluded any samples with inaccurate qPCR amplification plots.

4.3.3 Identification and Quantification of Bile Acids in Bile

Bile acids in piglet bile were identified and quantified by solid phase extraction and liquid chromatography-electrospray tandem mass spectrometry, as previously described.[17] The development and validation of this method, which identifies individual bile acids and their conjugates, are reported elsewhere.[18]

4.3.4 Statistical Analysis

As we have previously shown sepsis to be an important factor in the progression of IFALD[19], we excluded animals with confirmed sepsis from all analyses to limit confounding, and thus assessed the impact of PN lipid formulation in the absence of sepsis. We conducted statistical analysis using R, version 3.2.3,[20] with "car"[21] and "agricolae"[22] packages. To evaluate group differences in gene expression and bile acid composition, we performed ANOVA or

Welch's ANOVA for parametric data on homogeneous or non-homogeneous variance respectively, and Kruskal-Wallis ANOVA on nonparametric data. For post hoc testing we used Tukey's Honestly Significant Difference or pairwise comparisons for unequal variances with the Holm-Bonferroni Method for multiple inferences. Results are expressed as mean ± SD for normally distributed data, and median ± IQR for nonparametric data. To determine predictors of bile flow, we first performed univariate linear regression for each gene or bile acid species. We then conducted multivariate linear regression. Covariates with the largest coefficients on univariate regression were considered for inclusion in the model. We assessed for collinearity between covariates and chose the variable with largest coefficient. Due to sample size and statistical power considerations, we limited covariates in the model to a total of 4. We entered covariates into the model using a forward stepwise approach. We considered an alpha value ≤ 0.05 to be significant. 95% confidence intervals were included.

4.4 RESULTS

4.4.1 Animal Outcomes

Sepsis was confirmed in 1 piglet from the FO group, thus final data from 8 SO, 10 ML, 7 FO, and 10 SF piglets were analyzed. As was reported, there were no differences in mean age, baseline weight, weight gain, or average PN delivery between SO and FO piglets.[7] Compared to SO, ML piglets weighed more at baseline, and gained more weight each day.[8]

4.4.2 Gene Expression

To determine whether the PN lipid formulation alters bile acid metabolism, we measured the expression of genes involved in the regulation of bile acid synthesis and homeostasis. The cytochrome P450, family 7, subfamily A, polypeptide 1 (*CYP7A1*) gene encodes cholesterol 7 alpha-hydroxylase, the rate-limiting enzyme of the classic bile acid synthesis pathway.[23] As bile

acids are synthesized, they bind to and activate the bile acid receptor farnesoid X receptor (FXR),[24] which is encoded by the nuclear receptor subfamily 1, group H, member 4 (*NR1H4*) gene. Activation of FXR in turn regulates bile acid synthesis by inducing the expression of the small heterodimer partner (SHP),[25] encoded by the nuclear receptor subfamily 0, group B, member 2 (*NR0B2*) gene; SHP then represses CYP7A1, inhibiting bile acid synthesis.[25] We found the expression of *CYP7A1* was decreased in all groups compared to SF (p =0.04), but not different amongst any of the PN-fed groups (Figure 1A). The expression of *NR1H4* (FXR) was significantly higher in FO ($P < .01$) compared to all groups, but not different between any of the other groups (Figure 1B). There was a significant difference in the expression of *NR0B2* (SHP; $P < .01$), with expression being lower in ML than SO and FO groups, but not different from SF (Figure 1C). Although SO and FO groups showed similar expression of *NR0B2* (SHP), expression was only significantly greater in FO than SF piglets (Figure 1C).

The canalicular transport proteins are the major determinants of bile flow.[26] Thus, we measured the expression of the ATP-binding cassette, subfamily B member 11 (*ABCB11*) gene that encodes the bile salt export pump (BSEP), and the ATP-binding cassette subfamily C member 2 (*ABCC2*) gene that encodes the multidrug resistance protein 2 (MRP2).[26] We found a significant difference in gene expression of *ABCB11* (BSEP; $P = .04$); expression was greater in FO compared to SF, and greater in ML compared to SO groups (Figure 1D). We also found a significant difference in the expression of *ABCC2* (MRP2; $P < .001$); expression was increased in FO compared to all other groups, while gene expression was higher in ML compared to SO and SF, and not different between SO and SF piglets (Figure 1E).

Under cholestatic conditions, the expression of hepatic basolateral bile acid transporters organic solute transporter alpha (OSTA)[27] and multidrug resistance protein 3 (MRP3)[28] are

upregulated to allow efflux of bile acids into blood. The solute carrier family 51 alpha subunit (*SLC51A*) gene encodes OSTA, while the ATP-binding cassette subfamily C member 3 (*ABCC3*) gene encodes MRP3. We found a difference in expression of *SLC51A* (OSTA) amongst groups ($P < .001$); expression was highest in FO, followed by ML, SO, and SF groups (Figure 1F). We also found a significant difference in expression of *ABCC3* (MRP3; $P < .001$), with ML piglets showing the greatest expression, followed by FO; there was no difference in expression between SO and SF piglets (Figure 1G).

4.4.3 Bile Acid Composition

We identified and quantified the individual unconjugated and taurine- (t-) or glycine- (g-) conjugated bile acid species in bile secreted from the liver (Table 2). We could not detect the unconjugated forms of lithocolic acid (LCA), chenodeoxycholic acid (CDCA), or hyodeoxycholic acid (HDCA) in any group. However, we were able to determine the t-conjugates and/or g-conjugates of LCA, CDCA and HDCA to calculate the total amount of each of these bile acid species, as reported below.

We found a significant difference between groups in the concentrations of total bile acids in bile secreted from the liver (SF = 3729.39±821.71, SO = 806.58±555.58, ML = 1408.86±576.80, FO = 462.51±232.58 mcg bile acids/mL bile; $P < .001$; Figure 2). SF piglets secreted more bile acids than PN-fed piglets, while ML piglets secreted more than FO piglets. We then compared the percent of each total bile acid species (i.e. the total of all detectable unconjugated and conjugated forms of each bile acid species), as a proportion of secreted bile (Figure 3). Of the total amount of secreted bile acids, there was a greater percent of LCA conjugates present in ML and SF groups than in FO and SO groups ($P < .001$). There was a significant difference in the percent of CDCA conjugates in bile ($P < .001$); ML and FO piglets

secreted a higher percentage of CDCA conjugates than SF piglets. Both ML and FO groups secreted a lower percentage of cholic acid (CA) than SF ($P < .001$). Secretions of CA in SO piglets were highly variable, and not different from the other groups, nor was there was a difference between groups in the percent of HDCA conjugates secreted from the liver ($P = .6$). We found a significant difference in the percent of hyocholic acid (HCA) in secreted bile ($P < .001$). ML and FO piglets secreted a lower percent of HCA than SF; while the percent of HCA was lower in the FO than SO group, it was not different between ML and SO, or between SO and SF groups. It is important to note that HCA is the most prevalent bile acid in piglets,[29] while CA is the most prevalent in human neonates.[30]

4.4.4 Predictors of Bile Flow

Bile flow was previously reported.[7, 8] In the current study, we analysed potential predictors of bile flow. In univariate analysis of genes associated with bile acid homeostasis and transport, FXR, BSEP, MRP2, and OSTA were all independent predictors of bile flow (Table 3), while CYP7A1 ($P = .81$), SHP ($P = .74$), and MRP3 ($P = .2$) were not. Using proportional bile acid composition data, g-LCA, t-CDCA, g-CDCA, total CDCA conjugates, unconjugated CA, t-CA, total CA, unconjugated HCA, t-HCA, and total HCA were all univariate predictors of bile flow (Table 3). Only total LCA conjugates ($P = .49$), t-LCA ($P = .61$), t-HDCA ($P = .40$), g-HDCA ($P = .54$), and total HDCA conjugates ($P = .97$) did not predict bile flow. We included the 4 variables with the largest coefficients in the multivariate analysis. BSEP and unconjugated HCA together best predicted bile flow, giving a final model of 3.918 + (3.167 x BSEP) + (3.998 x unconjugated HCA) and overall model fit of R^2=0.559 ($P < .001$).

