PARENTERAL NUTRITION ASSOCIATED LIVER DISEASE: THE EFFECTS OF ALUMINUM CONTAMINATION AND LIPID COMPOSITION ON BILE ACID TRANSPORTERS

A Thesis
Submitted to the College of Graduate and Postdoctoral Studies
in partial fulfillment of the requirements
for the degree of Doctor of Philosophy
in the Program of Health Sciences
College of Medicine
University of Saskatchewan
Saskatoon, Saskatchewan

By:

Amanda Hall

PERMISSION TO USE STATEMENT

In the presenting of this thesis in partial fulfillment of the requirements for a Postgraduate degree from the University of Saskatchewan, I agree that the Libraries of this University may make it freely available for inspection. I further agree that permission for copying of this thesis in any manner, in whole or in part, for scholarly purposes may be granted by the following:

Gordan A. Zello, Ph.D.
College of Pharmacy and Nutrition

Grant Miller, M.D.
College of Medicine

In their absence, permission may be granted by the Dean of the College of Medicine. It is understood that any copying or publication or use of this thesis or parts thereof for financial gain shall not be allowed without any written permission. It is also understood that due recognition shall be given to me and to the University of Saskatchewan in any scholarly use which may be made of any material in my thesis.

DISCLAIMER

Reference in this thesis/dissertation to any specific commercial products, process, or service by trade name, trademark, manufacturer, or otherwise, does not constitute or imply its endorsement, recommendation, or favoring by the University of Saskatchewan. The views and opinions of the author expressed herein do not state or reflect those of the University of Saskatchewan, and shall not be used for advertising or product endorsement purposes.

Requests for permission to copy or to make other use of material in this thesis in whole or part should be addressed to:

Dean of the College of Medicine
Office 2D01 Health Sciences Building
Mail: Box 19, Health Sciences Building
University of Saskatchewan

Saskatoon, Saskatchewan, S7N 5E5
Canada

OR

Dean
College of Graduate and Postdoctoral Studies
University of Saskatchewan
105 Administration Place
Saskatoon, Saskatchewan, S7N 5A2
Canada

ABSTRACT

Rationale: Infants on parenteral nutrition (PN) risk developing parenteral nutrition associated liver disease (PNALD). The pathophysiology is not fully understood, but it is a multi-factorial disease that likely stems from sepsis, hepatic immaturity, pro-inflammatory (omega-6) lipids, and PN contaminants such as aluminum (Al). Dysfunction of bile acid transporters may be an important mechanism for the development of cholestasis and PNALD. The objective of this project is to examine the effects of Al on bile acid transporter proteins, both with and without pro-inflammatory lipids, thereby determining whether further efforts to reduce Al are needed for the treatment of PNALD.

Methods: We conducted four different experiments, including a clinical survey, two piglet PN models, and one rat hepatocyte model.

In our first experiment, we collected thirty samples of PN from a neonatal intensive care unit and analyzed the Al content using inductively coupled plasma mass spectrometry.

For the piglet work, we conducted two randomized control trials using a Yucatan miniature piglet PN model. Newborn piglets (aged three to six days) were placed into two groups of seven to eight animals each. All groups were maintained on a strict PN diet for two to three weeks. One group in each study received PN with 24μg/kg/day Al, while the other received 63μg/kg/day of Al in PN. The first trial used omega-6 lipids in the PN for all piglets, while the second study used a mixed lipid solution. We chose five bile acid transporters (Mrp2, Bsep, Mrp3, Ntcp, and Oatp8), a cytoskeletal protein (radixin), and a nuclear receptor (FXR) as targets important in bile flow. All transporters were examined by qPCR, but only Mrp2 and Bsep were studied by immunofluorescence confocal microscopy, while Western blot evaluated Mrp2 protein. The serum was analyzed for total bile acids and for C-reactive protein (a marker of inflammation). In the second study, we also conducted transmission electron micrography to evaluate hepatocyte ultrastructure.

Our last study used sandwich-cultured primary rat hepatocytes. The hepatocytes were placed into six groups to compare Al, omega-6 lipids, and mixed lipids, both individually and combined. After 60 hours of exposure to the Al and/or lipids, the hepatocytes were collected

and similar bile acid transporters were evaluated using qPCR, with additional Western blotting for Ntcp and Oatp2, as well as a cholyl-lysl fluorescein functional assay for Mrp2.

Results: Our clinical work demonstrated that 90% of neonatal PN samples had potentially unsafe levels of Al (mean: 14.02 (SD: 6.51) µg/kg/day), as compared to the FDA recommendations of <5µg/kg/day. In the piglet trial using omega-6 lipids, qPCR demonstrated more mRNA for Mrp2, Bsep, Mrp3, and Ntcp in the lower Al group, as compared to the higher Al group. In the piglet study with mixed lipids, qPCR showed more mRNA for only Oatp8, Ntcp, and Mrp3, in the lower vs the higher Al group. This second piglet study also had a greater rise in C-reactive protein ($p=0.03$) and shorter canalicular microvilli ($p=0.01$) in the group with higher Al contamination, as compared to the lower Al group. Neither study showed any difference in the amount of Mrp2 protein between the groups and there were minimal changes in immunohistochemistry. There was also no difference in serum bile acids in either study.

The hepatocyte experiment showed a wide array of mRNA changes following exposure to Al and/or lipids. For Western blot, there was no difference in Ntcp protein between any of the groups. However, there was significantly more Oatp2 protein in the isolated Al group as compared to the Al + omega-6 lipid group ($p=0.04$). Finally, the cholyl-lysl fluorescein assay demonstrated the least excretion in any group containing mixed lipids.

Conclusions: Neonatal PN still has significant Al contamination. In piglet studies, high amounts of Al in PN have a negative effect on the mRNA of many of the bile acid transporters, in addition to inducing structural changes, and increasing inflammation. The less-inflammatory mixed lipid solution only partially mitigates the effects of Al. The hepatocyte work also suggests that there may be a synergistic negative effect for Al and omega-6 lipids, at least for Oatp2. Overall, Al does have a negative effect on bile acid transporters and could plausibly contribute to the pathogenesis of PNALD. Further efforts to reduce the Al contamination in infant PN are warranted.

ACKNOWLEDGEMENTS

I would like to thank my supervisors Dr. Miller and Dr. Zello for their advice, guidance and patience. I am also grateful to Chris Arnold for his instruction and support. The weekly meetings between the four of us for the past four and a half years have taught me how to become an independent researcher.

I would like to thank the other members of my committee: Dr. Alcorn and Dr. Keith, who contributed generously of both their time and knowledge.

This project would not have been possible without the partnership of Dr. Bertolo and Dr. Brunton from the Memorial University of Newfoundland. They and their team of summer students, graduate students, and especially their lab manager Dr. Dodge-Lynch, welcomed me into their lab for two summers and guided me through the complexities of animal research.

I am grateful to the countless people who shared their technical expertise with me. This includes Eiko Kawamura, Larhonda Sobchishin, and Dr. Singh at the Western College of Veterinary Medicine, Dr. Desai from the Department of Pharmacology, Dr. Sergi from the University of Alberta, my labmates in 3D10, and the many other individuals who taught me. A special thanks goes to our lab technician Deborah Michel, and to our statistician Ha Le.

Lastly, I would like to thank my family, especially my husband, for believing in me and encouraging me through this process.

This project was generously funded by the University of Saskatchewan, Department of Surgery and by the Children's Hospital Foundation of Saskatchewan.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES AND EQUATIONS

LIST OF ABBREVIATIONS, SYMBOLS, AND ACRONYMS

Al: Aluminum

AA: arachidonic acid

AGA: Appropriate for gestational age

ANOVA: Analysis of variance

ASBT: apical sodium dependent bile acid transporter

ASPEN: American Society for Parenteral and Enteral Nutrition

ATP: Adenosine triphosphate

AVA: Average for gestational age

BA: Bile acids

BSEP/Bsep: Bile salt export pump

Cº: Degrees Celsius

CAR: Constitutive androstane receptor

CAT: Catalase

cDNA: Complementary deoxyribonucleic acid

CLF: Cholyl-lysyl fluorescein

cm: Centimetre

CRP: C-reactive protein

Ct: Cycle threshold

CYP7A1: Cholesterol 7 alpha-hydroxylase

CYP8B1: Sterol 12-alpha-hydroxylase

dL: Decalitre

d: Day

DHA: docosahexaenoic acid

DNA: Deoxyribonucleic acid

E: Efficiency

ELISA: Enzyme-linked immunoassay

EPA: eicosapentaenoic acid

FDA: Food and Drug Administration

FXR: Farsenoid X receptor

GAPDH: Glyceraldehyde 3-phosphate dehydrogenase

G: Gram

GSH: Glutathione

HNF-1a: Hepatic nuclear factor-1a

HPRT-1: Hypoxanthine-guanine phosphoribosyltransferase 1

IBABP: ileal bile acid binding protein

IC50: Half maximal inhibitory concentration

ICP-OES: Inductively coupled plasma optical emission spectrometry

IHC: Immunohistochemistry

IL-1/6/1β: Interleukin 1/6/1β

kDa: KiloDalton

kg: Kilogram

kV: Kilovolts

L: Litre

M: Molar

MDA: Malondialdehyde

mEH: microsomal epoxide hydrolase

mEq: Milli-equivalents

mg: Milligram

mRNA: Messenger ribonucleic acid

MRP1/Mrp1: Multidrug resistance-associated protein 1

MRP2/Mrp2: Multidrug resistance-associated protein 2

MRP3/Mrp3: Multidrug resistance-associated protein 3

MRP4/Mrp4: Multidrug resistance-associated protein 4

N: Number in group

Na: Sodium

NADH: Nicotinamide adenine dinucleotide

NEC: Necrotizing enterocolitis

n.d.: No date

NF-κB: Nuclear factor κB

NICU: Neonatal intensive care unit

nm: Nanometre

NTCP/Ntcp: Sodium-dependent taurocholic co-transporting polypeptide

OATP2/Oatp2: Organic anion transporting polypeptide 2

OATP8/Oatp8: Organic anion transporting polypeptide 8

OSTα/β: organic solute transporter α/β.

PFIC: Progressive familial intrahepatic cholestasis

PN: Parenteral nutrition

PNALD: Parenteral nutrition associated liver disease

PBS: Phosphate buffered saline

qPCR: Quantitative polymerase chain reaction

RNA: Ribonucleic acid

Rpm: Rotations per minute

RXR: Retinoid X receptor

ROS: Reactive oxygen species

SD: Standard deviation

SGA: Small for gestational age

TBST/TBS: Tris-buffered saline with/without tween

TNF-α: Tumour necrosis factor alpha

UDCA: Ursodeoxycholic acid

UK: United Kingdom

USA: United States of America

µg: Microgram

µm: Micron

µmol: Micromol

vs: Versus

1: INTRODUCTION

1.1 Rationale:

Parenteral nutrition associated liver disease (PNALD) is a multi-factorial disease with a poorly understood pathogenesis. [Beath, et al., 1996; Guglielmi, et al., 2008] It includes a spectrum of liver dysfunction, which starts with hyperbilirubinemia and potentially ends with cirrhosis and end-stage liver failure. [Naini & Lassman, 2011] Infants on long-term parenteral nutrition (PN) are at greatest risk, with the derangement of liver enzymes noted in approximately 25% for infants exposed to three weeks of PN [Beale, Nelson, Bucciarelli, Donnelly, & Eitzman, 1979; Arnold, 2004] as compared to almost all infants displaying some degree of hepatic dysfunction after three months. [Benjamin, 1981; Drongowski & Coran, 1989] Factors involved in PNALD include prematurity, an immature hepato-biliary system, sepsis, pro-inflammatory lipids, and contaminants such as aluminum (Al). To date, considerable progress has been made in improving the pro-inflammatory lipids traditionally used in infant PN, but the disease is still not reliably preventable or reversible with the new mixed lipids. [Cowan, Nandivada, & Puder, 2013; Tillman, 2013] Al contamination meanwhile, has not been addressed in North America and remains a significant concern. Al in PN is linked to an osteo-malacia type bone disease in infants, impairment in neurological development and exacerbation of anemia, [Gura, 2010; Courtney-Martin, et al., 2015] and we believe that it may contribute to PNALD in infants. [Alemmari, Miller, Arnold, & Zello, 2011; Arnold, Miller, & Zello, 2003] We will explore the role of Al and lipids in PNALD and compare the effects of Al contamination with both omega-6 and mixed lipids on bile acid transporters. We focused on bile acid transporters because they are crucial regulators of bile flow and can cause cholestasis when impaired. [Arresse & Ananthanarayanan, 2004] Animal models also suggest that dysfunction of bile acid transporters features prominently in the disease process of PNALD. [Li, Gong, Wu, Wu, & Kai, 2012; Tazuke, et al., 2004] We theorize that the best preservation of these transporters requires both a beneficial lipid environment and the reduction of Al.

1.2 Hypothesis:

We hypothesize that both aluminum and pro-inflammatory lipids cause dysfunction of bile acid transporters, through down-regulation and impaired functioning, thereby contributing to parenteral nutrition associated liver disease in infants. We theorize that switching from an omega-6 (pro-inflammatory) lipid solution, to a mixed omega-3 and 6 lipid solution (less-inflammatory) lipid solution without reducing aluminum contamination is insufficient to preserve normal bile acid transporter mRNA, protein, and activity. The best parenteral nutrition solution for bile acid transporter function is one with minimal aluminum contamination and with non-inflammatory lipids. We hypothesize that by using the least inflammatory lipid solution (ie: mixed lipids) and reducing aluminum contamination in parenteral nutrition, parenteral nutrition associated liver disease could be prevented or delayed.

1.3 Objectives:

1. Assess the current degree of aluminum contamination of parenteral nutrition used in a Canadian neonatal intensive care unit and review possible sources for this contamination.
2. Use the Yucatan miniature pig parenteral nutrition model to observe the effects of High (63µg/kg/day) vs Standard aluminum (24µg/kg/day) contamination, with either mixed or omega-6 lipids. Evaluate for changes to bile acid transporters by immunohistochemistry, polymerase chain reaction, and Western blotting techniques. Additionally, compare the total serum bile acids to detect early cholestasis.
3. For the piglet study with omega-6 lipids, compare High vs Standard aluminum in parenteral nutrition, in terms of its effect on serum C-reactive protein levels (a marker of inflammation), and hepatocyte morphology, as determined by transmission electron microscopy.
4. Isolate the effects of aluminum, omega-6 lipids, and mixed lipids on bile acid transporters using a primary hepatocyte model and determine if the effects of these factors are additive. Evaluate for changes to bile acid transporters by qPCR, Western blot and functional assay (where possible).

2: LITERATURE REVIEW

The following review will provide an introduction to parenteral nutrition associated liver disease (PNALD) and the bile acid transporters involved. The most important pathophysiologic factors, including aluminum contamination, and lipid emulsion, along with proposed management strategies, will also be examined in detail. Portions of this literature review were previously published in a modified format. Source: Hall AR, Arnold CJ, Zello GA, Miller GG. Parenteral nutrition associated liver disease: a current review focused on the infant. In: Lelio S, ed. *Parenteral Nutrition: Policies and Procedures, Safe Practices and Potential Complications*. Hauppauge, NY: Nova Science Publishers; 2014:49-84.

2.1 PNALD Overview:

Although parenteral nutrition (PN) has been an essential component of neonatal intensive care for decades, the complication of PNALD persists and its pathophysiology remains poorly understood. [Beath, et al., 1996; Guglielmi, et al., 2008] PNALD encompasses a spectrum of liver dysfunction including hyperbilirubinemia, cholestasis, fibrosis, and cirrhosis, [Naini & Lassman, 2011] with hyperbilirubinemia presenting first and preceding any histologic evidence of liver damage. [Benjamin, 1981; Touloukian & Seashore, 1975] Generally, PNALD is a diagnosis of exclusion, where the infant must display a direct bilirubin greater than 2mg/dL (>34μmol/L), be dependent on parenteral nutrition, and have no other reason for liver disease. [Koseesirikul, Chotinaruemol, & Ukarapol 2012; Mullick, Moran, & Ishak, 1994; Muhammed, et al., 2012] 40-60% of infants on long-term parenteral nutrition will develop some degree of cholestasis. [Kelly, 1998; Suita, Masumoto, Yamanouchi, Nagano, & Nakamura, 1999; Willis, et al., 2010] For those infants who progress to a conjugated serum bilirubin of >10mg/dL (171μmol/L) there is a 38% mortality rate. [Willis, et al., 2010] Fortunately, the incidence of PNALD has been decreasing over the last ten years, [Kubota, et al., 2000] and survival rates are slowly improving with the advent of multiple treatment modalities. [Guglielmi, et al., 2008, Tillman, 2013; Cowles, Ventura, & Martinez, 2010] The most definitive treatment is to stop the parenteral nutrition, but for many infants with short bowel syndrome and intestinal failure, this is not a feasible option. [Javid, et al., 2005]

Histology: PNALD in infants is histologically heterogeneous, but is usually characterized by cholestasis and fibrosis. The former is a result of bile accumulating in both the canaliculi and hepatocytes, and is one of the earliest and most frequent findings. [Zambrano, et al., 2004; Naini & Lassman, 2011; Postuma & Trevenen, 1979; Dahms & Halpin, 1981] It is suspected that bile stasis is one of the primary pathogenic mechanisms causing the subsequent histologic findings of fibrosis and ductal changes. [Benjamin, 1981] This fibrosis is also a relatively early characteristic of PNALD, and its perivenular location allows for differentiation from other causes of hepatic fibrosis. [Naini & Lassman, 2011] Eventually, the fibrosis may progress to bridging of fibrous tissue between portal areas. [Benjamin, 1981; Dahms & Halpin, 1981.]

Beyond cholestasis and fibrosis, there are often other signs of cellular and ductal injury, such as extensive ballooning and feathering of the hepatocytes; occasionally progressing to apoptosis. [Beale, et al., 1979; Naini & Lassman, 2011] Traditionally, ductal proliferation has been noted, [Benjamin, 1981; Zambrano, et al., 2004; Postuma & Trevenen, 1979] but more recent studies have noted ductal retraction and ductopenia in the earlier stages of fibrosis. [Naini & Lassman, 2011]

Not surprisingly, with this degree of cell injury and death, there is usually evidence of inflammation with predominantly lymphocytic peri-portal changes, and occasional giant cells, noted. [Dahms & Halpin, 1981] The immune response may progress to the point where extramedullary hematopoiesis is discovered. [Zambrano, et al., 2004; Postuma & Trevenen, 1979; Dahms & Halpin, 1981] Eventually, a micronodular pattern of cirrhosis can be found in end-stage disease. [Mullick, et al., 2004]

In general, children are more prone to developing fibrosis, while adults exposed to parenteral nutrition are more likely to develop steatosis of the liver. [Zambrano, et al., 2004; Naini & Lassman, 2011; Kelly, 1998] The severity of fibrosis is also inversely dependent on age, with a more frequent progression of fibrosis to cirrhosis seen in the youngest age groups. [Naini & Lassman, 2011; Mutanen, et al., 2013]

2.2 Risk factors: Implicated factors can generally be grouped into those related to lack of physiologic enterohepatic circulation/enteric intake, those related to toxicity directly from the

parenteral nutrition (PN) or its components, and those related to complications outside of the PN such as sepsis, need for gastrointestinal surgery, or other underlying co-morbidities. [Koseesirikul, et al., 2012; Beath, et al., 1996; Costa, et al., 2010; Guglielmi, et al., 2008]

2.2.1 Risk factors: Prematurity

PNALD is a multi-factorial disease and the most important risk factor is prematurity or size, as smaller infants are more susceptible to PNALD and are prone to worse outcomes. [Touloukian & Seashore, 1975; Shawn, et al., 2012; Beath, et al., 1996; Javid, et al., 2011] If birthweight is considered, the low birth weight infants or small for gestation age infants (SGA), are at higher risk than those appropriate for gestational age (AGA). [Zambrano, El-Hennawy, Ehrenkranz, Zelterman, & Reyes-Mugica, 2004; Christensen, Henry, & Wiedmeier, 2007] Multiple studies have found that cholestasis started earlier (requiring less PN exposure), was more severe and lasted longer in the SGA vs AGA. [Lee, et al., 2013; Robinson & Ehrenkranz, 2008] These smaller neonates (usually prematurely born) may be at higher risk because of immature metabolic functioning of the liver, decreased glycogen stores and other aspects of hepatobiliary underdevelopment. [Robinson & Ehrenkranz, 2008]

2.2.2 Risk factors: Surgery

Additionally cited risk factors for PNALD include infants undergoing surgical procedures or infants with necrotizing enterocolitis (NEC) [Shawn, et al., 1996; Beath, et al., 1996; Javid, et al., 2011], and often these populations overlap. One cohort study noted that 70% of infants undergoing surgery for NEC later developed PNALD. [Duro, Mitchell, Kalish, & Martin, 2011] However a direct causation between surgery, NEC, and PNALD may be overstated, because NEC requiring surgical resection is also one of the most frequent preceding diagnoses for infants with intestinal failure, implying long-term PN dependence. [Shawn, et al., 2012]

2.2.3 Risk factors: Prolonged PN exposure

The duration of parenteral nutrition exposure is instrumental in the development of this disease. The length of time required to induce PNALD is unclear, but multiple studies show that longer exposure increases the risks of cholestasis development. [Suita, et al., 1999;

Zambrano, et al., 2004; Beale, et al., 1979; Duro, et al., 2011; Naini & Lassman, 2011] Within 3 weeks approximately 23-25% of infants on PN will display early findings of PNALD. [Beale, et al., 1979; Arnold, 2004] At least one case report has demonstrated severe fibrosis in an infant exposed to PN for only 14 days. [Mercer, et al., 2013] Of greater concern is the finding that almost all infants with >60 days of exposure to PN will develop some degree of cholestasis. [Benjamin, 1981; Drongowski & Coran, 1989]

2.2.4 Risk factors: Immature biliary system

Bile acids (or bile salts) are a group of acidic steroids which share a similar structure. They provide numerous functions including emulsion of lipids, modulating pancreatic enzyme release and even antimicrobial activities. Cholesterol is the backbone of all bile acids and the process of transforming cholesterol into bile acids takes place by two main pathways (classical or alternative). The classical pathway, where the rate-limiting enzyme is CYP7A1, provides the majority of bile acids in healthy humans. [Monte, Marin, Antelo, & Vasquez-Tato, 2009]

Following synthesis in the hepatocyte, bile acids are secreted in the canaliculi; this is the rate-limiting step of bile acid circulation. [Arrese & Ananthanarayanan, 2004] From the biliary tree, bile acids enter into the small intestine for emulsification and other digestive activities and are then efficiently transported across the ileum into the portal blood, via the apical sodium-dependent bile acid transporter and the baso-lateral organic solute transporter alpha and beta. Bile acids travel through the portal blood bound to albumin and are reabsorbed through the sinusoidal membrane of hepatocytes via the Na-taurocholate co-transport polypeptide (NTCP) and the family of organic anion transporting polypeptides (OATP). [Monte, et al., 2009; Alrefai & Gill, 2007; Hagenbuch & Meier, 2003]

Neonates are born with an immature enterohepatic circulation of bile acids and, not surprisingly, preterm infants have an even greater difficulty handling these compounds. Regardless of term, the newborn infant has poorly functioning active transport of conjugated bile salts in the ileum and they are therefore lost into the gastrointestinal tract. If this is compounded by bowel loss or malfunction, reabsorption is further impaired. [Balistreri, Heubi, & Suchy, 1983] Even when reabsorption is successful, the bile acids are often trapped in the blood because of inefficient hepatic extraction. [Hofmann, 1995] As a result of these deficits,

the overall bile pool is proportionally much smaller than it should be, when compared to a healthy adult. Bile acids provide positive feedback for bile flow in the liver, so the smaller bile pool leads to hepatic bile stasis. [Rager & Finegold, 1975]

All of these issues are present in a preterm neonate, in addition to a mismatch between formation and secretion of hepatic bile salts. Animal studies indicate that the synthesis of bile salts is developed much sooner than the efficient secretion of them, leading to a functional cholestasis in the most immature infants. A more advanced secretion of bile acids has usually developed for those infants born at full term gestation, although it is still relatively incomplete, compared to an adult. [Balistreri, et al., 1983] All of these aspects of hepato-enteric underdevelopment place the infant at risk for PNALD, when exposed to other hepatic insults.

2.2.5 Risk factors: Lack of enteral feeding

As early as 1975, the lack of enteral stimulation was correlated with the development of cholestasis, and it was proposed that prolonged fasting contributes to disruptions in bile flow. [Benjamin, 1981; Rager & Finegold, 1975] Bile acid secretion is proportional to oral intake, and the lack of enteral stimulation also decreases many important gastrointestinal hormones such as cholecystokinin, gastrin, motilin, secretin, pancreatic polypeptide, glucagon, and vasoactive intestinal peptide. [Costa, et al., 2010; Hofmann, 1995] Cholecystokinin deficits, in particular, may lead to reduced gallbladder contractility and impediment of the enterohepatic circulation. [Guglielmi, et al., 2008; Hofmann, 1995] In addition, parenteral delivery means that the liver must adapt to changes in portal flow, because the nutrients are now delivered through the arterial system (in the portal triad) instead of through the portal vein. [Guglielmi, et al., 2008]

Multiple models have emphasized the importance of enteral feeding for normal biliary function. For example, enteral feeding in mice has been shown to restore blood flow to the small intestine and portal vein and may also trigger improved hepatic immune response. This same research group has also demonstrated that PN given enterally does not depress the hepatic immune response, supporting the idea that it is the absence of gut stimulation that is damaging. [Omata, et al., 2009] Cholestasis only resolves once the infant is solely on enteral feeds and no longer receiving parenteral nutrition. [Javid, et al., 2005] Switching to enteral

feeding is therefore the most effective treatment as it brings about clinical and biochemical improvement, often within 2 weeks. [Dahms & Halpin, 1981] Unfortunately, attempts to wean parenteral nutrition are fraught with problems due to the complicated medical conditions of the infants. Often these neonates cannot tolerate substantial enteral nutrition, and instead can only be given small amounts in hopes of stimulating the bowel. This has proven beneficial to gut and hepatic function [Omata, et al., 2009; McClure & Newell, 1999] and is commonly practiced in critical care units, but does not reliably prevent PNALD.

2.2.6 Risk factors: Aluminum contamination

While aluminum has no role in human physiology and aluminum deficiency does not exist, there are cases of aluminum toxicity; especially in PN-dependent patients. Overall, <1% is absorbed when aluminum is ingested orally, compared to the much higher amounts found in the blood stream following parenteral delivery. [Poole, et al., 2010] This is potentially dangerous, because high doses of aluminum have been shown to cause a variety of disorders. In adults on long-term PN, significant aluminum has been documented in long-bones and is associated with deleterious changes in bone structure. [Kruger, et al., 2014] Aluminum is known to cause anemia, neurologic disorders, and impaired bone metabolism in infants. [Gura, 2010; Courtney-Martin., et al., 2015] Rodent studies have found that even aluminum given orally damages the gastro-intestinal system both directly and by interfering with the growth of normal intestinal bacteria. [Vignal, Desreumaux, & Body-Malapel, 2016]

In 2004, the FDA recommended that aluminum intake should be < 5μg/kg/day [FDA, 2004], while the American Society for Parenteral and Enteral Nutrition (ASPEN) stated that 15-30μg/kg/day is unsafe, and 60 μg/kg/day toxic. [American Society of Parenteral and Enteral Nutrition, 1991] Notably, in Canada there are no rules limiting the amount of aluminum contamination in PN and Canadian manufacturers of PN components are not required to list the aluminum content on their products. [Courtney-Martin, et al., 2015]

Neonatal parenteral nutrition has aluminum levels 3-5 times higher than the FDA limit. [Poole, et al., 2010] Of concern, the highest levels of aluminum have been found in PN designed for the smallest infants. [Poole, et al., 2010] A wide collection of parenteral nutrition components are contaminated with aluminum, including amino acids, dextrose solutions, lipid

emulsions, trace elements, multivitamins, heparin, and insulin, but the majority is found in calcium gluconate, potassium phosphate, and sodium phosphate. (All of which are needed in higher amounts for rapidly growing infants). These compounds become contaminated by forming complex ions with the aluminum leeched out of glass containers used in the manufacturing process. [Poole, et al., 2010; FDA, 2011]

Since the early 1980s, researchers have identified high aluminum levels in infants with PNALD, but the link between the element and disease is not yet fully understood. [Alemmari, et al., 2011; Alemmari, et al., 2012; Arnold, et al., 2003; Klein, et al., 1984] Most recently, Courtney-Martin et al (2015) studied 27 PN-dependent children (mean age of five) and found that their average plasma aluminum levels were more than eight times higher than healthy children. [Courtney-Martin, et al., 2015]

Aluminum is toxic to many body systems, and is found in high concentrations in parenteral nutrition, and may plausibly contribute to the development of PNALD. [Alemmari, et al., 2012; Arnold, et al., 2003; Klein, et al., 1984] Much of the evidence to date for this theory is based on animal trials. For example, rats exposed to high doses of aluminum developed elevated bile acid levels, with evidence that sinusoidal uptake and canalicular excretion was impaired. [Gonzalez, Roma, Bernal, Alvarez, & Carrillo, 2004; Klein, Heyman, & Lee, 1998] Of note, these changes occurred despite enteral feeding provided to the animals. [Klein, et al., 1998] In other animal work, histological changes including portal inflammation, and aluminum-laden giant cells have also been caused by aluminum exposure, [Demircan, Ergun, & Coker, 1998; Bertholf, Herman, & Savory, 1989] but plasma membrane changes were not initially detected until very large doses (1500µg/kg/day) were used. [Alemmari, et al., 2011] These plasma membranes showed blunted bile canalicular microvilli, a decreased number of microvilli in the bile canaliculi, dilatation of the canaliculi, increased inflammatory cells, and lysosomes containing aluminum. [Alemmari, et al., 2011] Further evidence for aluminum affecting the plasma membrane comes from studies where rats exposed to high amounts of aluminum developed a 40% decreased expression of Mrp2, [Gonzalez, et al., 2004] and similar results have been replicated in the University of Saskatchewan studies, using piglets. [Unpublished results]

To determine whether histological changes developed with aluminum dosages closer to that observed in clinical settings, Alemmari et al (2012) conducted a piglet PN study with aluminum contamination of 38μg/kg/day. This level of aluminum significantly blunted the bile canalicular microvilli in the regular parenteral nutrition group vs the low aluminum group. [Alemmari, et al., 2012]

The majority of evidence suggests that aluminum would cause hepatic damage through pro-oxidant effects. It not only forms a superoxide radical ion, but it also acts synergistically with iron to participate in oxidative reactions. [Alexandrov, et al., 2005; Gonzalez, et al., 2007] In the mitochondria, aluminum causes a breakdown in critical cell energy pathways forcing the hepatocyte into anaerobic respiration. [Percy, Kruck, Pogue, & Lukiw, 2011; Mailloux, Lemire, & Appanna, 2011] In neurons, aluminum has also been found to accumulate in mitochondria and trigger apoptosis. [Gonzalez, et al., 2007] Interestingly, aluminum in HepG2 (hepatocyte) cells causes intracellular accumulation of lipids because their breakdown pathway (β-oxidation) is impaired. [Mailloux, et al., 2011] Animals exposed to aluminum not only demonstrate impaired bile flow but also significant increases in MDA (signifying lipid peroxidation) along with a decrease in the activities of the anti-oxidants GSH peroxidase, and CAT. [Gonzalez, et al., 2007; Gonzalez, et al., 2004; Guo & Wang, 2011] This aluminum-induced lipid peroxidation has been shown to be counteracted by the anti-inflammatory and anti-oxidant effects of vitamin E. [Gonzalez, et al., 2007] Of note, oxidative stress in these models is also linked to a decreased expression of Mrp2. [Gonzalez, et al., 2007; Gonzalez, et al., 2004; Guo & Wang, 2011]

Along with pro-oxidant actions, aluminum may also contribute to inflammatory processes in PNALD. In other disease situations, aluminum has been linked to the inflammatory cascade. For example, dialysis patients with high aluminum levels not only had decreased levels of anti-oxidants GSH, vitamin C, and vitamin E but also significantly increased C-reactive protein (an acute phase inflammatory marker), along with elevated cytokines TNF-α and interleukin-5. [Guo & Wang, 2011] Similarly, in human neuronal studies, aluminum has been found to induce pro-inflammatory genes. [Alexandrov, et al., 2005] Neither the oxidative nor the inflammatory effects of aluminum, however have been studied in a PNALD model.

Based on this combined evidence, it appears that aluminum does impede biliary flow and may do so through oxidative changes at the canalicular plasma membrane; but further research is needed to characterize the effects on bile acid transporters.

2.2.7 Risk factors: Lipids

One of the most important PN derived factors contributing to the development of PNALD is lipid content. Two theories have emerged to explain the hepatotoxic effects of lipids: the phytosterol content and the pro-inflammatory nature of omega-6 vs omega-3 fatty acids.

Phytosterols: Phytosterols are derived from plant-based lipid sources and are almost identical in structure to cholesterol, with matching nuclei but different alkylated side chains. [Iver, Spitz, & Clayton, 1998] Traditionally available intravenous lipids are soy-based (ie: Intralipid (Baxter/Fresenius Kabi, Deerfield, IL, USA)) and contain the phytosterols sitosterol, campesterol, and sigmasterol at a total concentration of approximately 1000µmol/L (40 mg/dL). [Bindl, Lutjohann, Buderus, Lentze, & v Bergmann, 2003] In comparison, fish oils (ie: Omegaven (Fresenius Kabi, Bad Homburg, Germany)) do not contain any phytosterols [Park, Nespor, & Kerner, 2011] and olive-oil based lipids have fewer than soy-based emulsions. [Kurvinen, et al., 2011]

Many studies have shown an increase in plant sterol levels in patients receiving PN. [Kurvinen, et al., 2011] Very preterm infants are especially susceptible to accumulating phytosterols, as opposed to more mature infants (sitosterol levels recorded as 2.60 vs 1.33 mg/dL). [Nghiem-Rao, et al., 2015] Likewise, those infants with cholestasis have the highest levels of phytosterols. [Nghiem-Rao, et al., 2015] However, it is difficult to isolate the effects of phytosterols vs inflammatory lipids and other factors in the PNALD disease state. [Zaloga, 2015] Case reports have shown at least some decrease in phytosterols with reduction of glucose rather than lipids, implying that these levels may be an indicator of liver dysfunction, (ie: the liver can no longer clear phytosterols), rather than lipid overdose. [Bindl, et al., 2000] In addition, a recent study examining the use of five different types of lipid emulsions in neonates also found no association between phytosterol intake and liver function at six weeks.

Of particular interest is the observation that in a group with the highest phytosterol intake, there were no cases of cholestasis. [Savini, et al., 2013]

Animal models have demonstrated a link between an increase in bile acids and infusion of phytosterols, with subsequent reversal of bilirubin following reduction of phytosterols. [Iver, et al., 1998; Carter, et al., 2007] In mice, phytosterols, even when added to omega-3 lipids significantly altered colonic bacteria until it resembled the flora found in similar animals with PNALD. They also activated hepatic macrophages and suppressed expression of canalicular bile acid transporters BSEP and MRP2 via interference with the nuclear receptor farsenoid X. [Kurvinen, et al., 2011; Harris, et al., 2014; El Kasmi, et al., 2013] In contrast, a piglet PN study found no correlation between phytosterol concentrations and liver injury, and the authors concluded that they could not link phytosterols to PNALD.[Vlaardingerbroek, et al., 2013] Overall, phytosterols are only excreted through the biliary system, but hepatic metabolism of these compounds is very slow. [Bindl, et al., 2000] During this prolonged exposure, phytosterols are thought to replace the cholesterol in the cell membranes and disturb the normal cholesterol-bile acid synthesis relationship, [Zaloga, 2015] as well as interfere with bile acid transport proteins. [Clayton, Whitfield, & Iyer, 1998] Generally, the role of phytosterols in PNALD has yet to be discerned, but preliminary evidence supports the theory that they may have some hepatotoxic effects.

Omega-6 fatty acids: Omega-6 fatty acids (as found in soy-based Intralipid) are pro-inflammatory in nature, and therefore a plausible trigger for PNALD. [Gura, et al., 2008; Cober & Teitelbaum, 2010; Cowan, et al., 2013] This group of polyunsaturated fats includes linoleic acid and arachidonic acid, compared to the omega-3 fatty acids group which includes docosahexaenoic acid, alpha-linolenic acid, and eicospentanoic acid. [Le, et al., 2009] The pro-inflammatory effects of omega-6 fatty acids are secondary to their production of platelet aggregating thromboxanes and immunosuppressing prostaglandin E1 (through arachidonic acid). (Of note, this pathway also creates ROS by-products, suggesting that omega-6 lipids may have a mild pro-oxidant effect). [Betteridge, 2000; Cober & Teitelbaum, 2010; Cowan, Nandivada, & Puder, 2013] See Figure 2.1 below for further detail of the fatty acid synthesis pathways and where particular lipid emulsions are derived. Soy-based preparations have very little α-tocopherol (absorbable vitamin E) to combat the inflammatory effects of this type of

fatty acids. [Klek, et al., 2013] Meanwhile, omega-3 fatty acids appear to be hepato-protective, with anticoagulating thromboxanes and the formation of prostaglandin E3 to assist immune function. [Park, et al., 2011; Cober & Teitelbaum, 2010; Cowan, et al., 2013] Omega-3 fatty acids may also help to regulate tumour necrosis factor-α, and inactivate the pro-inflammatory nuclear receptor NF-κB. [Park, et al., 2011; Tillman, 2013] In mouse models, omega-3 fatty acids were observed to preserve the functioning of macrophages when the liver was challenged with bacterial infection. (Interestingly, omega-3 seems to work by improving the functioning of macrophages, while omega-6 increases their numbers). [Moriya, et al., 2014] Leukocyte recruitment is a marker of inflammation, and includes capture and adherence of leukocytes to inflamed tissue. Multiple aspects of this process are inhibited in a mouse model using injections with mixed lipids containing omega-3, as compared to a mostly omega-6 solution. [Buschmann, et al., 2015] The same study also demonstrated that the omega-6 based lipid had a significantly greater recruitment of neutrophils as compared to a negative control with the same inflammatory stimulus, indicating that omega-6 lipids do trigger an over-active inflammatory response. [Buschmann, et al., 2015] Other mouse PN models have demonstrated that omega-6 lipids may adversely affect the gene expression of CYP7A1 and CYP8B1, crucial enzymes in the synthesis of bile acids. [Zhan, et al., 2016] Based on these and other findings, many groups have proposed a switch from the omega-6 fatty acids traditionally found in lipid emulsions, to a less hepatotoxic omega-3 formulation.

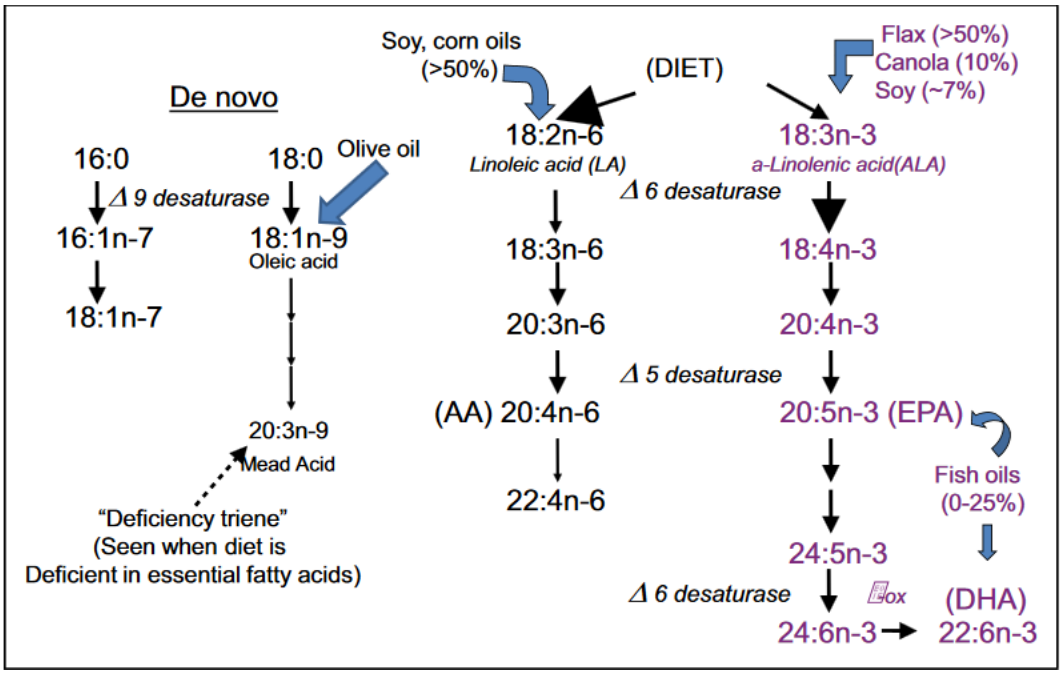


Figure 2.1: Fatty acid biosynthesis pathways, demonstrating where commonly used PN lipid emulsions are derived. Reproduced with permission from Gramlich L, Meddings L, Alberda C, Wichansawakun S, Robbins S, Driscoll D, Bistrian B. Essential Fatty Acid Deficiency in 2015: The Impact of Novel Intravenous Lipid Emulsions. *JPEN J Parenter Enteral Nutr* 2015; 39(1 Suppl):61S-6S. AA: arachidonic acid, EPA: eicosapentaenoic acid, DHA: docosahexaenoic acid

There are some issues regarding a switch to omega-3 fatty acids as they represent a markedly different composition than human milk lipids, [Savini, et al., 2013] and omega-6 fatty acids do play important roles in lipid synthesis pathways. [Cober & Teitelbaum, 2010] Both omega-6 linoleic acid and omega-3 alpha-linoleic acid are designated as essential fatty acids because they must be obtained from the diet and cannot be synthesized de novo. [Le, et al., 2009] Newer work however, suggests that in low-birth weight infants there may be additional essential fatty acids. These at-risk infants are unable to convert linoleic and alpha-linoleic acid to arachidonic acid, eicospentanoic acid, and docosahexaenoic acid and therefore a fish oil emulsion may be a better mix, as it provides these downstream fatty acids, although only small amounts of arachidonic acid. [Le, et al., 2009; Cowan, et al., 2013]

2.3 PNALD Pathophysiology:

There are many theories about the pathophysiology of PNALD, but inflammation and oxidative stress are strongly implicated.

2.3.1 Pathophysiology: Sepsis and inflammation

Sepsis has long been correlated with PNALD and it is often reported that those infants with multiple septic events have a higher incidence of cholestasis. [Suita, et al., 1999; Lambert & Thomas, 1985] Inflammation is a natural defense system employed by the body to destroy invading pathogens and stimulate healing. [Dinh, Drummond, Sobey, & Chrissobolis, 2014] It involves the release and activation of cytokines, chemokines, and other factors. [Dinh, et al., 2014] The liver plays a key role in the inflammatory response to invading pathogens, even when the primary site of infection is extrahepatic. [Trauner, Fickert, & Stauber, 1999] Kupffer cells, hepatocytes, and biliary epithelium all release cytokines (TNF-a, IL-1 etc), nitric oxide, and other mediators to boost the pro-inflammatory cascade. [Trauner, et al., 1999; Fuchs & Sanyal, 2008] Unfortunately, if this inflammation is unregulated, it can be damaging to the host. [Dinh, et al., 2014] For example, in animal models of inflammatory cholestasis, cytokines inhibit the gene expression of bile acid transporters Mrp2, Bsep, Oatp2 and Ntcp. [Trauner, et al., 1999; Kosters & Karpen, 2010] These transporters appear to be influenced at both the transcription and post-translational level. [Fuchs & Sanyal, 2008, Kosters & Karpen, 2010, Trauner, Arrese, Lee, Boyer, & Karpen, 1998] Since the inflammatory model of cholestasis is very similar to PNALD, they likely share a similar inflammatory cascade.