4.5 DISCUSSION

Our previous work in the piglet model has demonstrated that use of the FO-containing lipids, ML and FO, in the PN emulsion increases bile flow.[7, 8] This is consistent with reports in humans that FO-containing emulsions both treat and prevent IFALD.[5, 31] In the present study, we further explored the potential mechanisms for this effect, including potential differences in bile acid metabolism and in the composition of the bile acid pool. We found that expression of FXR was greater in FO compared to all other lipid treatment groups. We also found that PN reduces total bile acid secretion, but the use of FO is associated with altered bile acid composition.

Multiple explanations for altered FXR expression under lipid modulation are plausible. Firstly, of the variety of FXR antagonists and agonists, phytosterols are already recognized, including stigmasterol, which is present in SO. Stigmasterol has been shown to promote cholestasis in animal models purportedly via FXR antagonism.[32, 33] Therefore, it is plausible that FXR activity is not repressed in the groups administered FO-containing lipid because FO does not contain phytosterols. Alternatively, bile acids themselves are important FXR agonists and the composition of the bile acid pool, which did vary with the lipid treatments, is important. Not all bile acids activate FXR to the same degree. Specifically, in humans, CDCA has the strongest activating capacity, while LCA has the lowest.[34] Indeed, the FO group secreted a greater percentage of CDCA conjugates than any other group, and a lower percentage of LCA conjugates than SF and ML groups. Lastly, it was recently shown that FO reverses disrupted hepatic function, likely due to a lack of FXR signalling.[35]

Since FXR activity is increased in the presence of bile acids,[24] we would expect *de novo* bile acid synthesis to be decreased in the FO group, which had the highest FXR activation. This was indeed consistent with the decreased expression of CYP7A1 and increased expression of

SHP noted with the FO treatments. Normally, CYP7A1 expression is repressed by bile acids via FXR;[24, 25] therefore, it is somewhat counterintuitive that the reduced size of the bile acid pool in the PN-fed piglets did not drive increased bile acid synthesis in all groups. Bile acid species do differentially influence the expression of CYP7A1 and this may explain the difference between SO and FO containing treatments. Tauro-cholic acid (t-CA) and tauro-deoxycholic acid inhibited CYP7A1 enzyme activity in rats, but tauro-chenodeoxycholic acid (t-CDC) did not.[36] In mice, t-CA decreased CYP7A1 expression.[37] Finally, the more hydrophobic the bile acid pool, the more CYP7A1 enzyme activity is suppressed.[38] The FO group had the most hydrophobic bile acid pool concurrent with repressed CYP7A1 expression.

Increased canalicular transport of bile acids and organic solutes is likely to be a key factor in the differences noted in bile flow with the PN lipid treatments. Indeed, we found that BSEP and MRP2 each independently predicted bile flow. BSEP and MRP2 are both transcriptionally induced by FXR.[34] As such, we expected upregulated FXR to result in increased expression of both canalicular transport proteins. This was the case for FO group compared to SF, and for MRP2 in the ML group. Yet there was no significant increase in expression of FXR in the ML group. Furthermore, bile flow in both ML[8] and FO[7] groups was above the normal range for SF piglets[8], despite those groups secreting fewer total bile acids than the SF group. Therefore, we speculate that increased expression of MRP2 may explain the increased bile flow in ML and FO groups through increased transport of organic solutes, supported by our finding that MRP2 is an independent predictor of bile flow. Unfortunately, we did not measure organic solute transport and thus cannot test this hypothesis.

Although we found increased expression of the basolateral bile acid transporters OSTA in SO compared to SF piglets, we did not see an increase in MRP3, as would be expected in

cholestasis; this could be because bile flow in SO piglets was still within the normal range,[8] albeit at the lower end. A longer study duration may have resulted in more severe cholestasis. Nonetheless, we did see increased expression of OSTA or MRP3 in both the ML and FO groups, which are protective mechanisms for the prevention of cholestasis, consistent with clinical observations. However, we had not previously found increased serum bile acids with FO treatment when compared to SO treatment at a similarly restricted total dose.[7] We did see lower serum bile acids with ML treatment compared to SO at a higher dose.[8] The early increased expression of OSTA and MRP3 in the ML and FO groups in the absence of cholestasis is not readily explained and may be a unique regulatory effect of the omega-3 fatty acids. Regardless, it does support the role of both therapies in early prevention of IFALD.

As with FXR signalling, bile acid transporters also have differential function according to the substrates, including bile acid species. Yu et al.[39] demonstrated that BSEP expression is increased by CDCA, but decreased by LCA, through FXR. Further, in vitro treatment with CDCA increased expression of SHP, BSEP and OSTA.[27] Finally, Vlaardingerbroek et al.[4] found that BSEP expression increased dose-dependently in response to CDCA. Our study supports these findings in that the FO group had the greatest proportion of CDCA conjugates in bile, a low proportion of LCA conjugates, and the highest expression of SHP, BSEP and OSTA. Whether increased secretion of CDCA conjugates, along with decreased secretion of HCA, is due to preferential synthesis or transport would again be better understood by analysing bile acid concentrations in portal blood, liver, and serum.

Within the PN-fed groups, ML piglets expressed more BSEP, MRP2 and OSTA than SO piglets. Only BSEP expression was as high as in pure FO-treated piglets. Bile from ML piglets had similar proportions of CDCA conjugates and HCA as FO piglets, yet not statistically

different from SO piglets. The differences may simply reflect the lower total dose of FO delivered within the ML, as well as the presence and influence of a lower dose of phytosterols.

It is not unexpected that FXR, BSEP, and MRP2 univariately predicted bile flow given the role of FXR as a regulator of bile acid synthesis and transport, and the contribution BSEP and MRP2 make to the secretion of bile acid species and bile salt-independent flow respectively.[26] Similarly, it is not surprising that unconjugated HCA also univariately predicted bile flow, as this primary and hydrophilic bile acid would be expected to promote bile flow. Furthermore, it is likely that many of these univariate predictors are not independent of one another, which may explain why only BSEP and unconjugated HCA remained significant in the multivariate analysis. Although significant, each univariate predictor explains only a small portion of the model, as noted by low R^2 values, and approximately half of the variance in bile flow remains unexplained in the multivariate model. Thus, other important mechanisms in bile flow regulation remain to be discovered.

This study enables an examination of potential mechanisms associated with PN lipid doses as used in clinical practice; however, the lower PN lipid dose in the FO group is an acknowledged confounder. The 2-week study duration is another limitation, although given the rapid growth of piglets it must be remembered that this duration translates to approximately 4-6 months for a human infant, which is also equivalent to the age of weaning of piglets at 3 weeks.[40] Differences in the phytosterol and vitamin E content (Table 4) between lipid emulsions is another acknowledged confounder. While there is some data from rodent studies that does implicate specific fatty acids that are present in FO-containing emulsions, and absent in SO, as being hepatoprotective,[35] we cannot ascertain with our clinically determined study design if the effect is due to the presence of these fatty acids or difference in phytosterol or vitamin E content.