In infants with PNALD, sepsis and inflammation may be caused or be exacerbated by poor bowel function and subsequent immune function compromise (secondary to negative effects on the gastrointestinal lymphoid tissue). [Guglielmi, et al., 2008] With both bowel and immune stasis, the patient is at risk of bacterial overgrowth [Lambert & Thomas, 1985] and translocation. [Suita, et al., 1999; Costa, et al., 2010] The link between anaerobic bacteria and cholestasis has been found by researchers such as Lambert et al who described a rise in liver enzymes in adult patients on PN without metronidazole prophylaxis, compared to those who received the antibiotic. Given that metronidazole suppresses intestinal anaerobic bacteria, the authors postulated that this type of bacteria could contribute to hepatic injury. [Lambert & Thomas, 1985] Endotoxins and toxic bile salts released from intestinal bacteria can directly damage hepatocytes. [Trauner, et al., 1999] Specific work looking at Toll-like receptor agonists such as lipopolysaccharides has shown that both a lack of enteral feeding and a faulty intestinal barrier (secondary to bacterial overgrowth) are needed for these toxic substances to reach the liver. Once these agonists reach the liver in sufficient amounts they activate the Toll-

like receptors in Kupffer cells setting off the localized inflammatory cascade as described above. [El Kasmi, et al., 2012]

2.3.2 Pathophysiology: Oxidative stress

Both oxidative stress and inflammation plausibly play a role in the development of PNALD, but neither are well studied in this disease. In addition to cytokines, Kupffer cells also produce reactive oxygen species (ROS) such as superoxide and hydrogen peroxide to directly kill pathogens. [Dinh, et al., 2014; Fuchs & Sanyal, 2008] These ROS, or free radicals, have unpaired electrons that can cause mutagenesis. [Betteridge, 2000] Alternatively, ROS can help subdue an overactive inflammatory response. [Halliwell, 2007] Similar to the inflammatory cascade, the oxidant response can also cause host harm if unregulated. Anti-oxidants including reduced glutathione (GSH) and catalase (CAT) exist to facilitate the degradation of ROS into harmless components. [Betteridge, 2000; Basiglio, et al., 2014] When the oxidants overwhelm the supply of anti-oxidants, a situation of 'oxidative stress' is created and host cells are damaged. [Halliwell, 2007]

In cholestasis, increasing bile acids exacerbate the oxidative stress, as they appear to inhibit the activities of GSH and CAT. [Galicia-Moreno, et al., 2012] In addition, a common target of ROS is the lipid membrane of cells, a process called lipid peroxidation, which produces the oxidative stress marker malondialdehyde (MDA). [Betteridge, 2000; Galicia-Moreno, Favari, & Muriel, 2012] High serum MDA levels are found in people with non-PNALD cholestasis and in animal models where bile acid transporters are abnormally positioned intracellularly. [Basiglio, et al., 2014; Shoda, et al., 2001] Although PNALD specific models have not been examined, this evidence indicates that oxidative stress, as well as inflammation, is likely involved.

2.4 Treatments and preventative measures for PNALD:

Ursodeoxycholic acid (UDCA): The logical approach to faulty enterohepatic circulation is to directly stimulate bile secretion. Accordingly, agents such as ursodeoxycholic acid have been used, but with varying success. UDCA is taken up by the liver and conjugated into a functional bile acid, which is then secreted back into the bile and works to aid in fat digestion. It increases the bile acid-dependent bile flow and promotes enterohepatic circulation, and is

also believed to decrease the hydrophobic content of bile; making it less damaging to biliary epithelium. [Beuers, 2006] Early pilot studies in PNALD showed resolution of liver enzyme dysfunction and bilirubin elevation within 4-8weeks of UDCA, and rebound of enzymes when the UDCA was discontinued. It is important to note though, the dosage used in this study was higher than recommended, [Spagnuolo, Iorio & Vegnente, 1996] and subsequent clinical results have been mixed, with some showing no benefit and others modest improvement. [De Marco, Sordino, & Bruzzese, 2006; Heubi, Whiechmann, & Creutzinger, 2002; Teitelbaum, et al., 2005] These studies are often hard to interpret due to confounders such as the concurrent use of enteral feeding and small number of infants. [Shawn, et al., 2012] Some researchers are skeptical that an orally dosed medication would be absorbed well in infants with short-gut and other intestinal failure scenarios, [American Society for Parenteral and Enteral Nutrition, 2014] while others have raised concerns about the safety of administering doses large enough to be beneficial. [Hofmann, 1995] The most recent study has indicated that although UDCA does not prevent the development of cholestasis, it may delay the onset in newborns, with no adverse side-effects. [Simic, et al., 2014] The American Society of Parenteral and Enteral Nutrition cautiously agrees with this conclusion. Their 2014 guidelines admit that the evidence for UDCA use is very low quality but that there may be some benefit in neonates with PNALD and seemingly no harm. [American Society for Parenteral and Enteral Nutrition, 2014]

Cholecystokinin octopeptide: This enzyme causes gallbladder contraction and stimulation of bile flow, and was theorized to have positive effects in PNALD. Unfortunately, this has been refuted with a large trial demonstrating no prevention of cholestasis or gallstones in those infants on PN. [Teitelbaum, et al., 2005; Tsai, Strouse, & Drongowski, 2005] Although there are some reports of cholecystokinin preventing PNALD in the adult population, this may not translate into the neonatal population because of differences in hepatic maturity and enterohepatic circulation. [Tsai, et al., 2005]

Cycling: Another common practice in the management of PNALD is cycling PN. This involves providing the same daily amount of PN in a shorter time period, thereby allowing the liver an opportunity to recover and clear metabolites more efficiently. This cycling and rest pattern also better simulates the physiologic patterns of hormonal secretions observed with normal oral feedings. [Nghiem-Rao, et al., 2013] Cycling is frequently started once

hyperbilirubinemia is noted, but it may also be beneficial as a prophylactic measure, as one study has found prophylactic cycling led to fewer cases of hyperbilirubinemia and lower levels of direct bilirubin in those infants who did progress to cholestasis. [Nghiem-Rao, et al., 2013] The risks of cycling include hypoglycemia and hyperglycemia, which can develop very rapidly in the neonatal population when PN is altered. For example, Nghiem-Rao et al (2013) reported that in infants treated with prophylactic or treatment cycling of PN, 23% developed hypoglycemia and 7% developed hyperglycemia, but all episodes of glucose dysregulation were managed without adverse effect. [Nghiem-Rao, et al., 2013] Cycling is almost always used in combination with other treatments and the direct effect of this practice is unknown, but it appears to have benefits when included as part of a multi-faceted treatment plan. [Cowles, et al., 2010]

Antibiotics and anti-inflammatories: Empiric antibiotics are often initiated in situations of sepsis-associated cholestasis, [Fuchs & Sanyal, 2008] and metronidazole in particular has been proposed as a 'bowel decontaminant' to decrease bacterial overgrowth and translocation, as well as to prevent production of toxic, cholestatic bile salts. [Trauner, et al., 1999; Meehan & Georgeson, 1997] However, the American Pediatric Surgical Association feels there is currently insufficient evidence to support routine prophylactic use of antibiotics to prevent PNALD. [Shawn, et al., 2012] Meanwhile, the steroid dexamethasone, which inhibits the anti-inflammatory response (specifically cytokines TNF-α and IL-1) has been shown to halt cholestasis in rats, but is not routinely used in the neonatal PNALD population. [Trauner, et al., 1999]

Antioxidants: Animal models of cholestasis have demonstrated beneficial responses to the anti-oxidants vitamin E, [Betteridge, 2000; Gonzalez, Alvarez, & Pisani, 2007] vitamin C, [Hierro, et al., 2014] and N-acetyl cysteine, [Galicia-Moreno, et al., 2012] but none of these are currently used in the management of infant PNALD.

Lipid reduction: In the USA, Intralipid (omega-6) is the only lipid approved for neonatal PN, and Omegaven (fish oil) is available on a compassionate-use basis. [Nandivada, Fell, Gura, & Puder, 2016] Therefore, many institutions managing PNALD advocate reduction of lipid content in PN for those infants with hyperbilirubinemia, in an attempt to limit the damage caused by phytosterols and pro-inflammatory lipids. [Cober & Teitelbaum, 2010] The

standard dosing of lipids for neonates is 2-3gm/kg/day, but in a lipid reduction strategy, this is reduced to ≤1g/kg/d. Some researchers have found this to be effective, for example in Denmark, infants with established PNALD were switched to low-fat PN, where the lipids were exchanged for carbohydrates. Lipids with the necessary amounts of essential fatty acids were provided only a few times a week. In all ten infants, the bilirubin reversed to normal and no signs of essential fatty acid deficiency were observed. [Jakobsen, Jorgensen, Husby, Andersen, & Jeppesen, 2015] Of note, the infants in this study also had enteral feeding to varying degrees and different types of lipids were used for different patients. In a piglet study, lower doses of both omega-6 and omega-3 lipid emulsions preserved bile flow and decreased bilirubin as compared to high (standard dose) omega-6 lipids, indicating that a lower dose of lipids is beneficial regardless of lipid type. [Josephson, et al., 2015]

Reducing lipids is controversial however, because infants require this energy source (especially the omega-3 fatty acid docosahexaenoic acid (DHA)) for adequate neuronal development and the longterm neurologic effects of decreased lipid intake are unknown. [Nandivada, et al., 2016] For example, in a piglet study, it was found that those animals with low dose lipids (especially fish-oil based) had smaller brain size. [Josephson, et al., 2015] It is also difficult to detect deficiencies in specific fatty acids. [Nandivada, et al., 2016] At least one long-term follow up (2-5 years) of patients treated with soybean- based lipid reduction as infants for PNALD has shown reassuring results. On average, the 25 children studied were found to score 'not at risk' in multiple neurodevelopmental studies. For the small percentage of children who did not score perfectly, the authors found no correlation to any lipid related factor (ie: duration of lipid reduction, mean lipid dose etc). [Blackmer, et al., 2015]

Other studies have shown that reducing lipids had no effect on how quickly cholestasis developed or the overall incidence of cholestasis. In these cases, the authors felt that enteral feeding had a much greater impact on the reversal of hyperbilirubinema as compared to lipid reduction. [Nehra, et al., 2013] The American Society of Parenteral and Enteral Nutrition is cautious in their support for this strategy. Their recommendation states that this tactic may be considered, but only because there are no other lipid emulsions available in the USA. They feel that the evidence for lipid restriction is very low, with only small observational studies. [American Society of Parenteral and Enteral Nutrition, 2014]

Lipid modification: Concerns regarding soybean lipids have led to a variety of other lipid formulations in use world-wide. Many of these new emulsions are available only through compassionate use programs or investigational new drug applications. [Cowan, et al., 2013; Tillman, 2013] These formulations have shown encouraging results, but research into their use is still on-going. They include Omegaven (fish oil based, mentioned above), Clinolipid/Clinoleic (Baxter, Deerfield, IL, USA), which is 80% olive oil and 20% soy-bean oil [Zaloga, 2015] and SMOFlipid (Fresenius Kabi, Bad Homburg, Germany), which is a combination of soybean oil, medium chain triglycerides, olive oil and fish oil. SMOFlipid is unique in that it was designed to provide both traditional and conditionally essential fatty acids, in addition to containing a higher level of α-tocopherol to combat lipid peroxidation (200mg/L in SMOF, vs 38mg/L in Intralipid). [Klek, et al., 2013; Goulet, et al., 2010] The medium chain-triglycerides in SMOFlipid are also felt to be beneficial because they are cleared more rapidly from the bloodstream and are less prone to lipid peroxidation, as compared to polyunsaturated fatty acids. [Pitkanen, 2004] In the following sections, the evidence for the two most popular new lipids: Omegaven and SMOFlipid will be explored.

Fish-oil monotherapy: Omegaven (Fresenius Kabi, Bad Homburg, Germany) is a 10% fish-oil emulsion providing only polyunsaturated omega-3 fatty acids, and although it is currently difficult to access in North America, results of its use in PNALD are favourable. Omegaven has performed better than most other lipid formulations, including mixed lipids, in some animal studies. For example, when compared to SMOFlipid, Intralipid, Clinoleic, and a mixed olive oil/omega-6 lipid solution, only Omegaven maintained normal liver histology in a mouse model. Omegaven also had significantly lower serum alanine aminotransferase levels. [Meisel, et al., 2012]

In studies with fish oil as a monotherapy in infants with established PNALD, there has been reversal of serum direct bilirubin and improvement of clinical status for the majority of patients. [Park, et al., 2011; Gura, et al., 2008; Gura, et al., 2006; de Mijer, et al., 2010] For example, in a study of 67 infants with PNALD (direct bilirubin >2.0mg/dL) who were switched to fish-oil monotherapy, it was observed that 82.5% had normalization of their serum direct bilirubin levles and survived to be discharged from hospital. [Premkumar, Carter, Hawthorne, King, & Abrahms, 2013] In addition, overall morbidity and mortality for infants

with PNALD has been diminished for those on this formulation, as the risk of death or transplant was found to be 9.5% for fish oil vs 34.7% for soy-bean oil. [Puder, et al., 2009] One long term follow-up of 51 patients treated with Omegaven for cirrhosis reported that 76% had normalization of bilirubin over the next year and Pediatric End-Stage Liver Disease scores improved dramatically over the second year. Importantly, those infants who were still PN dependent retained stable Pediatric End-Stage Liver Disease scores, indicating that at the very least, the cirrhosis may be stable when treated with fish oil. [Nandivada, et al., 2015] There is also a case report of clinicians treating infant PNALD with fish oil and then returning to soy-bean oil emulsions once the bilirubin normalized, without further biochemical recurrence of PNALD for the following three years. [Calkins, et al., 2013] In a piglet PN study, Omegaven appeared to prevent PNALD in preterm animals, with significantly lower levels of serum bilirubin and limited hepatocellular inflammatory changes (as compared to an omega-6 lipid group).[Vlaardingerbroek, et al., 2013]

Some studies have attempted to combine the strategies of lipid reduction and fish oil lipids for treatment of PNALD. One such study found that when compared to a similar low dose of Intralipid, those infants switched to fish oil experienced a halt in their disease progression (as defined by rise of bilirubin). [Lam, et al., 2014]

Critics of this therapy have expressed concerns that an omega-3 emulsion alone would place an infant at risk of essential fatty acid deficiency. Omega-6 fatty acids are necessary for the functioning of all tissues, especially cell membranes. [Gramlich, et al., 2015] A triene/tetraene ratio is the most common biochemical marker of essential fatty acid deficiency, with >0.2 used to indicate early essential fatty acid deficiency. This ratio compares the atypical intermediate eicosatrienoic acid (a triene) to the expected, healthy intermediate arachidonic/eicosatetraenoic acid (a tetraene). [Gramlich, et al., 2015] However, although triene/tetraene ratios may be normal in many Omegaven studies, concentrations of individual fatty acids may still be dangerously low as fatty acid profiles are rarely reported. [Tillman, 2013] Researchers working with Omegaven in large retrospective studies have argued that careful monitoring for essential fatty acid deficiency has shown that the mixture of fatty acids is sufficient. [Le, et al., 2009; Cowan, et al., 2013] For example, more than 130 children in Boston have been treated with Omegaven and none of these patients developed clinical

(growth retardation or dermatitis) or biochemical evidence (elevated triene–tetraene ratio) of essential fatty acid deficiency. [Le, et al., 2009; Fallon, Le, & Puder, 2010] Omegaven solutions do have supplemented amounts of downstream omega-6 fatty acid products, in an attempt to prevent essential fatty acid deficiency. This may explain why essential fatty acid deficiency has not been observed to the extent that was predicted with clinical use of Omegaven. Additionally, it appears that these supplemented downstream products may be more effective than mixed lipid formulations for maintaining essential fatty acids. For example, when compared to four other lipid solutions (including SMOFlipid) in a mouse model, only Omegaven maintained a safe triene/tetraene ratio, preventing essential fatty acid deficiency. [Meisel, et al., 2012]

Regardless of these clinical findings, the manufacturer of Omegaven does not recommend the use of fish-oil based emulsions as a monotherapy, reiterating concerns about the low levels of the essential linoleic acid. [Le, et al., 2009] In addition, the treatment is not universally successful for infants with PNALD. [Tillman, 2013] In particular, it does not appear to be as successful with more advanced stages of disease, and premature infants may be less responsive. [Park, et al., 2011; Premkumar, et al., 2013; Sant'anna, et al., 2012; Nandivada, et al., 2016] At least one study comparing Omegaven to Intralipid (both with lipid reduction) showed no difference in liver enzymes and a surprisingly low incidence of cholestasis in both groups, indicating that the reduced lipid may be more important than the type of lipid provided. [Nehra, et al., 2014] In addition, a report of 6 children treated for PNALD with fish oil, who experienced normalization of bilirubin, still had persistent severe fibrosis on liver biopsies. In three cases, this fibrosis actually worsened with fish oil exposure and only in one case did it improve over time. [Mercer, et al., 2013] The American Society of Parenteral and Enteral Nutrition makes no recommendation on the use of Omegaven, because it is not routinely available in the USA, but they do caution that the overall evidence for fish oil lipids is very low quality and there are concerns regarding the effects on growth and development. [American Society of Parenteral and Enteral Nutrition, 2014]

Omega-3 and 6 mixture: The newest lipid emulsion attempts to address concerns about both essential fatty acid deficiency and pro-inflammatory omega-6 fatty acids. SMOFlipid provides a mixture of lipids from multiple sources, and it is rapidly gaining popularity. This

solution is the first to provide a 2.5:1 ratio of omega-6 to omega-3 fatty acids, which is the ideal balance for human physiology. [Deshpande, et al., 2014] In animal studies, SMOFlipid has demonstrated that it has anti-inflammatory properties. For example, in a murine model with lipo-polysaccharide induced inflammation, SMOFlipid injections produced similar recruitment of neutrophils as compared to a negative control. This was in contrast to an omega-6 based solution, which had significantly greater recruitment of neutrophils, indicating an over-active inflammatory response. [Buschmann, et al., 2015] However, the same study also found that SMOFlipid did not prolong survival in sepsis, whereas olive-oil based solutions did. [Buschmann, et al., 2015] Piglet work has also shown that SMOFlipid may preserve hepatic functioning, as compared to Intralipid (omega-6). In one recent article, a piglet PN model showed that the mixed lipid group had increased bile flow, ($p=0.008$), although both groups fell within the normal rate of bile flow for healthy piglets of similar age. The SMOFlipid group also had lower total bilirubin, and CRP, as compared to the Intralipid group. Only piglets in the Intralipid group developed serum markers and bile impairment that would be diagnosed as cholestasis. The authors concluded that the mixed lipid solution not only reduced inflammation, but also prevented liver disease. [Turner, et al., 2016] Other authors have speculated that the less inflammatory effects of SMOFlipid may be due to the vitamin E that is added to the solution; a strong anti-inflammatory. To further investigate this theory, Muto et al. conducted a piglet PN study where both groups received Intralipid, and one group also received activated vitamin E, in a similar amount as would be supplied with SMOFlipid administration. They found no difference in bile flow or in mRNA expression of genes associated with bile acid metabolism. The groups were also the same in regards to inflammatory and oxidative stress markers. Both groups had impaired bile flow, indicating developing cholestasis. [Muto, et al., 2017]

Initial studies in adults showed that SMOFlipid (as compared to an olive oil and soybean lipid) maintained normal liver enzymes and lower triglyceride levels, although the study was only 5 days in length. [Piper, et al., 2009] One earlier report focused on small groups of children with established PNALD on Intralipid, half of whom were then switched to SMOFlipid, with no reduction in the amount of lipid provided. At 6 months of treatment, cholestasis had resolved in 5/8 in the SMOFlipid group, compared to only 2/9 in the Intralipid

group. In fact, the mean bilirubin in the Intralipid group continued to increase throughout the study period by 79µmol/L compared to a decrease of 99µmol/L in the SMOFlipid group. Of note, multiple other treatment methods (cycling, UDCA etc) were also used, as is routine in that centre. [Muhammed, et al., 2012] Likewise, a separate trial in children over a shorter time period also found statistically significant decreases in total bilirubin in those children with the mixed lipid emulsion as compared to the soy-based, as well as increased levels of serum α-tocopherol in the former group. [Goulet, et al., 2010]

A study of very preterm infants (23-30weeks) compared SMOFlipid to Clinoleic (mostly omega-6 vs omega-3 (9:1 ratio)). The authors found that markers of oxidative stress (lipid peroxidation) were significantly reduced in the SMOFlipid group, while the anti-inflammatory vitamin E level was increased. [Deshpande, et al., 2014] This latter finding is not surprisingly, given that SMOFlipid has supplemented levels of vitamin E. Interestingly, the plasma CRP did not differ between the groups and the usual serum markers of PNALD, such as total and conjugated bilirubin, and liver enzymes remained in the normal range for both groups. [Deshpande, et al., 2014] Meanwhile, a pilot study of SMOFlipid, as compared to Intralipid, for preventing PNALD in neonates demonstrated that infants receiving SMOFlipid had a lower conjugated bilirubin than those who received Intralipid. The SMOFlipid patients were also more likely to have a return of serum conjugated bilirubin to 0µmol/L, as compared to the Intralipid group. The liver enzymes, however, were difficult to interpret, as some were significantly higher and others lower between the groups. Of note, this study was small, with only 12 patients in each arm, but it is one of the only to have an 8 week course, providing some of the first long-term results for SMOFlipid vs Intralipid. [Diamond, et al., ,2016] Lastly, a large study of mostly infants showed that SMOFlipid not only decreased liver enzymes and bilirubin, but also CRP. Of note, a small percentage of the studied patients still went on to develop cholestasis despite the SMOFlipid. [Pichler, Simchowitz, Macdonald, & Hill, 2014]

At least one mouse trial has found that SMOFlipid did not prevent PN induced liver steatosis, while Omegaven maintained normal liver histology. The same study also found that SMOFlipid exposure led to significantly elevated alanine aminotransferase. [Meisel, et al., 2012]

None of the trials to date have found SMOFlipid inferior to soybean oil lipids, at the worst, the outcomes are equivocal. [Zaloga, 2015] Similar to the case of Omegaven, because SMOFlipid is not approved for use in the USA, the American Society of Parenteral and Enteral Nutrition cannot make a recommendation on its use, and they feel that existing evidence is of very low quality. [American Society of Parenteral and Enteral Nutrition, 2014]

In summary, although switching lipids to either a pure omega-3 lipid emulsion or to a mixed lipid solution have promising initial studies, further research is needed. The design of many of the above studies make it difficult to determine if the type of lipid is solely responsible for the improvement in serum bilirubin and other markers of PNALD. For example, most of the studies include multiple other treatment approaches, (ie: cycling, and UDCA etc), some studies do not use standardized doses of lipids, while others include a population of children both pre and post-diagnosis of PNALD. It is unlikely that lipids alone will be the answer to the multifactorial disease of PNALD. Although the biochemical markers of cholestasis can be reversed by switching to omega-3 lipids, it appears that the fibrosis and hepatic structural damage persists and in some cases may worsen. [Tillman, 2013, Nandivada, et al., 2015, Mercer, et al., 2013] Likewise, other studies have shown that despite the normalization of bilirubin, the serum markers of oxidative stress remain elevated. [Goulet, et al., 2010] PNALD has existed in infants before the introduction of lipids in parenteral nutrition, [Arnold, Miller, & Zello, 2003] so other factors are likely involved.

Aluminum reduction: Given the concern for aluminum toxicity, multiple methods for decreasing aluminum exposure have been described. Package inserts in PN products with high aluminum contamination already advise about the risks of aluminum contamination, and pharmacists are generally aware of the risks of aluminum toxicity in infants on long-term PN. [Mirtallo, 2010; Pharmaceutical Partners of Canada, 2011] However, calculating the actual amount of aluminum exposure per patient is difficult, because PN products list the aluminum amount expected at product expiry, making the calculated result considerably higher than the recommended FDA limit. [Poole, et al., 2010; Smith, et al., 2007; FDA, 2011; Bohrer, et al., 2002] Calcium gluconate could be exchanged for calcium chloride, as the latter compound has considerably less aluminum contamination, but the increased chloride content may cause

acidosis and it precipitates easily with phosphate products, leading to PN compounding issues. [Bohrer, et al., 2002]

The most feasible method may involve restructuring the manufacturing and storage process to limit the amount of glass exposure. [Wier & Kuhn, 2012] Plastic vials for calcium gluconate provide significantly less aluminum than the traditional glass vials, although both packaging types do slowly increase in aluminum leeching over time. For example, one study quantified aluminum in samples stored in both types of containers and reported that in glass vials, there was an initial 5000µg/L concentration of aluminum, which increased 30-50% over 2 years. This was compared to an initial concentration of 20µg/L in the plastic product, which also increased by 100% over 2 years, but still remained significantly lower than the glass product. The same study also addressed the concern that plastic packaging releases phthalates which are toxic to endocrine and neurologic functioning. In this study, none of the plastic calcium gluconate packaging released any phthalates over the 2 years of study. [Yokel & Unrine, ahead of print] Some countries have therefore selected plastic vials as a method of managing aluminum toxicity, for example, the United Kingdom Medicine and Healthcare Regulatory Agency has ruled that pediatric calcium gluconate solutions be stored in polyethylene containers, instead of glass vials. [UK Medicines and Healthcare Regulatory Agency, 2010] Similarly, a new calcium gluconate PN product, stored in plastic has just been introduced to the American market. [Yokel & Unrine, ahead of print]

2.5 Bile acid transporters of interest: BSEP, MRP2, MRP3, NTCP, OATP and associated proteins:

Hepatocytes are polarized, meaning that there are distinct apical and basolateral surfaces of the epithelium with mostly unidirectional flow of bile acids between the ends of the cell and across the epithelium. The apical side of the hepatocyte epithelium faces the canalicular lumen, while the basolaeral side borders the space of Disse, (where plasma is filtered). [Alrefai & Gill, 2007] The polarity of a hepatocyte is maintained by both structural components (such as radixin cytoskeleton) and functional components, (the most important of which are the bile acid transporters themselves). Bile acid transporters are located on either the apical or basolateral membrane, but one type of transporter is never found on both regions. This strict positioning ensures that the apical and basolateral membranes have distinct

functions, the former for excretion and the latter for re-uptake. Other cellular elements such as the extracellular matrix, (which holds hepatocytes in place), tight junctions between cells and intracellular energy production assist in keeping the transporters functioning, thus maintaining polarity. [Gissen & Arias, 2015]

Bile acids (or salts) are synthesized from cholesterol in the hepatocyte through two different pathways (described above in the pathophysiology section) [Monte, et al., 2009]. Following synthesis, the bile acids enter hepato-enteric circulation in conjugated forms. They are transported across the apical membrane of the hepatocyte by BSEP and MRP2 and into the bile canaliculi [Arrese & Ananthanarayanan, 2004]. From there, the bile acids enter the small intestine to participate in emulsification and are then reabsorbed from the terminal ileum back into the portal blood stream. Basolateral transporters, such as NTCP and the OATP family, reabsorb bile acids from the portal blood, across the sinusoidal membrane and back into the hepatocyte. [Monte, et al., 2009; Alrefai & Gill, 2007; Hagenbuch & Meier, 2003] Bile acids themselves provide positive feedback for the flow of bile through the liver. [Rager & Finegold, 1975] This bile-dependent bile flow interaction is maintained by nuclear receptors, such as FXR, that regulate the activities of bile acid transporters in response to bile acid levels. [Kullak-Ublick, et al., 2004; Jung, et al., 2002] See Figure 2.2 for an overview of bile flow and hepato-enteric circulation.

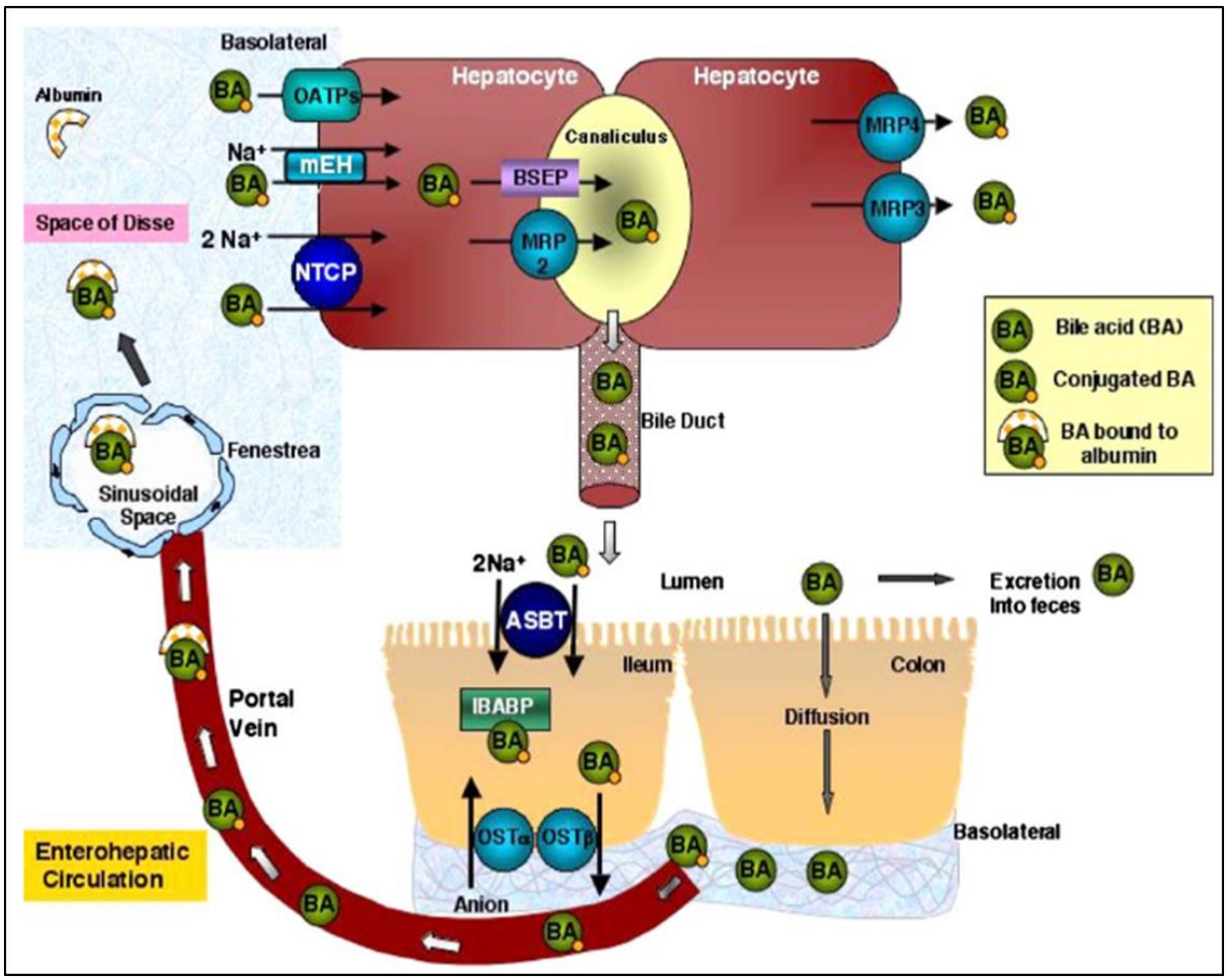


Figure 2.2: Enterohepatic circulation of bile acids, featuring the involvement of major bile acid transporters. ASBT: apical sodium dependent bile acid transporter; BA: bile acids; BSEP: bile salt export protein; IBABP: ileal bile acid binding protein; mEH: microsomal epoxide hydrolase; Mrp2/3/4: multi-drug resistance-associated protein 2/3/4; Na: sodium; NTCP: sodium taurocholate cotransporting polypeptide; OATPs: organic anion-transporting polypeptide; OSTα/β: organic solute transporter α/β. Reproduced with permission from W. A. Alrefai and R. K. Gill. Bile acid transporters: structure, function, regulation and pathophysiological implications. *Pharmaceutical Research* 2007; 24(10):1803-1823.

As described previously, neonates have been found to have deficiencies in almost all of the steps necessary for normal enterohepatic circulation of bile. This may be due to delayed development of bile acid transporter genes and the nuclear transcription factors that control them. [Tomer, Ananthanarayanan, Weymann, Balasubramanian, & Suchy, 2003] It is also possible that the bile acid transporters may be poorly positioned in more immature livers. [Chen, et al., 2005] Either insufficiency would negatively impact bile transport when it is needed most, ie: a time of cholestasis. The following section will discuss bile transporters that have been implicated in PNALD and other forms of neonatal cholestasis.

Interference at any of the stages of bile acid transporter synthesis can alter the amount or functioning of a bile acid transporter, potentially contributing to cholestasis. Alteration of transcription of mRNA from DNA is generally observed later in cholestasis, while changes to the protein after translation are usually an earlier event. [Anwer, 2004] The post-translational events are mediated by secondary messengers, while the earlier, transcriptional changes are controlled by nuclear receptors such as FXR. [Anwer, 2004, Ananthanarayanan, Balasubramanian, Makishima, & Mangelsdorf, 2001; Zollner & Trauner, 2009]

BSEP: Bile salt export pump (also known as ABCB11) is one of the most important bile acid transporters, as it features in the rate-limiting step of biliary flow: excretion into the canaliculi. [Arrese & Ananthanarayanan, 2004] It is found exclusively in the liver, on the canalicular membrane, and transports conjugated monovalent bile acids. [Arrese & Ananthanarayanan, 2004] Between the portal blood and canalicular space, the difference in bile salt concentration gradients is almost 1000-fold, so an active transporter such as BSEP is the only way to move bile salts out of the hepatocyte. [Kullak-Ublick, Stieger, & Meier, 2004] Studies of human fetal liver suggest that BSEP is expressed at 30-50% of adult levels but may not be properly localized until later in life. [Tomer, et al., 2003; Chen, et al., 2005]

BSEP responds differently to various cholestatic models through both transcriptional and post-transcriptional regulation. [Arrese & Ananthanarayanan, 2004] For example, in primary biliary cirrhosis, BSEP has an indirect upregulatory response, increasing both BSEP mRNA and protein levels. [Arrese & Ananthanarayanan, 2004] Other BSEP transporters are also recruited from an intracellular pool, acting on stimuli of cAMP, and hypo-osmolarity; in addition to the increased bile acids. [Arrese & Ananthanarayanan, 2004, Schmitt, Kubitz, Lizun, Wettstein, & Haussinger, 2001] This is likely an adaptation to improve clearance of bile acids from the hepatocyte. [Zollner, et al., 2003; Wolters, et al., 2002] However in other high bile acid scenarios, such as inflammation-induced cholestasis models, BSEP is decreased [Zollner, et al., 2001] and the preliminary studies looking at BSEP in PNALD appear to correlate more with this example. For instance, in murine models of PNALD, it was discovered that the mRNA expression of Bsep was significantly lower in the parenteral nutrition group as opposed to the non-PN group. [Li, et al, 2012] Although this is an interesting finding,

correlation in human studies is needed, as the murine Bsep does not seem to be quite as crucial as its human counterpart. [Alrefai & Gill, 2007]

MRP2 and MRP3: Multidrug resistance associated proteins, of which four members (1-4) are located in the liver, are also ATP dependent transporters. MRP3 and MRP4 are found on the basolateral membrane, while MRP2 is solely on the apical, canalicular membrane. (MRP1 is found in only very low levels in the liver). As a group, they export a variety of substrates into bile including glucuronidated (secondary) bile salts, estrogens, and glutathione conjugates of compounds. [Zelcer, et al., 2006; Gonzalez, et al., 2004] Of all the MRP transporters, MRP2 has the largest impact on biliary flow. Similar to BSEP, fetal liver studies show 30-50% adult protein expression in the second trimester. [Tomer, et al., 2003; Chen, et al., 2005] The fetal liver studies also found delayed positioning of the MRP2, but not as severe as fetal BSEP. [Chen, et al., 2005]

Despite its importance in hepatobiliary function, there are still many unanswered questions about the role of MRP2 in different types of cholestasis. A few authors have reported that MRP2 mRNA is increased in high bile states, [Zollner, et al., 2003; Zollner, et al., 2001] but most others have found it to be decreased. In particular, decreased mRNA has been found in a murine bile duct ligation model and in small population studies of primary biliary cirrhosis. [Zelcer, et al., 2006; Oswald, Kullak-Ublick, Paumgartner, & Beuers, 2001] Similarly, PNALD models in mice have shown significant down-regulation of Mrp2 mRNA. [Le, et al., 2012, Tazuke, et al., 2004] Interestingly, one was a seven-day model and although there were changes seen in the Mrp2 expression, there were no other signs of cholestasis. The authors hypothesized that if this change in mRNA persisted into a decrease in Mrp2 protein, then the Mrp2 changes might be one of the earliest precursors to PNALD. [Tazuke, et al., 2004]

Human MRP3 transports secondary, glucuronidated bile salts back out of the hepatocyte into the portal blood stream. [Kullak-Ublick, et al., 2004; Alrefai & Gill, 2007; Zelcer, et al., 2006] Rats have a much lower level of Mrp3 than humans, but it is upregulated in cholestatic scenarios and in response to decreased Mrp2 to prevent bile salts from accumulating in the hepatocytes. [Zelcer, et al., 2006] Mice without Mrp3 are surprisingly healthy until they are challenged with bile duct ligation, then they are unable to mount a high

serum conjugated bilirubin level. [Zelcer, et al., 2006] Human MRP3 is still under investigation, but it is believed that human MRP3 may also be upregulated in various types of cholestasis, including Dubin-Johnson syndrome and primary biliary cirrhosis, to excrete bile salts from hepatocytes. [Kullak-Ublick, et al., 2004; Alrefai & Gill, 2007; Elferink & Groen, 2002] There is limited data on human Mrp3 ontogeny, but both rats and mice have little or no expression of Mrp3 as fetuses, and the expression of this transporter increases gradually until it reaches adult levels after weaning (60 days for rats and 21 days for mice). [Zhu, Hou, Xu, Lu, & Liu, 2017; Maher, Slitt, Cherrington, Cheng, & Klaassen, 2005]

For those proteins that are successfully expressed there may be additional damage stemming from oxidative stress in diseases such as PNALD. There is preliminary evidence (extrapolated from other models of cholestasis) that bile export proteins such as MRP2 and BSEP may be internalized to a lysosomal compartment and possibly degraded. [Gonzalez, et al., 2004; Kojima, et al., 2003; Shoda, et al., 2001] The exact reasons for this relocation remain to be discerned, although the cytoskeletal protein radixin may be involved.

Radixin: This cytoskeletal protein connects the plasma membrane and transport proteins in the membrane to the actin cytoskeleton in bile canalicular microvilli. [Kojima, et al., 2003] Studies of cholestasis have shown that both radixin and MRP2 lose their normal co-localization, suggesting that the stabilization of radixin is necessary for normal canalicular positioning of export proteins. [Kojima, et al., 2003; Kojima, et al., 2008] Radixin's role as a stabilizer of export proteins has also been supported in experiments with mice lacking the gene for radixin. These animals developed the expected amount of Mrp2, however the protein was misplaced and the mice developed conjugated hyperbilirubinemia. [Kikuchi, et al., 2002]

NTCP: Sodium-dependent taurocholic co-transporting polypeptide is another important transporter, which may play a role in PNALD. Located in the basolateral membrane of hepatocytes, NTCP carries bile acids into the cell from the portal blood stream. [Alrefai & Gill, 2007] NTCP has the highest affinity for conjugated bile acids, but is also known to transport unconjugated bile acids, sulfated bile acids, and steroid sulfates. [Ho, et al., 2004] Its expression is altered in disease processes such as primary biliary cirrhosis and alcoholic cirrhosis, perhaps as a defensive mechanism to limit the amount of potentially toxic biliary products. [Zollner, et al., 2003; Zollner, et al., 2001] High levels of bile acids indirectly down-

regulate the expression of NTCP via nuclear receptors and other promoters, thereby limiting the amount of bile acids allowed into the hepatocyte. Similarly, pro-inflammatory cytokines and endotoxins (such as are found in PNALD models) indirectly down-regulate both mRNA and protein NTCP. [Arrese & Ananthanarayanan, 2004; Wolters, et al., 2002; Gonzalez, et al., 2004] This inflammation triggered down-regulation could feasibly play a role in PNALD, but evidence is currently lacking. [Alrefai & Gill, 2007] Of note, it is one of the bile acid transporters that is delayed in human fetal development, and is found to be expressed at only 1.8% of adult levels in the second trimester of gestation. [Chen, et al., 2005]

OATP: Organic anion transporting polypeptides, like NTCP are also found on the basolateral membrane, but differ in that they are responsible for the entry of unconjugated bile acids into the hepatocyte. [Alrefai & Gill, 2007; Hagenbuch & Meier, 2003] A collection of OATPs are found in the liver with subtly different functions. Generally, OATPs transport large anionic amphipathic molecules, but a few types can transport enzymes such as cholecystokinin 8 and even cations. [Alrefai & Gill, 2007; Hagenbuch & Meier, 2003] Based on rat studies OATPs may develop even later than BSEP and NTCP. [Hagenbuch & Meier, 2003]

Elevated bile acids have varying effects on the different members of the OATP family. For example, humans with primary sclerosing cholangitis have decreased expression of some subtypes of OATP, with increased expression of others. [Alrefai & Gill, 2007] OATP2 in particular has been studied extensively, and is decreased in later stages of primary biliary cirrhosis as well as inflammation-induced cholestasis. [Zollner, et al., 2003; Wolters, et al., 2002; Zollner, et al., 2001; Oswald, et al., 2001] Analogous to NTCP, researchers have theorized that OATP-type transporters likely play a role in PNALD and other types of acquired cholestasis based on its role in inflammation-induced cholestasis models. [Zollner, et al., 2001]

Congenital diseases with bile acid transporter mutations: Mutations in BSEP are the most detrimental of any of the bile acid transporter disorders. Alterations of this protein lead to the devastating disorder Progressive Familial Intrahepatic Cholestasis type 2, which progresses to cirrhosis and liver failure in the first ten years of life. Progressive familial intrahepatic cholestasis (PFIC, also known as Byler syndrome) includes at least three heterogenous diseases stemming from mutations in apical bile acid transporters. They are all autosomal recessive and present to varying degrees in childhood. PFIC type 2, caused by a complete absence of BSEP,

is the most severe of the group; with the earliest presentation and the quickest progression. It is also the only one to be associated with liver tumours. Histology demonstrates the absence of ductular proliferation, with severe portal fibrosis, and inflammation. Treatment for PFIC type 2 includes ursodeoxycholic acid, biliary diversion, and early liver transplantation. [Jacquemin, 2002; Arrese & Ananthanarayanan, 2004; Elferink & Groen, 2002]

Mutations in MRP2 meanwhile, create a surprisingly benign condition: Dubin-Johnson syndrome, characterized by conjugated hyperbilirubinemia with intact biliary excretion. [Alrefai & Gill, 2007] Dubin-Johnson syndrome is autosomal recessive and on histology, these patients have distinctive accumulation of black pigment in the lysosomes. Most patients are asymptomatic, but a few may complain of vague abdominal pain, weakness or nausea. Jaundice is not usually notable until puberty or by age twenty. The liver enzymes are characteristically normal, but the bilirubin (and subsequently the jaundice) fluctuate. Only in severe neonatal forms is treatment, such as ursodeoxycholic acid, required. [Lee, et al., 2006]

Absence or mutation of NTCP does not appear to cause a recognizable disease. [Ho, et al., 2004] Similarly, lack of OATP transporters may compound biliary stasis but minimal disease has been identified secondary to mutations in this transporter. [Alrefai & Gill, 2007] Only in very rare cases where multiple types of OATP transporters are missing, is there any deficit. For example, Rotor syndrome can develop when two OATP transporters are mutated. It shares many of the same characteristics as Dubin-Johnson syndrome, with mild neonatal jaundice. The distinguishing feature is the lack of intrahepatic pigment. [Gissen & Arias, 2015]

Farsenoid X receptor (FXR): FXR is one of the most influential nuclear receptors in bile acid synthesis and it regulates many of the genes involved in bile acid metabolism. [Ananthanarayanan, et al., 2001; Zollner & Trauner, 2009] Bile acids themselves activate FXR (which works in conjunction with retinoid X receptor (RXR) as a heterodimer) and this activated FXR/RXR complex then adjusts BSEP, MRP2, rat Ntcp, and OATP8 in hepatocytes. [Kullak-Ublick, et al., 2004; Jung, et al., 2002] The relationship between FXR and human BSEP is unique in that FXR directly induces gene expression of BSEP, [Ananthanarayanan, et al., 2001] and no other heterodimer of RXR can produce a similar effect. [Ananthanarayanan, et al., 2001] Murine studies have shown that when FXR is not present, the mRNA of Bsep is significantly reduced compared to normal, and the expected up-regulation of Bsep in response

to increased bile acids is impaired. [Ananthanarayanan, et al., 2001] Comparative studies of human fetal liver samples demonstrate that FXR is expressed at 75% of adult levels, suggesting that this receptor is present in substantial quantities very early in life and therefore likely active in infants with PNALD. [Chen, et al., 2005]

Most of the other interactions between FXR and bile acid transporters are indirect. For instance, FXR negatively controls NTCP through the work of an intermediary. [Zollner & Trauner, 2009] Strangely, in increased bile acid situations where NTCP is turned off by FXR, it appears that FXR turns on OATP8. This latter interaction between OATP8 is clearly stimulated by increased bile acids, but the reasoning for inhibiting one basolateral transporter and activating another is unclear. It may be that OATP8 is needed for ongoing peptide uptake or perhaps to export bile acids in a cholestasis environment. [Jung, et al., 2002]

Lastly, FXR also contributes to fibrosis by regulating the extracellular matrix production of hepatic stellate cells. [Zollner & Trauner, 20096] Acting indirectly again, FXR inhibits fibrosis production both by reducing collagen production and triggering apoptosis of the hepatic stellate cells; though this has only been shown in rat models so far. [Zollner & Trauner, 2009] Overall, FXR is clearly a key regulator of bile acid synthesis and responds to high levels of bile acids, but its role in PNALD is not known.