Finally, we have acknowledged that our study would have been enhanced by examination of ileal FXR, hepatic LXR, and signalling via FGF19. It would further have benefited from measurement of bile acid transport proteins, rather than assessment of gene expression alone.

In the current study, we were interested in studying the mechanisms for altered bile flow that we have previously shown with lipid emulsions as currently prescribed in neonatal practice to prevent or treat IFALD. These results show altered expression of genes involved in bile acid metabolism and transport, and altered bile acid composition with PN lipid modulation. FO is associated with increased expression of FXR, along with decreased expression of CYP7A1 for bile acid synthesis and increased expression of canalicular and basolateral bile acid transporters. Furthermore, FO-containing PN lipid resulted in the secretion of a more hydrophobic bile acid pool from the liver. Therefore, multiple, interrelated mechanisms appear to be implicated in bile flow up-regulation and hepatoprotective effects of FO. Based on comparisons between FO and ML, these effects appear to be dose dependent and support the current use of ML for prevention of IFALD and pure FO for treatment of severe, established IFALD.

Table 4-1: Sus Scrofa primers used for qPCR to analyze host gene expression in piglets.

Gene	Gene name	NCBI mRNA accession No.	Assay ID
ABCB11	ATP-binding cassette, sub-family B member 11	XM_003133457.3	AJFASAZ*
ABCC2	ATP-binding cassette sub-family C member 2	AF403247.1, DQ530510.1	Ss03373437m1
ABCC3	ATP-binding cassette sub-family C member 3	XM_003131575.4	AJVI4JV*
CYP7A1	cytochrome P450, family 7, subfamily A, polypeptide 1	NM_001005352.2	Ss03378689u1
HPRT1	hypoxanthine phosphoribosyltransferase-1	NM_001032.376.2	Ss03388274m1
NR0B2	nuclear receptor subfamily 0, group B, member 2	DQ002896	AJKAKZV*
NR1H4	nuclear receptor subfamily 1, group H, member 4	NM_001287412.1	AJKAKT2*
OSTA	organic solute transporter alpha	NM_001244266.1	AJMSHDY*

*Denotes custom-made assay using the Custom TaqMan® Assay Design Tool (Applied Biosystems, Carlsbad, CA, USA).

Table 4-2: Quantities of bile acid species in bile of PN-fed piglets.

Bile acid (mcg/mL)	SF (n=10)	SO (n=8)	ML (n=10)	FO (n=7)
Total bile acids	3729.39±821.71	806.58±555.58	1408.86±576.80	462.51±232.58
Total LCA	2.38 ± 0.93	0.27 ± .034	0.995 ± 0.78	0.13 ± 0.14
unconjugated LCA	n.d.	n.d.	n.d.	n.d.
t-LCA	1.48 ± 0.71	0.05 ± 0.10	0.40 ± 0.49	0.02 ± 0.05
g-LCA	0.90 ± 0.33	0.22 ± 0.26	0.59 ± 0.32	0.11 ± 0.099
Total CDCA	1012.26 ± 259.25	391.35 ± 372.35	781.46 ± 272.88	300.02 ± 123.87
unconjugated CDCA	n.d.	n.d.	n.d.	n.d.
t-CDCA	488.29 ± 112.35	78.25 ± 54.80	136.02 ± 121.93	34.06 ± 18.61
g-CDCA	523.96 ± 172.44	313.10 ± 319.42	645.44 ± 188.69	265.95 ± 114.56
Total CA	49.13 ± 29.21	6.80 ± 4.32	3.92 ± 3.71	0.80 ± 0.85
unconjugated CA	n.d.	0.55 ± 0.55	1.08 ± 0.60	0.35 ± 0.53
t-CA	48.29 ± 28.49	6.26 ± 4.69	2.84 ± 3.66	0.45 ± 0.61
Total HDCA	1101.18 ± 413.43	235.98 ± 192.91	416.13 ± 169.15	116.63 ± 75.90
unconjugated HDCA	n.d.	n.d.	n.d.	n.d.
t-HDCA	516.88 ± 188.84	15.31 ± 10.71	34.22 ± 33.65	7.42 ± 11.36
g-HDCA	584.30 ± 261.87	220.66 ± 182.75	381.91 ± 142.96	109.20 ± 66.99
Total HCA	1564.45 ± 569.29	172.18 ± 89.87	206.36 ± 182.79	44.94 ± 52.35
unconjugated HCA	15.52 ± 14.04	7.61 ± 7.67	13.36 ± 18.33	2.31 ± 2.10
t-HCA	1548.93 ± 557.87	164.56 ± 87.73	193.00 ± 180.32	42.63 ± 51.07

Mean ± standard deviation. n.d., not determined.

Table 4-3: Univariate predictors of bile flow in PN-fed piglets.

Predictor variable	Number of observations	Intercept	Coefficient	R^2	95% CI	*P* value
***Gene* (Protein)**						
NR1H4 (FXR)	34	7.696	1.612	0.169	0.323-2.902	0.02
ABCB11 (BSEP)	34	7.003	2.829	0.229	0.958-4.701	<0.01
ABCC2 (MRP2)	34	7.707	1.587	0.212	0.485-2.689	<0.01
SLC51A (OSTA)	32	9.088	0.053	0.254	0.019-0.087	<0.01
Bile Acid Species as a Proportion of Total						
g-LCA	35	7.576	123.762	0.123	6.583-240.941	0.04
t-CDCA	35	18.293	-0.734	0.254	-1.178-(-0.289)	<0.01
g-CDCA	35	7.102	0.104	0.162	0.0201-0.188	0.02
Total CDCA conjugates	35	6.422	0.096	0.116	0.002-0.190	0.05
Unconjugated CA	35	9.044	35.395	0.151	5.643-65.148	0.02
t-CA	35	12.442	-1.949	0.164	-3.508-(-0.390)	0.02
Total CA	35	12.521	-1.923	0.154	-3.523-(-0.324)	0.02
Unconjugated HCA	35	8.416	3.676	0.280	1.590-5.762	<0.01
t-HCA	35	13.341	-0.104	0.126	-0.200-(-0.007)	0.04
Total HCA	35	13.277	-0.098	0.112	-0.196-(0.000)	0.05

Table 4-4: Nutrient delivery to PN-fed piglets.

Nutrient	SO	ML	FO
Total energy, MJ/kg/d	1.1	1.1	0.9
Total lipid, g/kg/d	10	10	5
Soybean oil	10	3	0
Medium-chain triglycerides	0	3	0
Olive oil	0	2.5	0
Fish oil	0	1.5	5
Total protein, g/kg/d	16	16	16
Total dextrose, g/kg/d	29	29	29
Key fatty acids, g/kg/d*			
Palmitic acid (C16:0)	1.1	0.9	0.6
Oleic acid (C18:1 ω-9)	2.4	2.7	0.58
Linoleic acid (C18:2 ω-6)	5.3	1.8	0.16
α-Linolenic acid (C18:3 ω-3)	0.8	0.2	0.07
Arachidonic acid (C20:4 ω-6)	0.02	0.05	0.13
Eicosapentaenoic acid (C20:5 ω-3)	0	0.3	0.97
Docosahexaenoic acid (C22:6 ω-3)	0	0.2	0.93
Phytosterols, mg/kg/d*	22	10	0
Vitamin E, mg/kg/d* α-tocopherol equivalents	2.8	6.5	7.4

*Amounts provided by manufacturer (Fresenius Kabi, Bad Homburg, Germany) and are approximate due to variations in natural raw materials.