2.6 Piglet model:

PNALD is a complex, multifactorial disease that cannot be adequately replicated outside of a living organism, and this makes the piglet model invaluable. Through use of the piglet it is possible to mimic almost all of the conditions that are known to contribute to PNALD and then examine a disease in vivo. The piglet, as opposed to other animals, is the ideal choice because it most closely mimics infant physiology, anatomy, and metabolism. [Miller & Ullray, 1987; Baracos, 2004]

One of the most important features of a PNALD model is the ability to mimic a neonatal population, because this is the age group most frequently affected. Due to their size, both mouse and rat models of PNALD use mature animals as it is technically difficult to perform the surgical procedures required to create a short bowel model or insert central lines in rat or mouse pups. [Turner, et al., 2011, Sangild, et al., 2014] Both the human infant and the

piglet are born at a similar, immature stage of gastrointestinal/hepatic development. In fact, the piglet may be a closer model for the premature infant vs the term infant, given the lack of fat reserve in the new-born piglet. [Miller & Ullray, 1987] The pathophysiology of PNALD appears to depend on the presence of an underdeveloped enterohepatic system and therefore it is extremely important to have a model such as the pig that provides this feature. For example, Turner, et al., demonstrated in their piglet PN model that even with piglets born at term, there was increased intermediate filaments of liver epithelial cells, a finding indicating that ducts were still in the developmental phase. [Turner, et al., 2011] Piglets are also the animal of choice for other diseases of prematurity as well, such as necrotizing enterocolitis. [Sangild, et al., 2014]

Piglets share important immunological features with infants, where both rely heavily on antibodies received from colostrum and breast milk. Piglets are especially vulnerable, because they do not receive maternal immunoglobulins through the placenta. [Sangild, et al., 2014] This is in contrast to rats, because rat pups receive a large component of their maternal antibodies prenatally. The commensal flora is also similar between humans and pigs, in addition to susceptibility to many of the same bacterial and viral pathogens. [Butler & Singkora, 2007] Although the immune deficit may make the piglet model more accurate, it also makes the piglets harder to work with. Piglet PN studies often have a high attrition rate because of sepsis. [Turner, et al., 2011] To avoid attrition, many piglet studies administer maternal plasma to provide passive immunity. [Sangild, et al., 2014]

PNALD models often have a component of short bowel, and the piglet model produces a more translatable response to short bowel. For example, in most piglet PN models with short bowel, the animals are dependent on PN, whereas rodent models are not. [Sangild, et al., 2014] In one piglet PN model with short bowel, the authors reported hepatic cholestasis, rather than steatosis. Infants on PN are much more prone to cholestasis as opposed to steatosis and this implies that piglet livers respond to PN exposure in a similar way as infants. In comparison, other animal PN models, such as rabbits and rats more often produce steatosis, which indicates liver damage, but is not representative of neonatal PNALD. [Turner, et al., 2011]

Rodent models are often a good first-line model to test out a hypothesis, such as the role of intestinal growth factors. However, they are not as easy to translate to clinical PNALD

practice. Not only is it technically difficult to conduct surgery to create PNALD in rat pups, but the pups are born at a much more developed stage than human infants. [Sangild, et al., 2014] Rats do have an immature gastrointestinal system at birth, but the rest of their organ systems, including liver, kidney and nutrient metabolism are fully developed.[Sangild, et al., 2014] This is a key difference in a disease that requires hepatic immaturity.

Mice are unique in that they can survive massive bowel resection without requiring PN. This provides the opportunity to identify the molecular changes that allow the mouse to survive such considerable bowel loss. They also are more resistant to intestinal atrophy triggered by PN administration, similar to adult humans. [Sangild, et al., 2014] Studies using these features have generated considerable advances in the field of intestinal adaptation, a critical step to advancing enteral tolerance and weaning from PN. The mouse model also has the ability to use multiple different genetic strains, with or without genes of interest, a feature which is lacking in the piglet model. [Sangild, et al., 2014] This model still has many of the same limiting factors as described above for the rat model and a model using PN in mouse pups does not exist.

Neonatal piglets have a long history of acting as a model for a variety of newborn human formulas, [Book & Bustad, 1974] as the diet requirements between the two species are remarkably alike. [Miller & Ullray, 1987; Baracos, 2004] The parenteral nutrition requirements between the two species are equally similar, especially in terms of amino acid requirements. Piglets do require significantly more calories per day than human infants because of their expedited growth, but rapid development is advantageous. [Miller & Ullray, 1987] The growth rate of a piglet is generally 3-5 times that of a human. [Turner, et al., 2011] For example, the bowel in a piglet will double in length in only 10 days, whereas it takes 2-3 years for a human to do the same. [Turner, et al., 2011] This growth rate allows for rapid development of disease processes and quicker responses, such as bowel adaptation. In a large animal model, where housing and feeding is expensive, it is important to have a fore-shortened disease process. Research has shown that if a nutritional regimen can support the intense growth demands of a piglet, it should be sufficient for the slower developing infant. [Book & Bustad, 1974]

Our project examines bile acid transporters and therefore it is important that the ontogeny and function of these transporters be accurately represented in our model. There is

minimal data on the development of piglet bile acid transporters, but considerable evidence that rodents and humans have prohibitive differences in this area. For example, many of the transporters (including Mrp2, Mrp3, and Bsep) are minimally expressed in rodents at birth and take weeks to increase. [Zhu, et al., 2017; Maher et al., 2005; Tomer, et al., 2003; Chen, et al., 2005] This is in comparison to human infants, where 30-50% of these transporters are documented pre-natally. [Tomer, et al., 2003; Chen, et al., 2005] The adult functioning of some transporters may also differ between rodents and humans. This is true of Bsep, which is not as essential in rodents as it is in humans, [Alrefai & Gill, 2007] and Mrp3, which some rodents can lose completely and be unaffected. [Zelcer, et al., 2006]

Finally, as compared to rodents, piglets are also independently mobile shortly after birth, a feature which ensures recovery after surgery. [Butler & Sinkora, 2007] Considering these factors, the Yucatan miniature piglet was chosen specifically because it has a desirable growth rate as it develops a little slower than its domestic piglet cousin, but still much faster than a human infant. In addition, it is smaller, making it much easier to handle, house, and support nutritionally; and is hardy with few health issues. [Book & Bustad, 1974]

2.7 Rationale:

Due to the variety of factors contributing to the pathophysiology of this disease, the best results in treatment have also been multi-factorial. For example, larger treatment centers have reported success using a combination of PN cycling, lipid reduction, employing newer lipid emulsions, maximizing enteral stimulation, and use of UDCA. [Cowels, et al., 2010] Reducing aluminum is an important strategy to add to this list and will likely further improve the results. Overall, many questions remain about the pathophysiology of PNALD, especially regarding the importance of lipids and aluminum, and their effects on bile acid transporters.

3: TRANSITIONS

This thesis is comprised of five projects that progress sequentially, each one building on the findings of the preceding work. My roles were similar in all of the projects. For each one, I contributed to the experimental design, and I carried out the experiments and acquired data. I also contributed to data analysis and interpretation, and I was first author for drafting and revision of all manuscripts.

Firstly, we conducted a study examining Al contamination of infant PN in a Canadian NICU. Before examining the role of Al in PNALD, we needed to establish if Al contamination was still an issue in modern neonatal units. Our hypothesis was that Al is still at a potentially toxic level in neonatal PN and calcium gluconate contributes the majority of Al in PN. This study addressed objective 1: Assess the current degree of aluminum contamination of parenteral nutrition used in a Canadian neonatal intensive care unit and review possible sources for this contamination. This experiment was published in a modified form in the Journal of Parenteral and Enteral Nutrition: Hall AR, Arnold CJ, Miller GG, Zello GA. Infant parenteral nutrition remains a significant source for aluminum toxicity. *JPEN J Parenter Enteral Nutr*. 2016; pii: 0148607116638056. [Epub ahead of print].

With the knowledge that Al was still widely prevalent in infant PN, we proceeded to analyze the effects of high Al on the bile acid transporters essential for hepatic function. For this project, we conducted a randomized control trial using the piglet PN model. We compared the effects of Standard Al (similar to levels found in our NICU study) vs High Al (2.5 times more Al). We chose these amounts of Al because we wanted to amplify the effects of Al and have a better chance of identifying them in a relatively small study. All of the piglets in this study group had otherwise identical PN with an omega-6 based lipid. We hypothesized that Al contamination in PN would negatively affect bile acid transporters, causing both down-regulation and incorrect cellular localization. This study addressed objective 2: Use the Yucatan miniature pig parenteral nutrition model to observe the effects of High (63µg/kg/day) vs Standard Al (24µg/kg/day) contamination, with either mixed or omega-6 lipids. Evaluate for changes to bile acid transporters by immunohistochemistry, polymerase chain reaction, and Western blotting techniques. Additionally, compare the total serum bile acids to detect early cholestasis.

Based on our findings from the first piglet PN study, there was evidence that Al could alter bile acid transporters, but we felt that the pro-inflammatory omega-6 lipid might also be inhibiting bile acid transporters. We decided to repeat the randomized control piglet PN model and compare High vs Standard Al using a less-inflammatory mixed lipid solution. We hypothesized that both Al contamination and pro-inflammatory lipids in PN impair bile acid transporter proteins, and that a mixed lipid solution would only partially prevent the changes in transporters correlated with Al contamination. This study addressed objectives 2 and 3. 2: Use the Yucatan miniature pig parenteral nutrition model to observe the effects of High (63µg/kg/day) vs Standard Al (24µg/kg/day) contamination, with either mixed or omega-6 lipids. Evaluate for changes to bile acid transporters by immunohistochemistry, polymerase chain reaction, and Western blotting techniques. Additionally, compare the total serum bile acids to detect early cholestasis. 3: For the piglet study with omega-6 lipids, compare High vs Standard aluminum in parenteral nutrition, in terms of its effect on serum C-reactive protein levels (a marker of inflammation), and hepatocyte morphology, as determined by transmission electron microscopy.

Based on the second piglet PN study, we concluded that Al could still affect bile acid transporters despite the presence of a less-inflammatory lipid and we decided to explore the Al-associated changes further. Using liver tissue from the randomized control piglet PN model we compared the hepatic ultrastructure effects of High vs Standard Al with a less-inflammatory mixed lipid solution. We hypothesized that the hepato-protective nature of the mixed lipid solution would not completely protect the hepatocyte and Al induced structural changes would develop despite the use of the less-inflammatory lipid. This study addressed objective 3: For the piglet study with omega-6 lipids, compare High vs Standard aluminum in parenteral nutrition, in terms of its effect on serum C-reactive protein levels (a marker of inflammation), and hepatocyte morphology, as determined by transmission electron microscopy.

Our piglet work suggested that Al could be detrimental to bile acid transporters and that mixed lipids could partially prevent the Al-associated changes but we wanted to isolate the specific effects of both lipids and Al. We also wanted to further explore the combined effects of Al and lipids. We decided to conduct a trial using sandwich-cultured hepatocytes where we could study the effects of Al, omega-6 lipids, and mixed lipids, both individually and together.

We hypothesized that both Al contamination and pro-inflammatory lipids in PN could cause down-regulation and impaired functioning of bile acid transporter proteins and that the two factors might act synergistically. By addressing both concerns through the reduction of Al and the switch to a more balanced lipid formulation (mixed lipids), we theorized that there would be better preservation of bile acid transporters, as compared to either intervention individually. This project addressed objective 4: Isolate the effects of aluminum, omega-6 lipids, and mixed lipids on bile acid transporters using a primary hepatocyte model and determine if the effects of these factors are additive. Evaluate for changes to bile acid transporters by qPCR, Western blot and functional assay (where possible).

4: INFANT PARENTERAL NUTRITION REMAINS A SIGNFICANT SOURCE FOR ALUMINUM TOXICITY

Reprinted with permission of SAGE: Hall RA, Arnold CJ, Miller GG, & Zello GA. (2016). Infant parenteral nutrition remains a significant risk for aluminum toxicity. *JPEN J Parenter Enteral Nutr* 2016; pii: 0148607116638056. http://journals.sagepub.com

4.1 Abstract:

Background: Aluminum toxicity is associated with anemia, impaired bone metabolism, neurologic defects, and parenteral nutrition (PN)– associated liver disease. This element is a ubiquitous contaminant of PN components, especially in infant formulations. We assessed the current levels of aluminum contamination in infant PN at a level III neonatal intensive care unit.

Materials and Methods: Thirty samples of PN prepared in the same hospital for infants aged <30 days (mean [SD] weight, 1.54 [0.71] kg) were collected from discarded solution. Each sample was analyzed for aluminum content via inductively coupled plasma mass spectrometry. The components of PN (from label) and measured aluminum content were then compared using linear regression and 1-way analysis of variance.

Results: The mean (SD) aluminum contamination of infant PN was 14.02 (6.51) mcg/kg/d. Only 3 samples were <5 mcg/kg/d. Aluminum levels and infant weight were not associated. Linear regression revealed a significant correlation between aluminum and both calcium gluconate ($P < .0001$) and phosphate ($P = .05$), with a trend between aluminum and potassium ($P = .07$).

Conclusions: Aluminum contamination in infant PN remains almost 3 times higher than the advised maximum exposure (<5 mcg/kg/d, Food and Drug Administration 2004). Unexpectedly, an association between infant weight and aluminum exposure was not apparent, likely due to the homogeneity of our population. Isolating the source of aluminum contamination is difficult, as multiple components appear to be involved. Calcium gluconate is likely still a major contributor, but further investigations into individual components are warranted to promote the reduction of aluminum in infant PN.

Clinical Relevancy Statement:

Despite heightened awareness of the dangers of aluminum in infant parenteral nutrition (PN), potentially toxic levels appear in current PN injectable products. Further action to reduce aluminum in PN injectable products should be undertaken, and many strategies are available, especially to decrease the contribution of calcium gluconate to aluminum contamination.

4.2 Introduction:

Aluminum (Al) has been identified as a substantial contaminant of parenteral nutrition (PN) for over 20 years. [Klein, et al., 1982; Klein, et al., 1984] The Al contamination of PN injectable products is particularly concerning for infants due to a higher risk of toxicities associated with renal Al accumulation. [Koo, et al., 1989; Koo, et al., 1986] Bohrer et al (2010) have demonstrated that even in infants with normal renal function, approximately 56% of Al introduced via PN is not excreted. Unfortunately, premature infants (often with underdeveloped kidneys) are also those exposed to the highest levels of Al in PN because premature infants require proportionally greater amounts of the 2 most contaminated components: calcium (usually calcium gluconate) and phosphate. [Bohrer, et al., 2010; Poole, et al., 2010; Alvarez, Rebollido, Fernandez-Lorenzo, Cocho, & Fraga, 2007; Moreno, Dominguez, & Ballabriga, 1994]

Al provides no nutritional benefit and no deficiency has ever been described, but in excess it is associated with a variety of pathologies. [American Academy of Pediatrics Committee on Nutrition, 1986] Deposits of Al have been found in the liver, brain, kidneys, and developing bone. [Koo, et al., 1989; Koo, et al., 1986; Bertholf, et al., 1989] In the bone, Al interferes with vitamin D, parathyroid hormone, and osteoblast activity, causing an osteomalacia-type bone disease. [Klein, et al., 1982; Koo, et al., 1989; Klein, et al., 1995] The long-term effects of this impaired bone metabolism are evident as late as 13–15 years after PN exposure. [Fewtrell, et al., 2011] Bishop et al (1997) reported that preterm infants exposed to Al-contaminated PN (as opposed to low Al PN) had impaired neurodevelopment at 18 months. [Bishop, et al, 1997] The possible contributions of Al to liver damage and cholestasis in infants receiving PN have recently been reviewed by our group, [Hall, Arnold, Zello, & Miller, 2014] and multiple studies support its hepatotoxic nature. [Alemmari, et al., 2011; Alemmari, et al.,

2012; Arnold, et al., 2003; Klein, et al., 1988; Demircan, et al., 1998] Furthermore, Al also contributes to anemia in the infant. [Gura, 2010]

Article	Al Contamination, Mean	PN Components with Highest Al Contamination
Bohrer et al, 2010[5]	15.2 mcg/kg/d	Individual components not examined
de Oliveira et al, 2010[8]	Not provided	Calcium and phosphate salts
Poole et al, 2010[6]	17.6 mcg/kg/d (calculated on published results)	Calcium gluconate
		Potassium phosphate
		Sodium phosphate
Alvarez et al, 2007[9]	105 mcg/L per PN bag[a]	Calcium gluconate
		Phosphate
		Sodium bicarbonate
Speerhas et al, 2007[26]	9.5 mcg/kg[a]	Individual components not examined
Advenier et al, 2003[11]	2.16 mcg/kg/d	Calcium
		Trace elements Amino acids
		Glucose
Moreno et al, 1994[12]	16.73 mcg/kg/d	Calcium gluconate

Al, aluminum; PN, parenteral nutrition.

[a]Unable to calculate mcg/kg/d, as rate ± infant weight not provided.

Table 4.1: Review of articles examining Al contamination in infant PN

In 2004, the Food and Drug Administration (FDA) issued a recommendation that infants should be exposed to no more than 5 mcg/kg/d Al. [Federal Register, 2011] However, multiple investigations into both individual components and the resulting PN solution have demonstrated that Al contamination of infant PN remains well above 5 mcg/kg/d (Table 4.1). [Bohrer, et al., 2010; Poole, et al., 2010; Speerhas & Seidner, 2007] Unfortunately, considerable variation exists between these studies. These differences include the methods of Al measurement and analysis, the ages of the patients, and the degree of investigation into individual components. To determine if current methods to decrease Al levels in PN have been successful in North America, we measured the Al contamination in infant PN at our institution.

4.3 Methods:

Thirty samples of PN were obtained from discarded solutions, prepared for 18 different patients, at a single hospital pharmacy for our level III neonatal intensive care unit (NICU). At our institution, all neonatal PN is provided as a 2-in-1 admixture consisting of amino acids, dextrose, and other additives in a single bag. A lipid emulsion containing soybean oil, medium-chain triglycerides, olive oil, and fish oil (SMOFlipid) is provided as a separate infusion based on recommendations of the SMOFlipid manufacturer (Fresenius Kabi, Richmond Hill, ON, Canada) and national organizations such as the American Society for Parenteral and Enteral Nutrition (A.S.P.E.N.). [Boullata, et al., 2014, Fresenius Kabi, 2015]

Each PN sample was taken from the bag of amino acid and dextrose solution once it had been de-identified and discarded from the infant's bedside and was no longer needed for active treatment. The separate bag containing SMOFlipid was not examined. Two samples of sterile water were also analyzed as a control. Those infants selected had been treated with PN for at least 7 consecutive days, and 14-day intervals were used for those cases where repeat samples were obtained from the same infant's formulation. All infants receiving PN were aged <30 days, and their mean (SD) weight was 1.54 (0.71) kg. This study was approved by the University of Saskatchewan Biomedical Research Ethics Board prior to starting this study. No information was collected about the identity or health of the infants receiving PN, and therefore consent was not required.

A sample volume of 30–50 mL was collected from each PN bag and transferred to a sterile polyethylene 50-mL centrifuge tube. These samples were stored at 4°C for up to 1 month before they were analyzed. The 32 samples (PN and water) were analyzed for Al content via inductively coupled plasma optical emission spectrometry (ICP-OES; Model iCAP 6500 Duo Analyzer; Thermo Scientific, Waltham, MA) using standardized procedures recognized by Environment Canada, the US Environmental Protection Agency, and the Canadian Centre for Mineral and Energy Technology.

First, nitric acid was used to digest the sample. The resulting solution then underwent pneumatic nebulization and was passed through the argon-based plasma beam of the ICP-OES. Following additional exposure to high temperature and radio-frequency, individual atoms and ions in the sample became excited and emitted light. The light was then passed through a prism and Echelle grating to separate the light by wavelength and order, respectively. The separated

photons were then measured at specific emission lines (atomic line 396.7 nm for Al) by the solid-state charge injection device. All equipment involved in the digestion and analysis of the samples was Al free. An internal sample was run concordantly for all analyses, and runs were repeated if 70%–125% of the internal standard was not recovered. The ICP-OES instrument used has a lower detection limit of 0.005 mg/L for Al. [American Public Health Association, 1999; Saskatchewan Research Council, 2016]

Each bag was labeled with the weight of the infant, the PN administration rate, and a listing of the individual PN components with their amounts.

Statistical Analysis

All statistical analysis was carried out using SPSS version 22 (SPSS, Inc, an IBM Company, Chicago, IL) and SAS (SAS Institute, Cary, NC). Infant weight and PN Al content were compared using linear regression and 1-way analysis of variance (ANOVA). For the 1-way ANOVA, infant weight was classified as quartiles and also as 3 groups (<1 kg, between 1 and 2 kg, and >2 kg).

To examine the relationship between PN components and Al content, both univariate analysis and multivariate analysis were performed. The former method compared each PN component individually to Al content (in mg/L and mcg/kg/d) using regression analysis, while the latter method included all significant components together in a single model. Components identified in the univariate analysis to have significance of $P < .20$ [Bursac, et al., 2008] were included in the multivariate analysis (also 2 models for Al in mg/L and mcg/kg/d). Significance level was set as $P < .05$.

4.4 Results:

The mean (SD) Al contamination was 14.02 (6.51) mcg/ kg/d, with a range of 1.16–27.18 mcg/kg/d. Only 3 samples contained <5 mcg/kg/d Al, while 3 samples contained >25 mcg/kg/d (Figure 4.1). In the 2 water samples, the Al contamination was below the detectable limit of 0.005 mg/L, indicating that the centrifuge tubes had not contributed any Al.

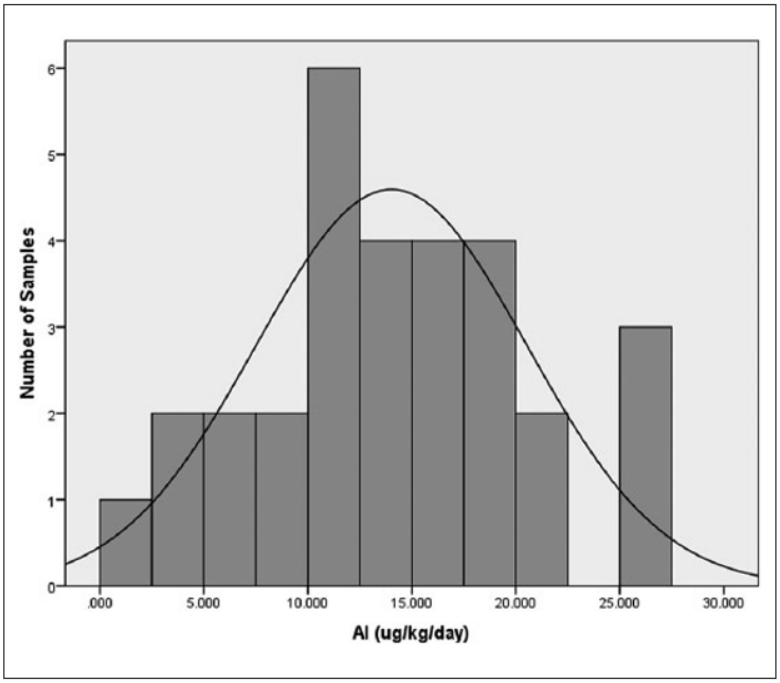


Figure 4.1: Histogram of Al content in 30 PN samples

Al and infant weight were not related when the 2 factors were compared by linear regression (P = .21). One-way ANOVA also resulted in no significant correlation between weight and Al. For the quartile classification, the resulting P value was .22, while for the 3 weight groupings, the P value was .70.

Results of the comparison between the proportion of each individual PN component and Al contamination of the total PN sample are shown in Table 4.2. The number of components for each PN sample was not consistent throughout the PN study samples. For example, selenium was found in only 3 samples. In the first univariate model, where Al was measured in mg/L, both calcium and phosphate were significantly related to Al content, while potassium and multivitamins showed a trend toward significance. In the second univariate model, where the Al was measured in mcg/kg/d, calcium was still significant, along with amino acids. Also, the multivitamins showed a weak trend toward significance, but phosphate and potassium were no longer significantly related to Al contamination.

PN Component (Manufacturing Information)	Component Concentration in PN	Al (mg/L) vs Component[a] P Value	Al (mcg/kg/d) vs Component[a]

	Sample, Mean (SD)		P Value
Amino acids, % (PRIMENE 10% [amino acid injection]; Baxter, Mississauga, ON, Canada)	4.01 (0.93)	0.17	0.002
Dextrose, % (70% dextrose injection, USP; Baxter, Mississauga, ON, Canada)	17.50 (2.16)	0.94	0.55
Sodium, mEq/L (sodium phosphate injection, USP [Sandoz, Boucherville, QC, Canada]; sodium acetate injection, USP [Omega, Montreal, QC, Canada]; sodium chloride injection, USP [Fresenius Kabi, Richmond Hill, ON, Canada])	61.00 (38.00)	0.21	0.86
Potassium, mEq/L (potassium phosphate injection, USP; Fresenius Kabi, Richmond Hill, ON, Canada)	33.33(18.26)	0.07	0.58
Magnesium, mEq/L (magnesium sulfate injection, USP, 50%; Fresenius Kabi, Richmond Hill, ON, Canada)	3.33 (1.67)	0.44	0.80
Phosphate, mmol/L (sodium phosphate injection, USP [Sandoz, Boucherville, QC, Canada]; potassium phosphate injection, USP [Fresenius Kabi, Richmond Hill, ON, Canada])	12.70 (6.34)	0.05	0.30
Chloride, mEq/L (sodium chloride injection, USP; Fresenius Kabi, Richmond Hill, ON, Canada)	45.61 (47.56)	0.60	0.38
Acetate, mEq/L (sodium acetate injection, USP; Omega, Montreal, QC, Canada)	37.80 (31.41)	0.27	0.33
Calcium, mEq/L (calcium gluconate injection, USP, 10%; Fresenius Kabi, Richmond Hill, ON, Canada)	23.07 (4.42)	<0.0001	0.05
Multivitamins, mL/kg (MULTI-12/K1*Pediatric [Multivitamins for infusion], Sandoz, Boucherville, QC, Canada)	2.23 (1.01)	0.07	0.08
Trace elements, mL/kg (MICRO+*6 Pediatric [6 Trace elements injection, USP], Sandoz, Boucherville, QC, Canada)	0.09 (0.03)	0.11	0.97

Zinc, mg/kg (MICRO Zn [Zinc Sulfate injection, USP]; Sandoz, Boucherville, QC, Canada)	0.23 (0.10)	0.26	0.58
Carnitine, mg/kg (CARNITOR Injection, USP [Levocarnitine]; Sigma-Tau Pharmaceuticals, Gaithersburg, MD)	5.33 (5.07)	0.70	0.54
Selenium, mcg/kg (MICRO Se [Selenious acid injection, USP]; Sandoz, Boucherville, QC, Canada)	0.10 (0.31)	0.79	0.26

Table 4.2: Univariate Analysis of Al Content vs Individual PN Components. Al, aluminum; PN, parenteral nutrition; SD, standard deviation; USP, United States Pharmacopeia. **a**: Significant at P < .05

For the first model of multivariate analysis (with Al measured in mg/L), amino acids, phosphate, calcium, potassium, trace elements, and multivitamins were included, but only calcium trended toward a relationship with Al. The second multivariate model (with Al measured in mcg/kg/d) included amino acids, calcium, and multivitamins. Although phosphate, potassium, and trace elements did not meet the numeric inclusion requirements in the initial univariate model, they were still added to this multivariate model because of their clinical importance in the literature. Previous studies have identified high Al contamination in these 3 components (as per Table 4.1), and they were therefore included to obtain a complete clinical model. In this second multivariate model, only amino acids displayed a significant relationship with Al (Table 4.3).

	Al vs Component,[a] *P* Value	
Component	Model 1 (mg/L)	Model 2 (mcg/kg/d)
Amino acids	.10	.002
Potassium	.22	.25
Phosphate	.45	.25
Calcium	.07	.75
Multivitamins	.95	.96
Trace elements	.53	.81

Al, aluminum; PN, parenteral nutrition.

[a]Significant at $P < .05$.

Table 4.3: Multivariate analysis of PN components vs Al content

4.5 Discussion:

Al contamination in our collection of infant PN was almost 3 times higher than the recommended limit, and only 10% (3 samples) of the PN study samples were compliant with the FDA-recommended Al contamination guideline. In 1991, the American Society for Clinical Nutrition (ASCN) and American Society for Parenteral and Enteral Nutrition (A.S.P.E.N.) released a statement that <2mcg/kg/d is the safe limit for aluminum in parenteral nutrition solutions. If our results are interpreted using this guideline, then only 1 of our 30 samples has a safe level of aluminum contamination. [American Society for Parenteral and Enteral Nutrition, 1991] On the basis of these findings, most infants receiving prolonged PN are at risk of Al-related complications. The mean PN Al contamination from this investigation of 14.02 mcg/kg/d is similar to the findings reported by other authors in 2010. [Bohrer, et al., 2010; Poole, et al., 2010] A similar study was conducted in the same NICU as the current study in 2005 by Li (2005), and at that time, it was found that the mean (SD) Al contamination was 21.6 (5.2) mcg/kg/d. However, the PN Al content of the 2005 study was estimated based on extrapolation of individual PN component measurements and not on actual measurement of the total PN Al concentration. [Li, 2005]

In contrast to other investigations, our study demonstrated no relationship between amount of Al exposure and infant weight. [Bohrer, et al., 2010; Poole, et al., 2010] Compared with these previous works, our population was more homogeneous and composed of smaller infants (mean [SD], 1.54 [0.71] kg). This lack of weight variation would have made it difficult to detect weight-dependent differences. In addition, the infants received similar amounts of calcium gluconate, which is the component containing the highest amount of Al.

The range of calcium gluconate provided per milliliter was 0.00–24.00 mEq/L, but the standard deviation was only 0.004. When measured in kg/d, the calcium gluconate ranged from 0.00–2.84 mEq/kg/d with a standard deviation of 0.72.

Our univariate analysis was comparable to other studies and showed that calcium, phosphate, potassium, multivitamins, and trace elements were still the major sources of Al contaminants. Upon initial examination, calcium appeared to be very significant ($P < .0001$)

but, with the exception of 3 samples, every bag of PN included the same 24.0 mEq/L of calcium.

This lack of variability in calcium dose complicates determining the relationship between calcium and aluminum contamination. Interestingly, though, the only bag with no calcium added to it was also the one with the lowest Al contamination at 1.20 mcg/kg/d.

The results of our multivariate model (with Al measured in mcg/kg/d) revealed only amino acids as a significant contributor to Al contamination, which is not consistent with previous investigations. [Advenier, et al., 2003] This finding may be secondary to the ratio of high amino acids to low PN volume given to the smallest neonates. In our study population, weight and percentage of amino acids in PN are inversely related (P = .002). This ratio is created in an attempt to maximize nutrition and restrict volume for the most premature infants but may distort the apparent relationship between Al and amino acids. These findings may also indicate that there are other, unexplored factors complicating the relationship between the individual components such as PN product packaging and age of components. [de Oliveira, et al., 2010; Bohrer, Bortoluzzi, do Nascimento, de Carvalho, & de Oliveira, 2009; Bohrer, et al., 2003; Bohrer, et al., 2001]

Multiple strategies have been recommended to reduce Al contamination in PN formulations. PN products with high Al contamination (such as calcium gluconate) often include a pack- age insert warning of the risks of Al toxicity, and pharmacists are advised to consider the risks associated with Al contamination when compounding PN for infants receiving long-term PN. [Mirtallo, 2010; Pharmaceutical Partners of Canada Inc., 2011] However, because PN product labels often list the amount of Al found at expiry, calculating the total amount of Al is highly inaccurate and inevitably results in a final predicted Al content considerably higher than the FDA-recommended amount. [Poole, et al., 2010; Smith, et al., 2007; Speerhas, et al., 2007; Canada, 2005] It would be useful if the product expiry date was instead the date when Al started accumulating at dangerous levels. This information could be extrapolated from studies examining Al leaching from glass. [Bohrer, et al., 2001; Bohrer, et al., 2003; Bohrer, et al., 2009] Another tactic would be to prioritize the newest vials for the high-risk neonate population, with the remainder of the vials to be used for adults based on standard expiration protocols. Poole et al (2008) have demonstrated the difficulty in meeting

the FDA-recommended Al content for patients weighing <50 kg when using estimated calculations. Therefore, measuring the actual amount of Al in PN, instead of estimating amounts based on calculations, may be a more accurate reflection of true Al content in PN formulations. [Canada, 2005]

Alternatively, calcium chloride could be substituted for calcium gluconate, but there is a theoretical risk of acidosis from the increased chloride content. Some authors argue that this acidosis could be ameliorated by substituting other chloride salts with nonchloride alternatives (ie, potassium acetate). [Bishop, et al., 1997] However, calcium chloride is also difficult to combine with phosphates, due to problems with appropriate phosphate availability and precipitation with some phosphate products. [Canada, 2005] For a short time period, a sodium glycerophosphate product (Glycophos; Fresenius Kabi) [American Society of Health-Systems Pharmacists, 2015] was available in North America, as a temporary replacement for inorganic salts. If this product were available permanently, it would allow pharmacists to balance the chloride (from calcium chloride/acetate ratio) with the simple addition of sodium acetate and potassium acetate. Other, lower Al content calcium gluconate products are available, but they are not approved for human use (Calcium gluconate; Sigma-Aldrich, St Louis, MO).

Most PN contamination is due to leaching from the glass containers frequently used for packaging. [Bohrer, et al., 2002] This process is time dependent, which results in Al concentrations that are directly related to shelf-life. [Li, 2005; Bohrer, et al., 2001; Bohrer, et al., 2003; Bohrer, et al., 2009] The transfer of Al from the glass vial to the injectable solution within is also exacerbated if the vial and contents are sterilized with heat. [Bohrer, et al., 2003] The current 10% calcium gluconate injection is a super-saturated solution [Pharmaceutical Partners of Canada Ltd., 2015] that requires additional heating and processing to create. These additional manufacturing steps increase the Al leaching from glass. [Bohrer, et al., 2003] If calcium gluconate were produced as a 5% solution, the Al leaching could be possibly decreased and the resulting product would be useful in PN formulations, with small modifications. Some European countries have taken a much more proactive approach to decrease the Al stemming from glass storage. For example, in the United Kingdom, the UK Medicine and Healthcare Regulatory Agency has mandated that calcium gluconate injectable solutions for use in pediatric patients be stored in polyethylene containers instead of glass vials.

[UK Medicines and Healthcare Regulatory Agency, 2010] Storage in polyethylene containers is one of the most effective methods for reducing Al contamination of calcium gluconate because it has negligible Al content, even when sterilized. [Bohrer, et al., 2003] Binding agents such as silica have also been investigated as a potential remedy to reduce the amount of Al available in PN solutions. [Bohrer, et al., 2009] Last, new compounding equipment using chelating resins for Al is currently under development and shows promise. [Yokel, et al., 2014]

4.6 Conclusion:

Our work has shown that Al contamination remains a major concern for infant PN in Canadian hospitals, with the average Al content almost 3 times higher than the FDA-recommended limit. Multiple PN components appear to contribute to Al contamination of PN. Calcium gluconate is still likely a leading source, although further investigation into the contribution from other PN injectable products and other factors associated with manufacturing and storage is warranted. Strategies for reduction of Al contamination in PN injectable products should be considered because of the continued risk for Al toxicity in infants.

5: EXAMINING THE EFFECTS OF ALUMINUM ON BILE ACID TRANSPORTERS USING A PIGLET PARENTERAL NUTRITION MODEL WITH OMEGA-6 LIPIDS

5.1 Abstract:

Background/Purpose: Aluminum (Al) is a known contaminant of parenteral nutrition (PN) with adverse effects on bone metabolism and neurological development. We hypothesize that it is also hepato-toxic and may contribute to the multi-factorial PN associated liver disease (PNALD). The objective of this study was to assess the impact of Al on bile acid transporters in an effort to better understand PNALD.

Materials and Methods: A randomized control trial was conducted using a Yucatan miniature piglet PN model. Newborn piglets (aged 3-6 days) were placed into two groups of seven animals each. The high aluminum (High Al) group received PN with Al contamination of 63μg/kg/day, while the standard aluminum group (Standard Al) received PN with a lower Al contamination (24μg/kg/day). All piglets were maintained on a strict PN diet (containing omega-6 lipids) for 3 weeks. Serum and liver samples were collected for analysis. We chose five bile acid transporters (Mrp2, Bsep, Mrp3, Ntcp, and Oatp8), a cytoskeletal protein (radixin), and a nuclear receptor (FXR) as targets important in bile flow. These were examined by qPCR, immunofluorescence confocal microscopy, and Western blot. The serum was analyzed for total bile acids.

Results: qPCR demonstrated statistically more mRNA for Mrp2, Bsep, Mrp3, and Ntcp in the Standard Al group, as compared to the High Al group ($p<0.05$). The amount of mRNA for Oatp8, FXR, and radixin, was not significantly different between the groups. Mrp2 protein, as evaluated by Western blot, did not differ between the groups. Immunofluorescence demonstrated that radixin maintained co-localization with both Bsep, and Mrp2, but there was a significantly denser signal for the Mrp2 protein in the lower Al group. There was no difference in serum bile acids.

Conclusions: High Al has a negative effect on the mRNA of the bile acid transporters Mrp2, Bsep, Ntcp, and Mrp3, but further research is required to understand the relationship between Al and PNALD.

Clinical Relevancy Statement:

Al remains a significant contaminant of PN and it may have a negative effect on bile acid transporters. If the mRNA changes are translated into protein deficit, then Al could contribute to PNALD, especially in those infants on long-term PN and prone to hepatic disease. Strategies to decrease Al should be added to the armamentarium of treatments for PNALD.

5.2 Introduction:

PNALD refers to a spectrum of liver dysfunction that develops in neonates following prolonged exposure to PN. [Benjamin, 1981; Touloukian & Seashore, 1975] It is a multi-factorial disease with contributing factors including sepsis, infant prematurity, pro-inflammatory lipids, and contaminants of PN. Considerable advances have been made in improving the outcomes for infants with PNALD, [Guglielmi, et al., 2008; Tillman, 2013; Cowels, et al., 2010] but it remains problematic.

Al is a ubiquitous contaminant of food, medications, and PN components. It has been found in dextrose solutions, amino acids, potassium salts, and even heparin. [Poole, et al., 2010; Wier & Kuhn, 2012] The highest levels of contamination are usually found in calcium gluconate PN injectables. [Poole, et al., 2010; Wier & Kuhn, 2012] Calcium gluconate is an important additive in PN for the premature infant because these infants are lacking the calcium deposition and bone mineralization that normally occurs in the third trimester, and are at risk for osteopenia of prematurity. [Harrison, Johnson, McKechnie, 2008] Premature infants are also more likely to have renal insufficiency, which hampers the clearance of Al. [Bohrer, Oliveira, Garcia, Nascimentio, & Carvalho, 2010; Advenier, et al., 2003] This combination of increased need for Al-contaminated calcium, and the difficulty in excreting Al, places neonates at highest risk for Al toxicity from PN.

Calcium gluconate becomes contaminated with Al through the manufacturing and storage process. Generally, calcium gluconate is stored in glass, where Al leeches into the injectable solution in a time-dependent manner. [Poole, et al., 2010; Wier & Kuhn, 2012] This transfer of Al is exacerbated with the heat-based sterilization common to calcium gluconate manufacturing. [Bohrer, do Nascimento, Binotto, & Becker, 2003]

Al in PN has been implicated in a variety of neonatal disorders. It disrupts normal bone metabolism and may interfere with parathyroid functioning, leading to osteomalacia that is problematic for years after the PN exposure. [Klein, et al., 1982; Koo, Kaplan, Krug-wispe, Succop, & Bendon, 1989; Klein, 1995; Fewtrell, Edmonds, Issacs, Bishop, & Lucas, 2011] Al may also cause delays in neurodevelopment, and worsen anemia of prematurity. [Gura, 2010; Courtney-Martin, et al., 2015] For those infants on long-term PN, Al accumulates in the serum and liver. [Courtney-Martin, et al., 2015, Alemmari, et al., 2012, Arnold, et al., 2003, Klein, et aal., 1984] Animal studies have also found that Al impairs biliary excretion and may alter the structure of canalicular microvilli. [Gonzalez, et al., 2004; Klein, et al., 1988; Alemmari, et al., 2011] For these reasons, it is hypothesized to play a role in the development of PNALD.

5.3 Objectives:

We hypothesize that Al contamination in PN can negatively affect bile acid transporters, causing both down-regulation and abnormal cellular positioning. Our objective is to use the Yucatan miniature pig PN model to observe the effects of high Al vs a standard amount of Al contamination in PN. The standard amount of Al in PN used for the latter group is similar to the contamination of Al found in neonatal PN samples in Canadian hospitals. This comparison of a High vs Standard Al groups will allow us to isolate and potentially magnify the negative effects of Al, rather than risk missing a small, but significant, effect (similar to a toxicity study). The effects of Al will be determined by the mRNA expression of bile acid transporters, as measured by polymerase chain reactions, in addition to evaluating the protein expression and cellular positioning of a few of the transporters, as determined by Western blotting and immunohistochemistry.

5.4 Methods:

Animal Work: All animal work and protocols were reviewed and approved by both the University of Saskatchewan Animal Research Ethic Board and the Institutional Animal Care Committee at Memorial University of Newfoundland. Live animal work was conducted at the Memorial University of Newfoundland vivarium. Yucatan miniature piglets, aged 3-6 days, were obtained from the herd of Yucatan pigs managed by Memorial University and were placed randomly into one of two groups. If at all possible, the piglets were pair-matched with a littermate of similar size and matching gender, to ensure comparable group demographics.