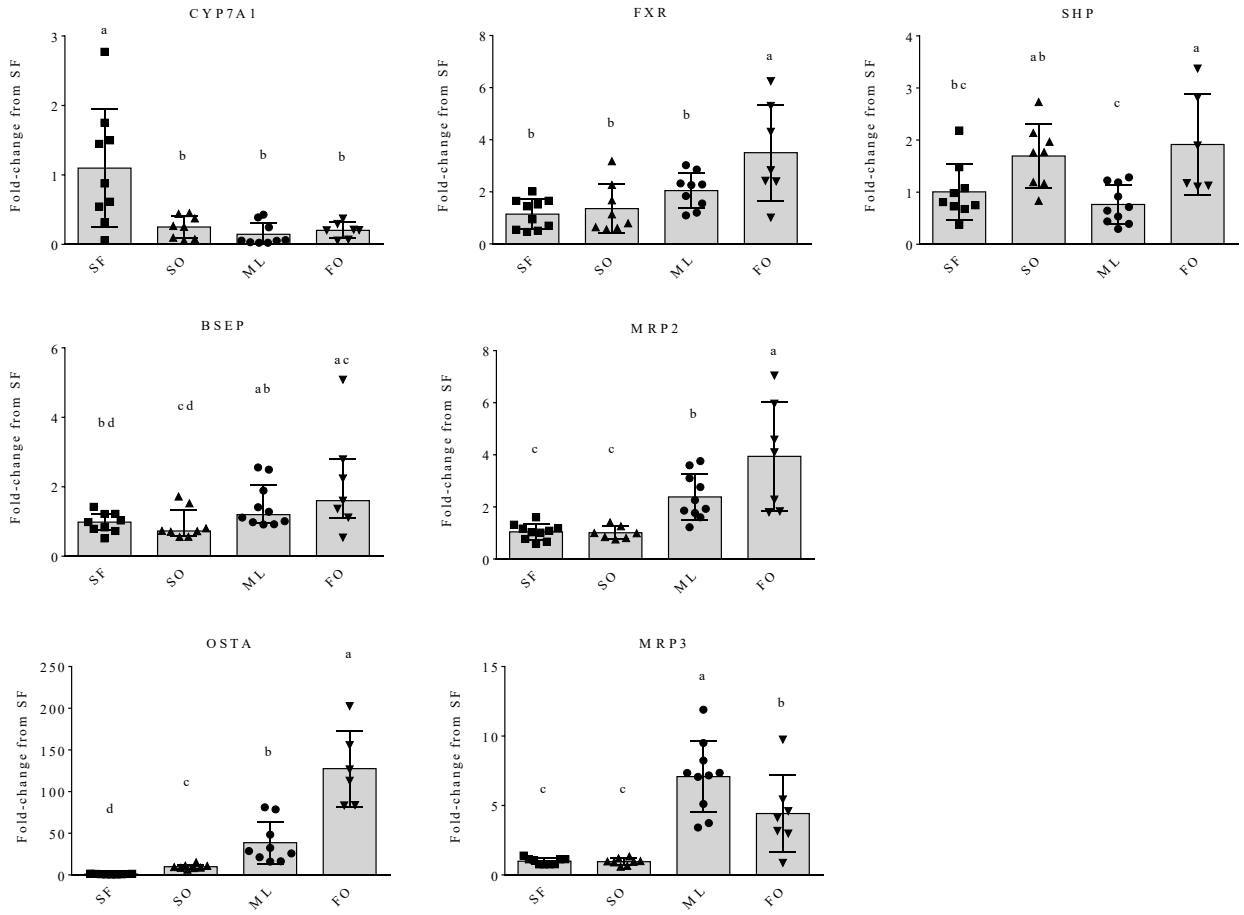

Figure 4-1: Relative expression of genes involved in bile acid metabolism in SF and PN-fed neonatal piglets.

Relative gene expression of (A) CYP7A1 (p =0.04), (B) FXR (p <0.01), (C) SHP (p <0.01), (D) BSEP (P = .04), (E) MRP2 (p <0.001), (F) OSTA (p <0.001), and (G) MRP3 (p <0.001). Data are mean ± standard deviation for CYP7A1, FXR, MRP2, OSTA, and MRP3 (analyzed by Welch's ANOVA); mean ± standard deviation for SHP (analyzed by ANOVA); and median ± interquartile range for BSEP (analyzed by Kruskal Wallis ANOVA). Post hoc statistical significance $p \leq 0.05$. a,b,c,d: between-group differences are noted by different letters.

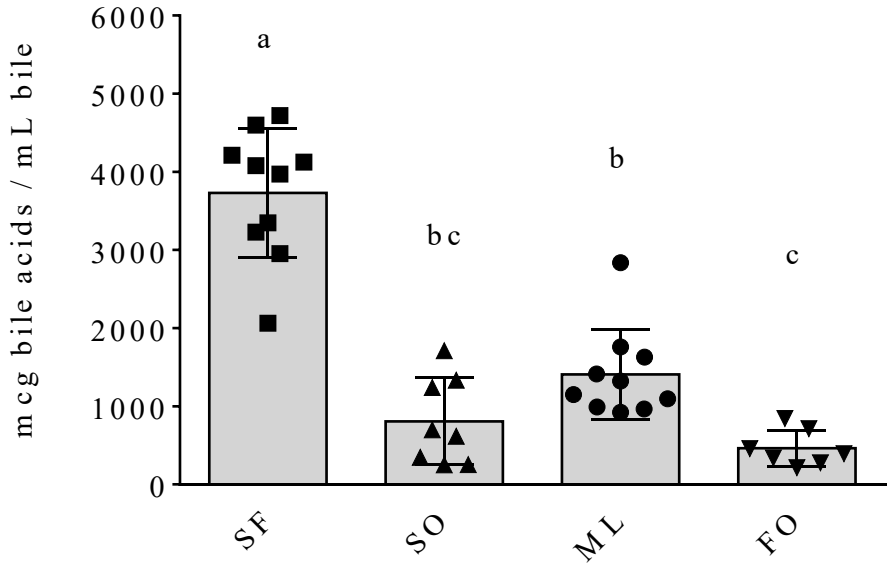

Figure 4-2: Bile acids excreted in bile of SF and PN-fed neonatal piglets.

There were significant differences in the total bile acids excreted from the liver in bile (p <0.001). Data are mean ± standard deviation. Post hoc statistical significance $p \leq 0.05$. a,b,c: between-group differences are noted by different letters.