(Reasons for selecting the piglet as a model are outlined in Chapter 2). The High Al group (N=7) received PN with Al contamination of 63µg/kg/day, while the Standard Al group (N=7) received otherwise identical PN with Al contamination of 24µg/kg/day. The Al contribution of our PN components is outlined in Table 5.1.

PN Component**	Al content (unit varies)	Al content¶ (µg/kg/day)
Calcium gluconate ($C1_2H_{22}CaO_{14}$)	2.50 µg/g	3.83
Monopotassium phosphate (KH_2PO_4)	<0.50 µg/g	0.01
Dipotassium hydrogen phosphate (K_2HPO_4)	1.00 µg/g	0.38
Potassium acetate ($C_2H_3KO_2$)	0.80 µg/g	0.28
Glucose ($C_6H_{12}O_6$)	0.90 µg/g	19.46
Trace elements: zinc, copper, manganese, chromium, selenium and iodine. Made in lab.	0.054 mg/L	0.05
	Standard Al group total= Approx. 24 µg/kg/day	
Aluminum ($AlCl_3 \cdot H_20$) Added only to High Al group	111.75 µg/g	38.00
	High Al group total= Approx. 63 µg/kg/day	

Table 5.1: Al contamination of piglet PN components*

*Values obtained from 2015 PN components, but identical products were used in 2013 at the time of this study. **All PN components except for those made in lab are products from Sigma-Aldrich, Oakville, ON, Canada. ¶Calculated by multiplying the amount of component added per bag of PN x rate x hours. Ex: Calcium gluconate = 2.5µg/g x 6.41g/L= 16.03µg/L of Al (from calcium gluconate) in dextrose/amino acid solution. 16.03µg/L x 0.75L (amount of dextrose/amino acid solution added per PN bag) =12.02µg per bag of PN. 12.02µg / 902 mL (total volume in bag) = 0.013µg/mL x 12mL/kg/hr. x 24 hours/day= 3.83µg/kg/day.

On day zero, the piglets underwent general anesthesia consisting of inhaled Isoflurane (1-2%) delivered in oxygen (1.5 L/min) (Piramal Critical Care, Inc., Bethlehem, PA, USA), preceded by the intramuscular injection of the anaesthetic adjuncts Acepromazine (VetOne, Boise, Idaho, USA) 0.5mg/kg, Ketamine (Putney Inc., Portland, ME, USA) 22mg/kg, and

Atropine (Vedco, Saint Joseph, MO, USA) 0.05mg/kg. They were also given their first intramuscular dose of the analgesic buprenorphine hydrochloride, dosed at 0.03mg/kg (Temgesic, Indivior UK Limited, Slough, UK). Three central venous catheters (made in lab) were then implanted using an open approach. One catheter was placed in the internal jugular vein (for infusing PN) and the other two in the femoral veins (for extracting blood samples and as back-up catheters). The catheters were tunneled out through the back of the piglet and secured. See Figures 5.1 and 5.2 below. Each piglet was fitted with a vest which prevented them from chewing on the surgical sites or catheters. The catheters were passed out through an opening in the dorsal aspect of the vest. Following surgery, local antibiotic cream was applied to each surgical incision site (Polysporin, Johnson and Johnson Inc.) and the piglet was given their first intravenous dose of the antibiotic trimethoprim sulfadoxine, dosed at 20mg trimethoprim/ 200mg sulfadoxine (Borgal, Intervet Canada Corp).

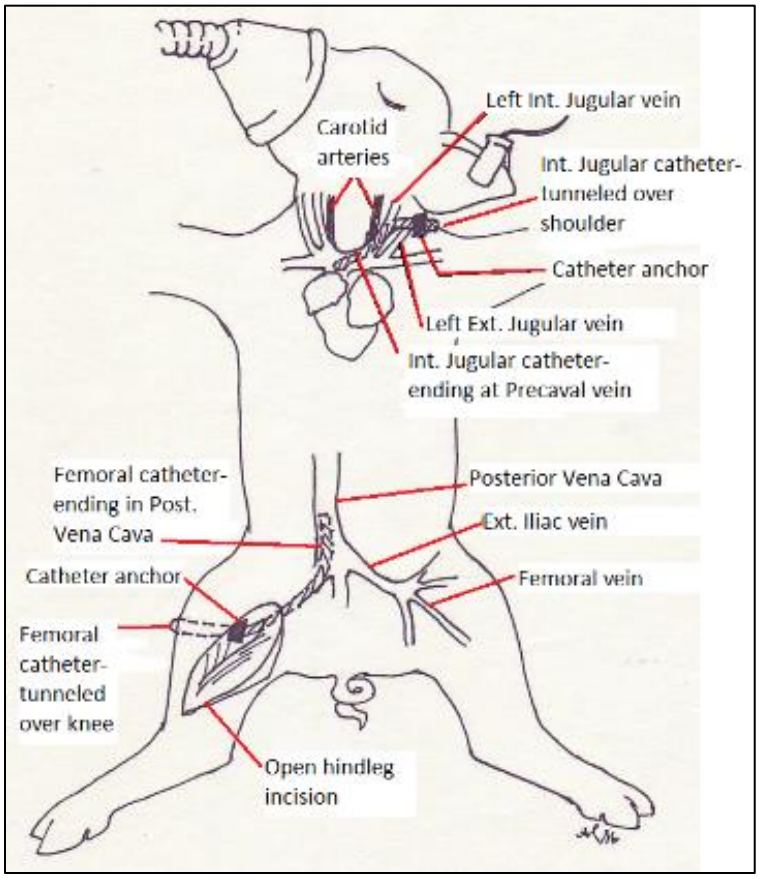


Figure 5.1: Catheter implantation surgery. Ventral view of piglet, demonstrating the cannulated vessels and sites of catheter insertion

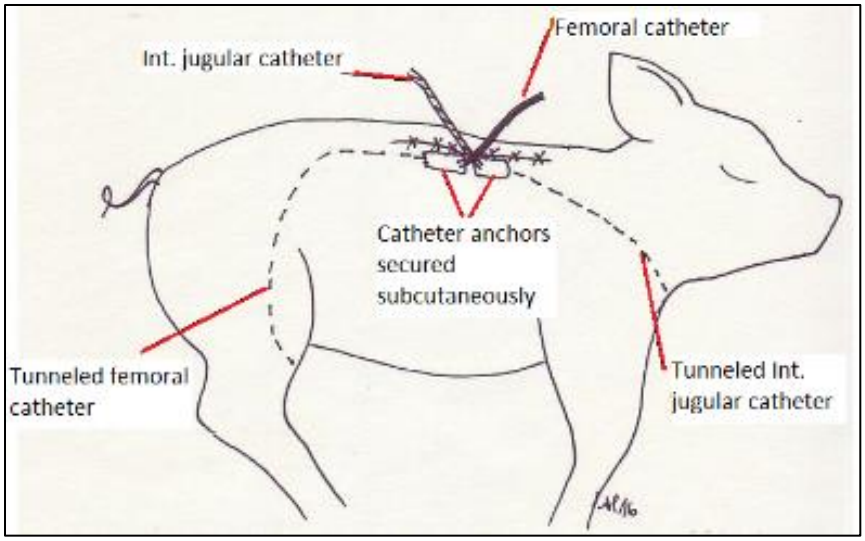


Figure 5.2: Piglet lateral view: depicting catheter tunneling and exit sites of catheters

Immediately post-op, the piglets were moved to individual metabolic cages, where the catheters were secured in a tether from the roof of the cage. This tether allowed the piglet full movement in the cage but kept the catheters secure. PN was infused via the catheters as they passed down through the roof of the cage, along the tether and into the piglet. PN was started on the day of surgery, at 50% of the goal rate, and increased incrementally over 24 hours to a goal rate of 12mL/kg/hour. In the first 48 hours post-operatively, the piglets were given intravenous doses of Temgesic every 12 hours as necessary, based on their behavior (ie: guarding or lethargy). Borgal antibiotic dosing was given prophylactically every second day. The piglets were exposed to standard 12-hour light/dark cycles and the room was kept at 26-28ºC with additional heat lamps provided for each cage. Environmental enrichment consisted of toys, music and human physical contact; additionally, the piglets had aural and visual contact with each other.

Figure 5.3: Photo of tether and vest set-up for piglet while receiving PN

All piglets were kept on a strict PN regimen for the duration of the study (17-21 days). The PN received by the Standard Al group mimicked the neonatal formula used in many Canadian hospitals in 2013; our own work has verified that some infants receive up to 25µg/kg/day of Al. [Book & Bustad, 1974; Also, see previous chapter for Al results in infant PN]. The High Al group received approximately 2.5 times this amount of Al contamination, but the remaining constituents of PN were identical between the two groups. Both groups received all lipids in the form of Intralipid 20% (omega-6 lipid). In total, the piglets received 0.98 MJ/kg/d and 13.3g amino acids/kg/day with non-protein energy supplied in an approximately 50:50 ratio of carbohydrate and fat. The amino acid-dextrose solution was infused at a rate of 10.1 mL/kg/h (242.4 mL/kg/d) and the lipid emulsion at a rate of 1.9 ml/kg/h (9 gm/kg/day). In previous similar studies carried out by this lab and others, this PN regimen had been sufficient to support the normal growth and development of these piglets. [Alemmari, et al., 2012] Every second day, the piglet was weighed and the PN rate was adjusted accordingly. For complete PN recipes, refer to Appendix C.

Throughout the study period, the catheters were flushed with heparinized saline daily, and if a catheter could not be flushed it was capped and left in place, but no longer used. 5mL of blood was collected every four days, including on the day of initial surgery and the necropsy day.

The blood was spun for 10 min at 10,000 rpm and the resulting serum was decanted and frozen at -20ºC until analysis.

If a piglet began displaying signs of infection, including lethargy, increased temperature, or panting, an additional dosage of Borgal was determined by a veterinarian. In cases where the piglet did not improve in the following 24 hours, it was euthanized with an overdose of pentobarbital sodium (Euthanyl, BiMeda-MTC, Cambridge, ON, Canada).

At the conclusion of the study, the piglets were anaesthetized with Isoflurane, the abdominal cavity opened, all major liver vessels clamped, and the liver removed intact. Excess blood was blotted from the liver, it was weighed, and then cut into small blocks and snap-frozen in liquid nitrogen. Following removal of the liver, euthanasia was performed with a cardiac injection of pentobarbital sodium.

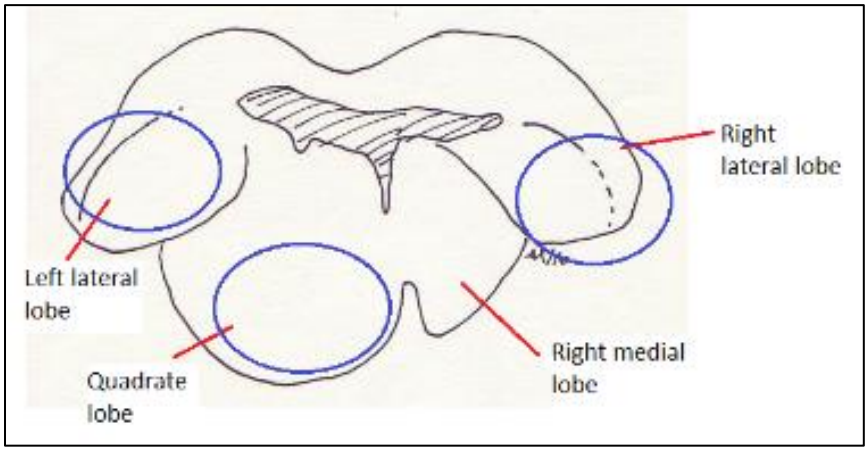


Figure 5.4: Dorsal view of piglet liver. Blue circles indicate our three sites of sample collection, to ensure random sampling from each piglet.

Quantitative Polymerase Chain Reaction (qPCR): qPCR was used to evaluate the amount of mRNA transcribed for the bile acid transporters Bsep, Mrp2, Mrp3, Ntcp, Oatp8, the cytoskeletal protein radixin, and the nuclear transcription factor FXR. Firstly, mRNA was extracted from the frozen liver samples using a lysing agent (Trizol, Thermo Fisher Scientific, Waltham, MA, USA), mechanical disruption, and repeated filtrations. The RNeasy Mini Kit (Qiagen, Hilden, Germany) was used for this step. The resulting purified mRNA was quantified using a NanoVue spectrophotometer (GE, Boston, MA, USA). An A260/A280 ratio of 1.8 to 2.0 was used as the limit of acceptable mRNA purity. It was then normalized with nuclease free, sterile water to ensure identical amounts of RNA in each sample. Following the normalization

step, the VILO Superscript cDNA Synthesis kit (Thermo Fisher Scientific, Waltham, MA, USA) was used to produce cDNA from the mRNA template. For this process diluted mRNA was combined with the VILO reaction and enzyme mix and subjected to heating in a thermocycler. It was incubated at 25ºC for ten minutes, followed by heating at 42ºC for one hour, and the reaction was terminated with five minutes of exposure to 85ºC heat. The resulting cDNA was diluted 20 fold and kept on ice. Diluted cDNA was then added to nucleotides and a fluorescent probe (Power Mastermix, Thermo Fisher Scientific, Waltham, MA, USA) along with both forward and reverse primers (Table 5.2). This solution was pipetted in 14.5µL replicates (each containing 2µL of diluted cDNA) into a 96 well PCR plate, sealed and placed in a Real Time cycler (7300 Real Time PCR Systems, Applied Bio Systems, Foster City, CA, USA). The cycler followed a protocol of 30 minutes of incubation at 95ºC, followed by repeated cycling from 95ºC to 60ºC to induce annealing, and synthesis. A dissociation curve was added to the end of each reaction to assess for unwanted primer-dimers and other artifacts. The Ct value was calculated based on the number of cycles required for adequate detection of the probe, allowing for extrapolation of the original amount of mRNA. Primers were designed with and obtained from Integrated DNA Technologies (Coralville, IA, USA). All primers were tested for efficiency before their use and all primer pairs demonstrated efficiency between 90-110%. [Taylor, Wakem, Dijkman, Alsarraj, & Nguyen, 2015] Each sample was run in triplicate.

Target	Forward primer 5'-3'	Reverse primer 5'-3'
Mrp2	CAC AGA GAA GAT CGG TGT AGT G	ACA CAA AGG ACT TGA GGT GAG
Bsep	GTG TCC AGG TTT ACC GAC TAT G	AAG GGT TCC TGC TGT GTA TTC
Ntcp	CCC TAT GGC AGC ATT GTG ATA	AAG CCG GTG AAA GGC ATA A
Oatp8	GGC GAA TGC CCA AGA AAT G	TGA GAT GGT TCC CAA TGA AGA G
Mrp3	CCC TGC ACA GCC TAG ATA TTC	GGC TAC AGA GCC CTT CAT ATA C
Radixin	GCT GAG GCT AGT GCT GAA TTA T	CTG TCG CAG AGT CTT GTA CTT ATC
FXR	GCC TGC CAA AGG TGT ACT AA	GGT AGA AAC CCA GGT TGG AAT AA

Cyclophilin A	GGT GAC TTC ACA CGC CAT AA	GCT CCA TGG CTT CCA CAA TA

Table 5.2: Primers for qPCR

Cyclophilin A, an enzyme important in intracellular signalling and apoptosis, was used as the reference gene. It was selected after testing a collection of reference genes for their stability across the samples. Using Cyclophilin A as a reference, the qPCR results for each pair of piglets were compared with the Pfaffl method to obtain a relative quantitation (explained below in statistical analysis).

Western Blot: Western blotting was used to evaluate the amount of Mrp2 protein in the liver samples.

Protein was extracted from frozen liver specimens using the PARIS kit (Thermo Fisher Scientific, Waltham, MA, USA), where tissue is chemically lyzed and mechanically disrupted. The resulting protein pellet was then quantified with a Bradford assay (Thermo Scientific Pierce BCA Protein Assay, Thermo Fisher Scientific, Waltham, MA, USA) and normalized with sterile water to produce samples with identical total protein quantity. Protein samples were denatured and loaded into a pre-cast 8% Bis-Tris polyacrylamide gel with 1xMOPS buffer solution (Thermo Fisher Scientific, Waltham, Ma, USA). Electrophoresis of samples was conducted at 150V for 40 minutes to separate proteins by size. The separated proteins were then transferred to a 0.2micron nitrocellulose membrane with exposure to 10V for 60 minutes. Transfer of proteins was confirmed with a ponceau stain.

The nitrocellulose membrane was cut in half to separate the heavier Mrp2 protein bands (185kDa) from the lighter Glyceraldehyde 3-phosphate dehydrogenase (GAPDH, 37kDa). GAPDH, an important enzyme in the glycolytic pathway, was selected as the reference protein for this study because it had shown stability throughout the samples in both qPCR and Western blot experiments and because it is highly abundant in hepatocytes.

The cut membrane was then blocked for one hour with 3% bovine serum albumin in 1xTBST (tris-buffered saline with tween). It was washed with TBST and incubated with the primary antibody for one hour at room temperature, washed with TBS, and incubated again for an hour with the secondary antibody at room temperature. Finally, it was washed once more and a chemiluminescent solution was added to bind to the horseradish peroxidase in the secondary

antibody. The chemiluminescent signal was imaged in an AlphaImager Fluor FC2 (Cell Biosciences, Santa Clara, CA, USA).

The Mrp2 primary antibody was a custom-designed crude serum polyclonal antibody created in a rabbit host by Thermo Fisher (Pierce Custom Services, Thermo Fisher Scientific, Waltham, MA, USA). It was used in a 1:500 concentration and diluted in a 9:1 solution of TBST and 3% bovine serum albumin +TBST. The secondary antibody was goat anti-rabbit horseradish peroxidase conjugated, (Abcam, Cambridge, UK, ab6721) used in a 1:2500 dilution (diluent was a 9:1 solution of TBS and 3% bovine serum albumin +TBST). The GAPDH primary antibody was a monoclonal mouse anti-pig antibody (Proteintech, Chicago, IL, USA, 60004-1-Ig), used in a dilution of 1:10,000 with the same diluent as used for Mrp2. The secondary antibody was a horseradish peroxidase conjugated donkey anti-mouse product (Novex/Thermo Fisher, Thermo Fisher Scientific, Waltham, MA, USA, A16017) used in a dilution of 1:2000, with the same diluent as for the Mrp2 secondary antibody. Although multiple attempts were made, a custom-made Bsep antibody was not suitable for this application and so Bsep could not be examined by Western blot in this project.

The digital images obtained from the AlphaImager were inverted and analyzed using the open source program ImageJ. (https://imagej.net/Downloads) Densitometry studies were conducted on the bands, and each Mrp2 band was normalized to its corresponding GAPDH reference band.

Immunofluorescence and Confocal Microscopy: Immunofluorescence was used to characterize the position of the apical bile acid transporters Mrp2 and Bsep. They were also individually co-localized with radixin to explore the relationship between this cytoskeletal protein and the two bile acid transporters.

Frozen liver was sectioned with a cryotome to achieve 5 micron-thick specimens. The tissue was immediately fixed in acetone for 20 minutes. The acetone was then washed off and the sections were blocked with 8% skim milk in 1xPBS (phosphate-buffered saline) for 30 min at room temperature. After another washing, the slides were incubated with the primary antibody diluted in 0.08% skim milk + 1xPBS at room temperature for two hours. The secondary antibody, diluted in the same solution, was applied and the slides incubated at room temperature for 30 minutes before a final washing. Finally, the slides were sealed with a mounting media

containing the nuclear stain DAPI (Abcam, Cambridge, UK). For both Mrp2 and Bsep the primary antibody was a custom crude serum polyclonal antibody, created in a rabbit host based on our selected peptides (Pierce Custom Services, Thermo Fisher, Waltham, MA, USA). Both Bsep and Mrp2 antibodies were used at a dilution of 1:100. The primary radixin antibody was a monoclonal mouse anti-pig product (Abcam, Cambridge, UK, ab50007) and was used at a dilution of 1:250. The secondary antibody for both Mrp2 and Bsep was donkey anti-rabbit (AlexaFluor 647, Abcam, Cambridge, UK, ab150075) diluted 1:200. The radixin secondary antibody was goat anti-mouse (AlexaFluor 488, Abcam, Cambridge, UK, ab150113) diluted 1:300.

The slides were then viewed under the confocal microscope (Leica SP5, Wetzler, Germany) and the three areas of clearest staining were photographed with a resolution of 49.2 x 49.2 microns. These images were analyzed using ImageJ. The area, density, and width of staining for Mrp2, Bsep, and radixin were mapped and calculated.

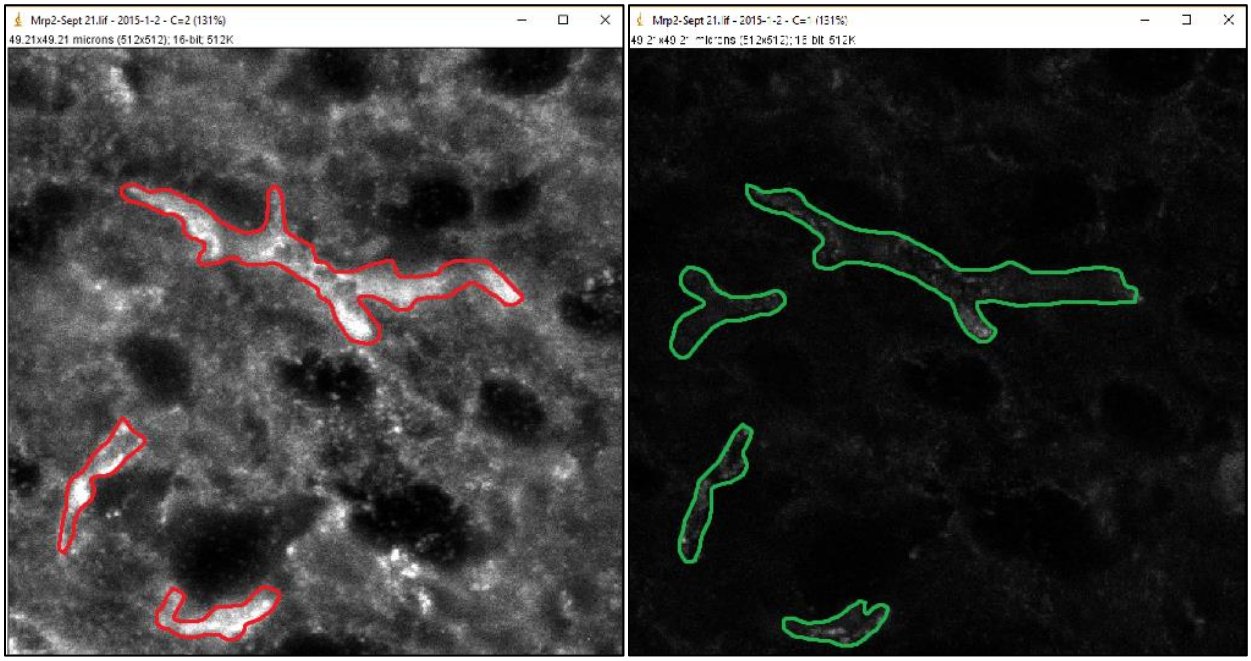


Figure 5.5: Mapping technique used for confocal images. A sample image has been split to isolate two antibody staining patterns. Areas of brightest white staining are outlined. Image on left displays Mrp2 staining outlined in red. Right-sided image demonstrates radixin staining outlined in green. The area inside the outlines is analyzed for area, density, and width.