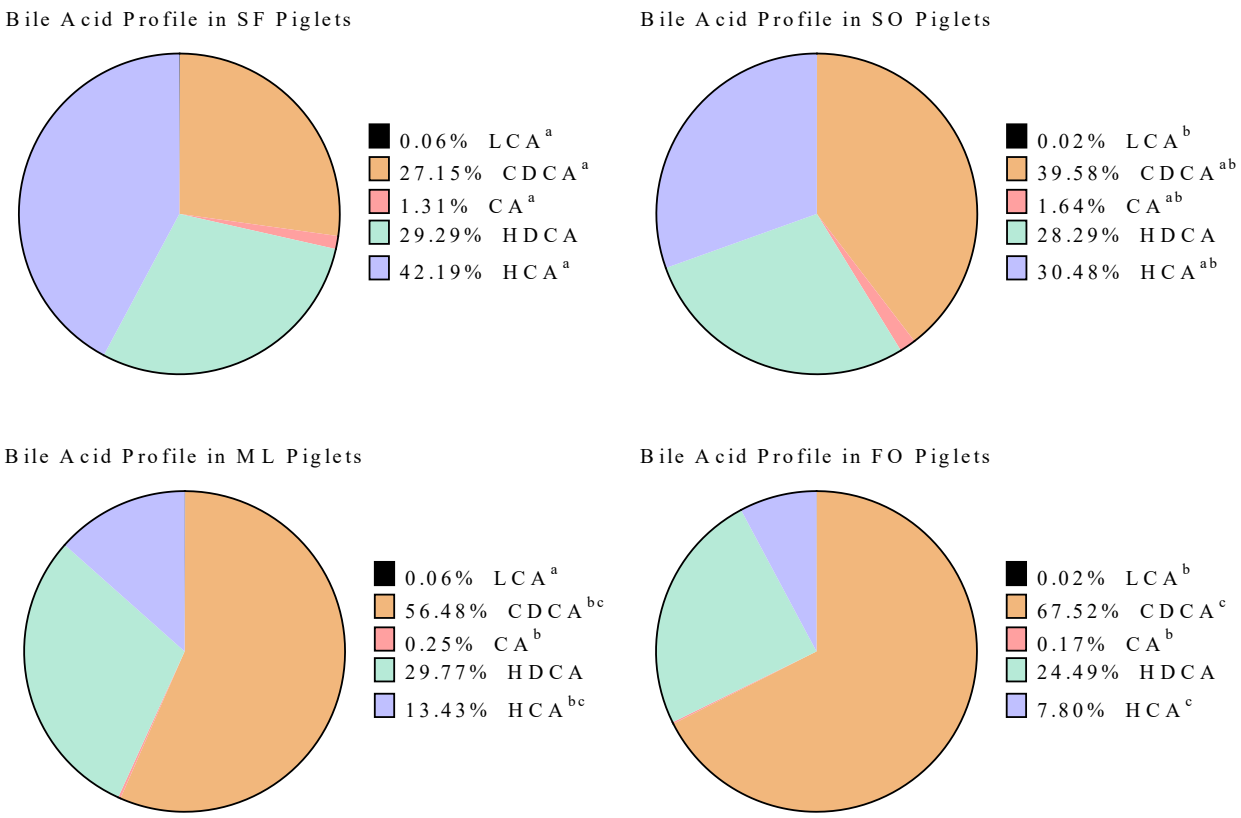

Figure 4-3: Bile acid composition in bile of SF and PN-fed neonatal piglets.

Proportion of total LCA, CDCA and HDCA conjugates, and total unconjugated and conjugated CA and HCA in excreted bile. Bile acid species are listed in order of most to least hydrophobic from LCA to HCA. Analyzed by ANOVA for LCA and HDCA, and Welch's ANOVA for CDCA, CA, and HCA. Statistical significance $p \leq 0.05$. a,b,c: between-group differences are noted by different letters.

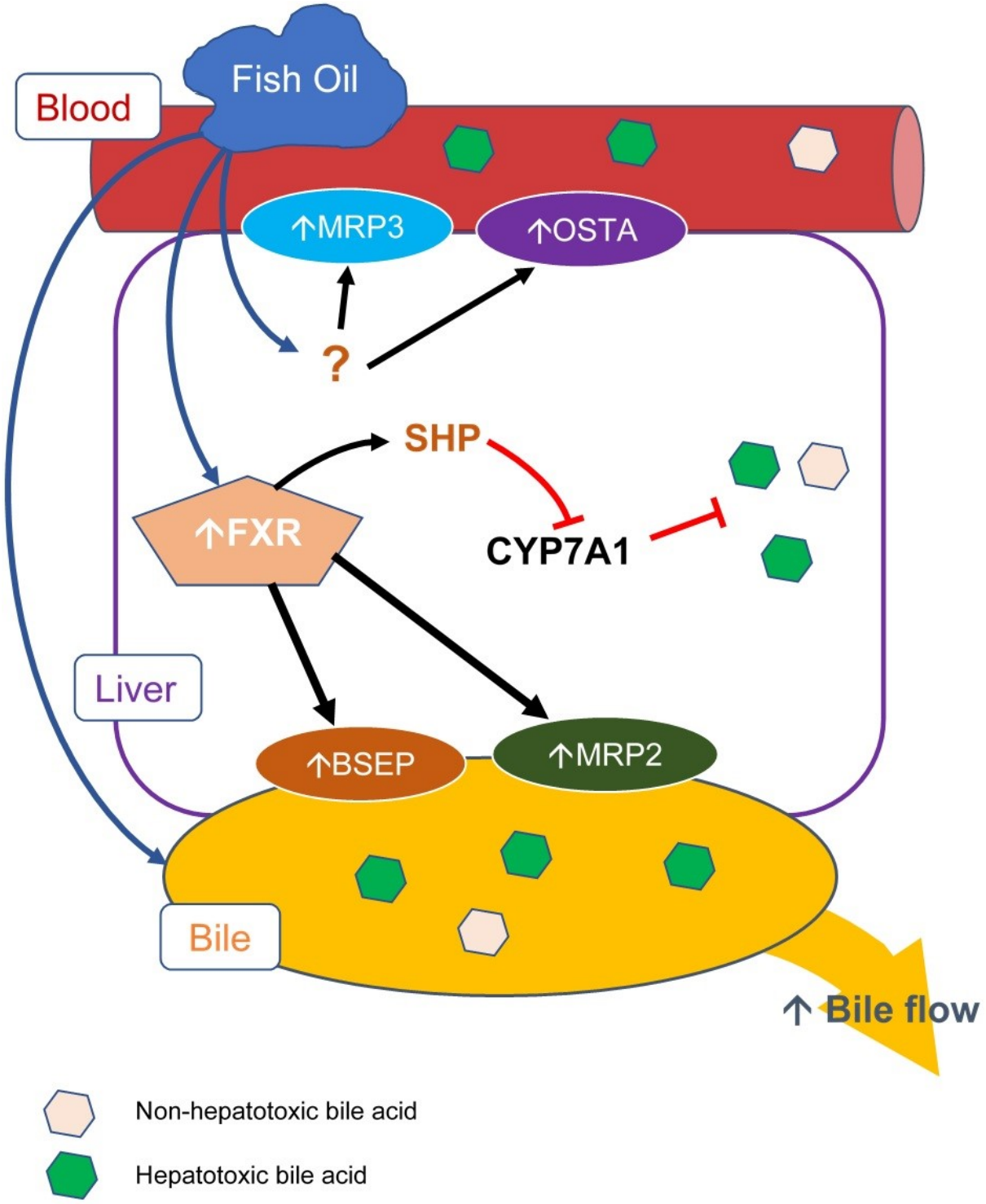

Figure 4-4: Mechanisms contributing to increased bile flow with FO-containing PN lipid.

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CHAPTER 5: Conclusion

5.1 SUMMARY OF THE STUDIES

Neonatal IFALD is a devastating disease with a risk of end stage liver disease with fibrosis, even in this era where the use of fish oil emulsions has improved outcomes.[1-4] Our limited understanding of the mechanisms of IFALD, especially in neonates, has resulted in a paucity of evidence-based treatments for the disease. The research in this thesis is novel in that it is the first to examine the role of remnant SBS anatomy in the progression of neonatal IFALD, in the setting of 100% PN, including the effects on bile flow, EBF, and bacterial translocation. Additionally, this thesis is significant because it adds to our knowledge of the effects of PN lipid modulation on EBF, GI immune function, the gut microbiota, and bile acid metabolism in neonatal IFALD.

Resection of the ileum and ICV in SBS have been considered factors for increasing severity of IFALD; yet, biomedical evidence explaining the mechanisms for this increased risk is limited. Further, loss of the ICV is believed to increase bacterial translocation despite evidence to the contrary in adult animal models. To aid in filling this gap in evidence, the objective of Chapter 2 was to determine the impact of remnant SBS anatomy on the progression of neonatal IFALD in the setting of 100% PN. We measured bile flow and clinical markers of liver disease, and evaluated liver pathology to determine the presence and severity of liver disease. Additionally, we studied the effects of remnant anatomy on EBF by assessing changes in intestinal structure and by measuring intestinal permeability through Ussing chamber methodology and the expression of intestinal tight junction proteins. We further compared bacterial translocation in the presence and absence of the ileum and ICV.

As expected, PN resulted in decreased bile flow with increased serum bilirubin, GGT, and serum bile acids, and worse liver pathology scores. Surprisingly however, and contrary to

our hypotheses, the progression of IFALD was not worse when resection included that of the ileum and ICV. None of the indicators of IFALD, including bile flow, clinical indicators of liver disease, and liver pathology scores, were different between resected SBS anatomies. Serum bile acids were highest in the non-resected, PN-fed group, but that was the only difference in markers of IFALD between the PN-fed groups. The findings suggest that PN itself is a more important risk factor for IFALD than intestinal anatomy, which would not be a modifiable factor regardless. The role of remnant SBS anatomy, such as presence of an ICV proposed as being protective for IFALD, would simply be that of potential for intestinal adaptation, and hence autonomy from PN. This does fit with the evidence that human patients[5] and piglets[6] without an ICV take longer to adapt to EN, and the likelihood of adaptation is decreased. Going forward the focus needs to be understanding the exact mechanisms for PN in causing IFALD so that more modifications can be made to prevent this complication.

Unexpectedly, although jejunal villi length was shorter in PN-fed groups, intestinal permeability, as measured by Ussing and assessed through the gene expression of tight junction proteins, did not differ with PN or between intestinal anatomies. It is unclear why increased permeability was not associated with PN. However, a longer study duration may have identified differences in intestinal adaptation and permeability between remnant anatomies. It is also plausible that the PN-related changes to intestinal morphology and function found in adults[7] simply don't occur in the same manner in the developing neonatal GI tract. Further, given that gut permeability is initially high in neonates, and decreases over time,[8, 9] it is possible that incremental PN-related increases in permeability may not be to a sufficient degree to quantify notable differences. Finally, it is possible functional adaptation in the period following surgical resection may provide an initial protective effect in SBS; thus, differences in permeability may

not be noted for some time post-operatively. This is supported by a study of EN-fed mice in which increased intestinal adaptation was found in the ileum of gut-resected mice compared to sham operated mice at 3, 7, and 14 days post-operatively.[10] Further, ileal permeability was lower, and membrane resistance was higher, 3 days post-operatively in gut-resected mice. By 7 days post-operatively, there were no differences in permeability or resistance between the groups.

Bacterial translocation to the lymph nodes was indeed associated with sepsis. Yet, again contrary to our hypothesis, remnant SBS anatomy did not impact bacterial translocation. Thus, our results provide evidence in a neonatal model that absence of the ICV does not increase bacterial translocation. Importantly, we did discover that sepsis contributed more to the progression of IFALD than intestinal anatomy. In fact, PN without resection resulted in the highest incidence of sepsis. Although we cannot explain this increased incidence, we were able to decipher the effect of sepsis, regardless of intestinal anatomy, on IFALD. Sepsis was associated with decreased bile flow, increased bilirubin serum bile acid levels, longer villi, increased jejunal permeability as measured by Ussing methodology, and greater bacterial density in the lymph nodes. The importance of this finding will be determining still further modifiable factors that can reduce sepsis during PN, in order to prevent IFALD.

In previous work, our laboratory found that use of a FO-containing ML in the PN emulsion prevented liver disease and reduced systemic inflammation.[11] We hypothesized this could be due to the anti-inflammatory[12] and antimicrobial[13-15] properties of the LC-PUFAs contained in the ML. As such, we were interested in whether ML mitigates microbial dysbiosis and the host immune response. In Chapter 3 we explored the impact of PN lipid formulation on EBF, immune function, and the gut microbiota in a neonatal model.

While PN lipid modulation did not impact intestinal structure, it did affect the expression of genes related to EBF. PN per se was associated with increased gene expression of tight junction protein CLDN-1, antimicrobial peptide beta defensin-2, and glycoproteins *Muc1*, *Muc2*, and *Muc4*, and the expressions of all these genes were highest with SO. The increased expression of IL-8 with SO indicates an increased inflammatory response. This supports our earlier finding that ML reduces inflammation.

It was not surprising that PN altered the gut microbiota. This may be attributed to the lack of EN, and thus lack of nutrients to support the gut microbiome. However, notably we also showed that the PN lipid formulation altered the gut microbiota, which is a novel finding. Although the microbiota of all groups was different from one-another, the ML group was more similar to healthy controls than the SO group was to healthy controls. Remarkably, these results indicate that the gut microbiome can be altered by changing the composition of nutrients provided systemically, irrespective of the lack of EN. Whether these changes in the microbiome are a cause or effect of concurrent alterations in EBF, such as changes in the mucus layer, has yet to be elucidated.

Our laboratory has previously shown that PN lipid modulation alters bile flow.[11, 16] The purpose of Chapter 4 was to investigate potential mechanisms for this altered bile flow. To do so, we examined expression of genes related to bile acid metabolism and transport in response to PN lipid modulation, in the setting of 100% PN. This study is the first to examine such potential mechanisms associated with PN lipid formulations and doses that are currently used in clinical practice. Indeed, PN resulted in the altered expression of hepatic genes involved in the regulation, synthesis and transport of bile acids, as well as in a reduced bile acid pool size, and altered bile acid profiles. PN lipid modulation also altered gene expression and bile acid profiles.

Although we would have expected the reduced size of the bile acid pool with PN to drive increased de novo synthesis, PN-feeding led to decreased expression of CYP7A1, and thus decreased synthesis. Given that the FO group was the only PN-fed group associated with increased hepatic FXR expression, the role of ileal signalling to FXR in the regulation of CYP7A1 should be considered.

The increased expressions of canalicular bile acid transport genes associated with FO was expected given, the increased FXR expression in that group. These transporters are likely crucial determinants for increased bile flow, as each BSEP and MRP2 univariately predict bile flow. Alternatively, the reason for increased expression of basolateral transport genes with FO-containing PN lipid is unclear since increased serum bile acids were not found in those piglets. Finally, a novel finding, which was contrary to our hypothesis, was that FO-containing PN lipid was associated with a more hydrophobic bile acid pool. It seems the hepatoprotective effect of FO may involve reduced bile acid synthesis along with upregulation of canalicular and basolateral transporters to limit the build-up of toxic bile acids in the liver, but the reason for increased hydrophobicity of the bile acid pool is unclear. The mechanisms, including the role of the gut microbiota, for altered bile acid composition with FO-containing lipid should be further explored.

Using the results of our molecular and metabolic analyses, we employed linear regression to determine predictors of bile flow. The expression of several genes, and the proportion of many bile acid species univariately predict bile flow in our model. However, in multivariate analysis we determined that BSEP and unconjugated HCA best predict bile flow. Given the small portion of bile flow that univariate and multivariate predictors explain, there are yet other contributors to bile flow regulation that remain to be elucidated. It seems these mechanisms are interrelated and

likely dose-dependent. Although our results are contrary to our hypotheses, it is important to consider that the lack of EN in the setting of 100% PN may contribute to a molecular and metabolic phenotype that deviates entirely from that expected when enteral stimulation is provided. Regardless, our results do support the use of FO-containing PN lipid for the treatment and prevention of IFALD.

5.2 LIMITATIONS OF THE STUDIES

There are limitations to address in the studies regarding experimental design and confounding factors. Firstly, we considered piglets raised with their sows to be the gold standard against which to compare our variables of interest. These sow-fed control animals were raised under standard barn conditions, which we recognize is a substantially different environment than what the treatment animals were exposed to. Thus, the results we have noted between sow-fed control and treatment animals may be an effect of the environment and of both surgical stress, and the stress of life in single-housed metabolic cages, over and above treatment effects. The variables for which environment and stress could reasonably have a large impact are the gut microbiome, mucosal immunity, and permeability.

Housing and handling in metabolic cages, parenteral lines and antibiotic use to avoid central line infections are all likely to have major impact on the microbiome, which in some ways is akin to hospitalization of infants with intestinal failure. Sow-fed animals are raised alongside the rest of their litter, in close contact with their sow, whereas treatment animals are raised in individual cages, without direct exposure to their sow or siblings. These differences in environmental exposures are known to affect the gut microbiome.[17] Hospitalization is also known to impact the development of the infant microbiome.[18] The reality for neonates with IF is that they will be hospitalized for prolonged periods and will frequently be exposed to courses of

antibiotic, and both of these factors would impact the microbiome, just as in the piglet model. In our study design, treatment animals were raised under the same environmental conditions, although differences in antibiotic exposures did occur. However, we recognize that we could have strengthened the study design by including a control group of formula-fed piglets raised in individual cages and given the same antibiotic treatments. Future studies should consider the benefits of including such a control group against the additional direct and indirect costs associated with doing so.

In prior surgical models sham piglets received an abdominal incision for gastric catheter insertion, and bowel handling to measure bowel length, but no resection. However, we saw high mortality rates in those sham piglets and believe this was likely due to increased risk of adhesive bowel obstruction due to the rapidly growing intestine in the sham. Since piglets in the sham groups in the current studies did not require gastric catheter incision, they instead had surgery for jugular catheter placement but did not receive an abdominal incision. As such, we acknowledge there was likely considerably less surgical stress likely in the sham.

Our study designs provided 14 days of continuous PN, which translates to 4-6 months for human babies.[19] At this point, only the early markers of IFALD indicate presence of the disease, and intestinal adaptation may only be in the early stages. The study duration may be too limited to examine the impact of adaptation on the progression of IFALD in varying SBS anatomies. Our laboratory did previously experiment with 21-day trials, but found that due to the rapid growth rate of piglets, they outgrew their jugular catheters before the end of the study. The need for surgery to insert a second catheter mid-trial would have added further confounding factors, such as surgical stress impacting the host immune response. Thus, we limited our study duration, such

that we are observing the early emergence of IFALD and not its progression to an end stage disease.

Our assessment of EBF included the gene expression of tight junction proteins, beta defensins, and mucins. Although the expression of these genes is commonly measured as an indicator of the integrity and function of the epithelium, we recognize that the protein expression of these can at times be decreased, despite increased gene expression. The use of Ussing chamber methodology, as was the case in Chapter 2, allows for a more functional analysis, and would have added value to the study in Chapter 3.

We did observe large variations in both the gene expression levels and functional permeability results. Previous work in our laboratory detected similar variation.[20] Given that the variability was noted across measurement variables and treatment groups, in both our current and previous work, we suspect the variability is inherent in this neonatal piglet model. Although permeability data from rat and mouse models tends to be less variable, those studies are in adults with a fully developed GI tract.[21-24] Thus, the variability observed in the present work may be a factor of the developing neonatal GI tract. Measuring protein expression and visualizing tight junction proteins would greatly improve the study design, and may help to explain the variability seen in our results.

We also recognize there are numerous immune markers and tight junction proteins that we did not assess in either Chapter 2 or 3. As such, we can only conclude that no differences were noted in barrier function or permeability only in the specific factors that we did measure. It is possible that other immune markers or tight junction proteins would have show differences. Thus, ideally the studies would have benefited with the measurement of additional immune markers and tight junction proteins to strengthen the conclusions.

There are also known limitations of Ussing methodology that must be considered as factors for the lack of permeability differences between groups in this study, particularly given the large within-group variations. This includes tissue specific factors, such as the impact of limited tissue viability over time, edge damage, edema and differences in tissue handling at the time of terminal laparotomy.[25] The integrity of ex vivo intestine deteriorates over time;[25] thus, the time from excision to processing of the tissue is critical. Any differences in this length of time between samples can alter the results. We did transport samples in the same manner with similar transport time, but there may well have been differences in the operation time to procure the sample that could have impacted tissue viability. Edge damage, which is an artifact of the procedure and results from the extrusion of a portion of the mucosa around the aperture while under Ussing investigation, can artificially increase and P_{app} and TER.[25] Other limitations that may have impacted our Ussing chamber findings were the presence of edema, which can develop during PN feeding,[7] and poor tissue integrity resulting from the handling to release adhesions that develop after abdominal surgery.[26] Edema has been linked to changes in permeability measured in the Ussing chamber.[27] As we did not have an accurate measure of total body water, we cannot be certain that our permeability data were not affected by edema. Further, local inflammation and adhesions may have contributed further to the high within-group variability that was noted in this study. While larger sample sizes may provide sufficient power to control for such factors in the statistical analyses, but this must be carefully considered against the ethical dilemma of utilizing more animals in the research process and the costs of a large animal model.

Given shorter villus height between PN-fed compared to SF groups, the impact of overall epithelial surface area should be considered in interpreting the permeability results. With shorter

villi, it is expected that the overall exposed epithelial surface area in the Ussing chamber would be reduced in the samples from PN-fed piglets, dependent on mucosal atrophy or adaptation. Additionally, just as injury to the tissue will reduce tissue integrity, as mentioned above, injury to the mucosa may also reduce the overall absorptive surface area, and this injury is more likely to be noted in the SBS piglets than Sham or SF. Thus, since a similar amount of a given probe permeated the tissue in PN-fed piglets, despite a smaller overall surface area, the jejunum of PN-fed piglets may indeed be more permeable than that of SF piglets. That septic piglets had increased villus height, and thus greater overall epithelial surface area, along with greater permeability to mannitol supports this suggestion.

Finally, the size[28, 29] and structure[28] of probes used in the Ussing experiments are also important considerations. Molecules of smaller molecular weight (MW) are generally more permeable to the epithelium than those of greater MW.[28, 29] However, inulin (~5000 MW) was shown to have similar permeability as PEG 900 (~900 MW), suggesting the permeability of inulin is due to the structure of the molecule. In our experiments, we used two probes of relatively small MW: PEG 400 (~400 MW) was used as a paracellular marker and mannitol (~180 MW) as a transcellular marker of permeability. Although PEG 400 is a common marker for intestinal permeability,[30] employing a greater variety of probes of varying sizes, such as inulin and PEG 2000, may have yielded differences in the permeability results.

The study in Chapter 4 employed a design in which PN-fed piglets were treated with lipid doses based on current clinical practice. SO and ML were provided at the equivalent of what would be a conventional dose in human infants, whereas FO was provided at a lower dose, as is the case in standard practice.[31-34] In human infants, additional carbohydrate can be provided to replace kilocalories when lipid dose is reduced. As explained elsewhere[16], in pilot studies we

attempted to provide piglets isocaloric diets by adding more dextrose to low-dose lipid groups. Due to the much higher energy needs of neonatal piglets, the amount of dextrose needed to meet their needs resulted in a nephropathy causing high mortality. As such, we were not ethically able to match kilocalories in the low-dose lipid group to the conventional-dose groups. Additionally, even if PN diets could be made isocaloric by increasing dextrose, we would not be able to decipher whether any effects seen in the FO-treated group were due to the lipid or to the additional carbohydrate. A study design that provide all PN lipids at the higher dose, as well as at the lower dose, would eliminate this limitation. It would allow a comparison of impacts on molecular mechanisms at equivalent lipid doses, as well as an examination of potential dose-responses.

Other important factors that confound results are the development of sepsis, and antibiotic use to manage it. To reduce the risk of sepsis, all PN-fed piglets were given prophylactic antibiotics in the 4 days immediately post-surgery, and again from day 8 to 12, as our previous experience has shown this to be when sepsis typically occurs when antibiotics are not given. Unfortunately, sepsis is still a risk despite this prophylactic treatment. We carefully monitored the piglets for signs of sepsis, which include lethargy, fever, and vomiting. Because a piglet's status can deteriorate quickly in the case of sepsis, we drew blood for cultures at the onset of these signs and immediately treated with additional antibiotics. From that point, the piglet was treated with an individualized antibiotic regime, dependent on its clinical response. Initial culture results were reported after 24 hours, but full results were often only available after 7 days. This meant that piglets would be treated for sepsis, before being confirmed as septic. In some cases, the culture results were negative. As such, that piglet was included in analyses as non-septic, despite having been treated with additional antibiotics. Additionally, it is possible

that culture results for some piglets were falsely negative, as it is possible that not all bacteria may be cultured with the standard techniques used. Nevertheless, the decision to include a piglet in analyses as either septic or non-septic must be based on the evidence available, therefore we considered septic animals to be those that were confirmed septic by a positive blood culture. Given the samples sizes in our studies, we did not have sufficient power to control for the varying antibiotic regimes. Although we compared septic to non-septic animals, and we cannot discern whether any differing effects were a result of sepsis or of antibiotic treatment. Sadly, sepsis and antibiotic treatment are also common in PN-fed human neonates, and it may not be feasible to increase sample sizes to control for sepsis and varying antibiotic treatment.

5.3 FUTURE DIRECTIONS

This thesis has begun to elucidate some of the molecular mechanisms of host-microbial interactions and bile acid metabolism in IFALD, but there is still much to be learned. Besides implementing the changes in study designs mentioned above, research in the following areas will improve our understanding of the mechanisms and potential treatments for IFALD.

A broader examination of the mechanisms involved throughout the enterohepatic circuit would further our understanding of alterations that occur with PN lipid modulation, and may help explain why the secretion of hydrophobic bile acids is promoted with FO-containing PN lipid. Thus, future studies should analyse the bile acid composition and size of the bile acid pool in portal blood, the liver, and serum, in addition to these analyses in bile. In Chapter 4, we examined molecular mechanisms in the liver. However, FGF19 signalling from the ileum also acts to regulate bile acid synthesis,[35] and ileal bile acid uptake impacts the size of the bile acid pool.[36] Hence, it would be beneficial to examine the impact of PN lipid modulation on ileal FGF19 and on the expression of ileal bile acid transport proteins.

In Chapter 3 we determined that the PN lipid formulation does indeed affect the bacterial composition in the gut, but the mechanisms by which this occurs are unknown. Additionally, we learned in Chapter 4 that bile acid composition is altered by PN lipid modulation. Thus, given that bile acids can alter the gut microbiota,[37] it is plausible that PN lipid modulation alters the gut microbiota through the resulting changes in bile acid composition and metabolism. Alternatively, since the microbiome affects bile acid composition,[36] microbial dysbiosis certainly has the potential to alter bile acid composition and metabolism to a phenotype that contributes to the progression of IFALD. As such, it would be pertinent to investigate associations between PN lipid formulation, bile acid metabolism, and the microbiome.

In Chapter 2 we concluded that SBS anatomy per se contributes minimally to the early development and progression of IFALD with the use of SO as the PN lipid, in the setting of 100% PN. However, an examination of other factors, including bile acid metabolism, the immune response, and the microbiome under varying SBS anatomies could bring to light underlying mechanisms that may impact the progression of IFALD in the long term. As mentioned above, microbial dysbiosis has the potential to alter bile acid composition and metabolism, and the bacterial colonies that proliferate can increase the risk of sepsis, thereby increasing the risk of IFALD. Moreover, we have suggested that SBS anatomy may influence gut adaptation, and thus indirectly increases the risk of IFALD through prolonged dependence on PN in the absence of adaptation. Recent evidence suggests that the microbiota plays a role in adaptation following surgical resection.[38] Further, a study of preterm piglets investigated the effects of intravenous compared to oral antibiotics on the progression of NEC;[39] as such, the piglets were first provided PN, followed by rapidly advanced volumes of EN formula to induce NEC. The authors concluded that enteral antibiotics enhanced gut function, whereas intravenous

antibiotics did not. Although the results of the study are not directly applicable to 100% PN-fed infants, they do highlight the potential impact that oral antibiotics could have on gut adaptation in IF. As such, quantification and characterization of bacteria in the remnant intestine, as well as analysis of associations between gut microbiota and bile acid metabolism, will significantly improve the body of knowledge regarding IFALD, and could potentially lead to the development of probiotic or antimicrobial treatments. Our lab has collected data in this regard and we are currently conducting the related analyses. The piglets in our studies were treated with intravenous antibiotics, but once the gut microbiota has been characterized, a future trial could compare the use of oral versus intravenous antibiotics in the setting of 100% PN.

5.4 CONCLUDING STATEMENT

This thesis provides novel evidence of the impact of remnant anatomy on the development of IFALD, EBF, and bacterial translocation in a neonatal model of SBS, in the setting of 100% PN. It offers experimental evidence that absence of the ICV is not associated with increased risk bacterial translocation in neonatal SBS, at least in the short term. Further, this thesis provides in vivo evidence that PN lipid modulation alters the gut microbiota, EBF, GI immune function, bile acid metabolism, and bile acid composition. Importantly, this work provides biomedical evidence of the molecular mechanisms of bile acid metabolism in IFALD. Perhaps the most notable contribution of this thesis is the finding that both the gut microbiota and the bile acid composition of secreted bile can be altered by changing the composition of lipid provided systemically, in the absence of EN. This may be the first step in discovering a PN formulation with either probiotic or antimicrobial effects that could potentially reduce the incidence of IFALD.

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