Bile Acid Assay: A colorimetric kit was used for the total bile acid assay as per the manufacturer's instructions (BQKits, San Diego, CA, USA). In this assay, the oxidation of bile

acids produces NADH, which then reacts with nitrotetrazolium blue to form a coloured dye. A spectrophotometer was used to detect the amount of dye formed by measuring absorbance at 540nm and the amount of dye is directly proportional to the quantity of bile acids present in the sample. Each serum sample was tested twice and any sample with variance greater than 10% was re-tested.

Statistical Analysis

All statistical analysis was carried out using SPSS version 22 (IBM, Armonk, NY) and SAS (SAS Institute, Cary, NC). The bile acid assay results were examined using a repeated measurement model for longitudinal analysis. In this comparison, a p value of <0.05 was considered statistically significant. The basic demographic data (ie: weight) was compared using a repeated measure model and t-tests, with significance indicated by a p value of <0.05.

For the qPCR data, the fold difference between each pair of samples was calculated using the Pfaffl equation [Pfaffl, 2001]:

$$Fold\ induction = \frac{E\ target^{[\Delta Ct\ target\ (control-treatment)]}}{E\ reference^{[\Delta Ct\ reference\ (control-treatment)]}}$$

Equation 5.1

Where *E* represents the primer efficiency and *Ct* represents the cycle threshold value (the number of cycles of heating and cooling required until there was sufficient target DNA for the fluorescent probe to be detected). The mean and median fold difference and standard deviation were then calculated for each bile acid transporter. Due to the small sample size, the data was not normally distributed. To accurately account for this, a non-parametric Wilcoxon-Signed-Rank test was performed for each bile acid transporter. This non-parametric test was able to determine if the median fold difference was equal to, or greater than, two (a standard cut-off for significant fold difference in qPCR). [University of Montreal, "Information on qPCR results", n.d.] A p-value of <0.05 was used as statistically significant for the Wilcoxon-Signed-Rank test. Given that the data did not have standard distribution, the median was a more reliable value than the mean.

The data obtained from immunohistochemistry was compared between the two groups using T-tests, as were the densitometry measurements from the Western blots for Mrp2. In both of these comparisons, a p value of <0.05 was considered statistically significant.

5.5 Results:

Basic demographics: A repeated measurement model demonstrated that there was no difference in weight gain between the two groups (p=0.76), although females generally gained more weight than males did (p=0.043). On average, both groups experienced significant weight gain every day. T-tests indicated that there was no difference between the two groups in the initial weights of the piglets (p=0.91) nor in the final liver weight (p=0.49).

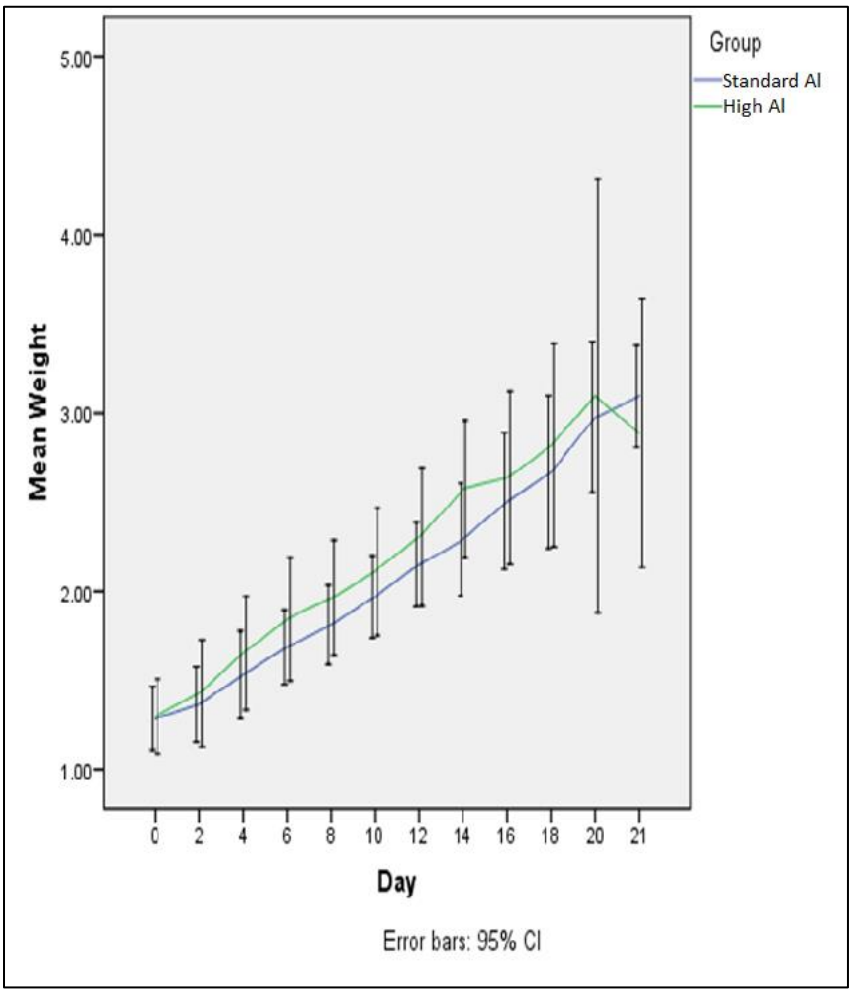


Figure 5.6: Mean weight gain for both piglet groups. (Weight in kg)

qPCR: There was more mRNA for both Mrp3 and Bsep: mean fold differences 3.5 (SD 3.5) and 3.1 (SD 2.4) respectively, in the lower Al (Standard) group as compared to the High Al group. Mrp2 had statistically more mRNA in the Standard Al group than the High Al group (mean fold difference 1.8 (SD 0.8), and so did Ntcp (1.4, SD 1.1). Since the data is not normally distributed, a non-parametric analysis of median values is most accurate and all four of these bile acid transporters demonstrated a median value equal to or greater than 2.0. There was no significant fold difference for any of the other remaining targets. See Figure 5.7 and Table 5.3.

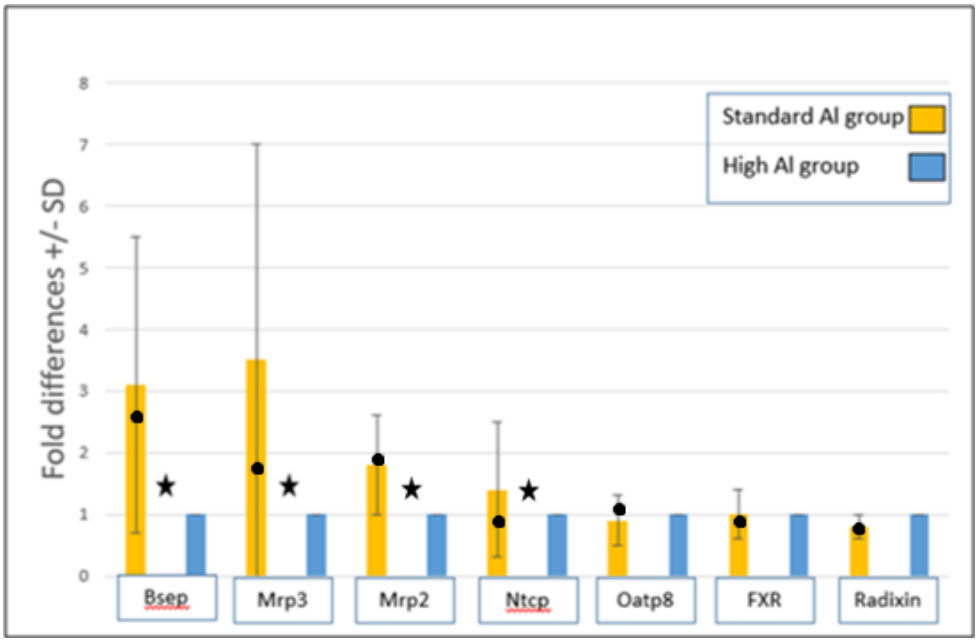


Figure 5.7: qPCR fold differences: High vs Standard Al. Mean fold differences +/- standard deviations. High Al group arbitrarily set at '1' to demonstrate difference between groups (not an actual value). Median values indicated by black dot. Star symbol designates statistically significant fold difference (greater than or equal to 2 by non-parametric test).

	Null Hypothesis	Test	Sig.	Decision
1	The median of Mrp2 equals 2.000.	One-Sample Wilcoxon Signed Rank Test	.735	Retain the null hypothesis.
2	The median of Bsep equals 2.000.	One-Sample Wilcoxon Signed Rank Test	.470	Retain the null hypothesis.
3	The median of Oatp8 equals 2.000.	One-Sample Wilcoxon Signed Rank Test	.018	Reject the null hypothesis.
4	The median of Ntcp equals 2.000.	One-Sample Wilcoxon Signed Rank Test	.055	Retain the null hypothesis.
5	The median of FXR equals 2.000.	One-Sample Wilcoxon Signed Rank Test	.018	Reject the null hypothesis.
6	The median of Radixin equals 2.000.	One-Sample Wilcoxon Signed Rank Test	.018	Reject the null hypothesis.
7	The median of Mrp3 equals 2.000.	One-Sample Wilcoxon Signed Rank Test	.499	Retain the null hypothesis.

Asymptotic significances are displayed. The significance level is .05.

Table 5.3: Hypothesis summaries for Wilcoxon-Signed-Rank test

Western Blot: There was no significant difference in Mrp2 protein when comparing the Standard Al group to the High Al group (p=0.98). For the Standard Al group, the mean protein optical density was 0.63 (SD 0.34), as compared to the High Al group, where the mean was 0.62 (SD 0.30). (These values do not have units because they are density measurements generated by the ImageJ program, and normalized to their own reference band).

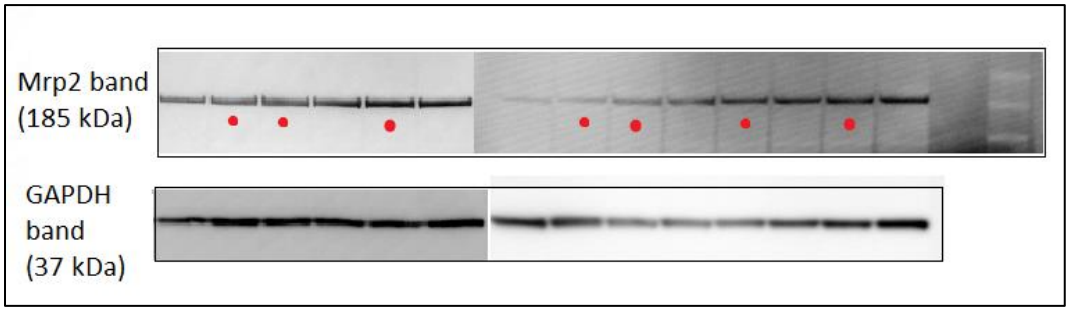
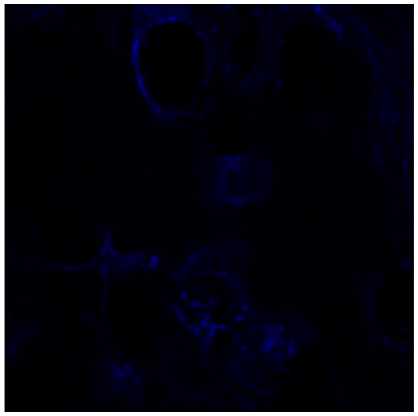
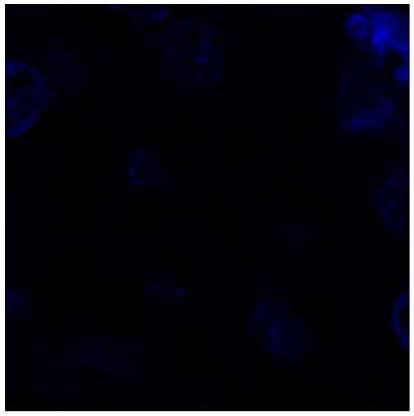


Figure 5.8: Western blots of Mrp2 and GAPDH (2 membranes used). Each sample is run next to its pair-matched control. Red dots indicate Standard Al group samples.

Immunofluorescence and Confocal Microscopy: Overall, the co-localization of both apical bile acid transporters Mrp2 and Bsep was maintained with radixin, regardless of Al exposure. In almost all slides, the antibodies for both Mrp2 or Bsep and radixin were on top of each other, and subjectively it appeared that the co-localization relationship was not disturbed. As we were unable to identify a cell membrane marker, it is impossible to determine if these bile acid transporters and radixin were drawn intracellularly, or if they both remained normally at the cell membrane.

The density of Mrp2 signal was significantly higher in the lower Al group (Standard Al), as compared to the High Al group ($p=0.009$), however the area and width of the region covered by the protein was the same between groups ($p=0.30$ and 0.84 respectively). When examining the radixin signal on these slides, the area covered by radixin in the High Al group was much greater ($p=0.003$) than the Standard Al group, but the density and width of signal did not have a significant difference between groups ($p=0.30$ and 0.15).

	High Al	Standard Al
Nuclear stain (DAPI)		

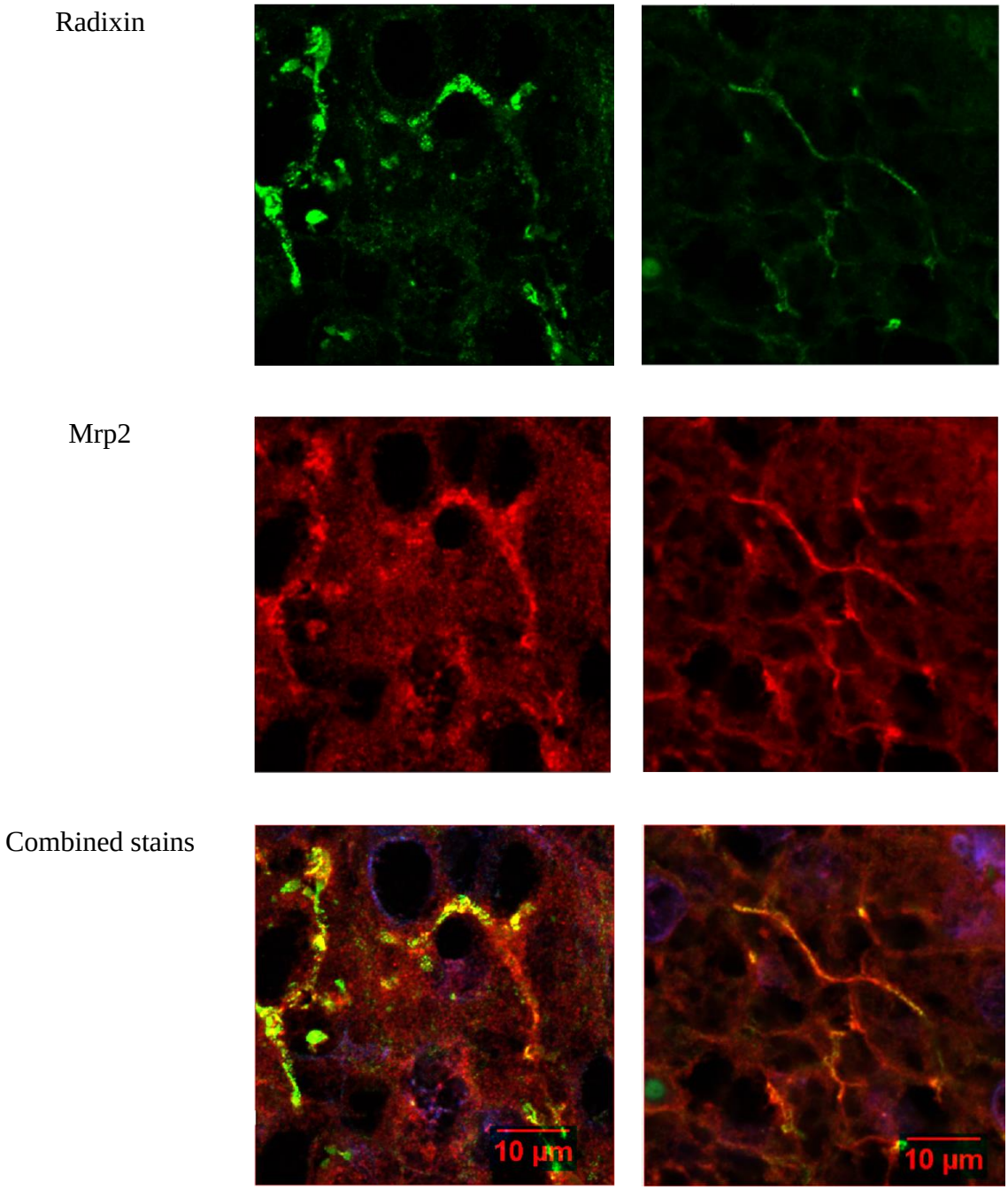
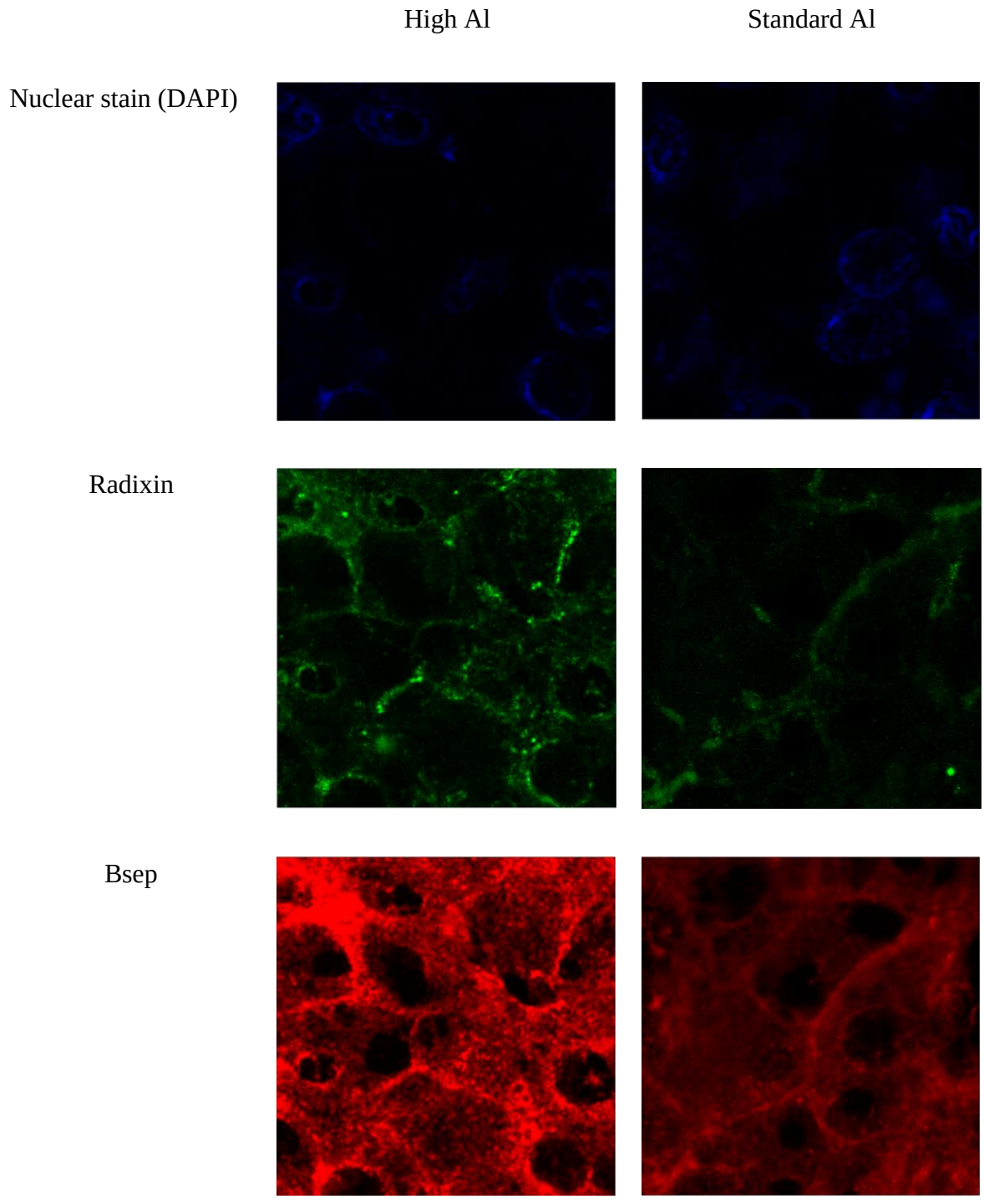


Figure 5.9: Comparison of immunohistochemistry images for Mrp2/radixin. Images demonstrate denser Mrp2 staining in Standard Al group and dispersed radixin staining in the High Al group. Both groups show tight overlap of radixin and Mrp2 signal.

The examination of Bsep did not display any significant difference between the groups in terms of area, density, or width of signal generated by the Bsep antibodies. The p values for area, density, and width were 0.54, 0.15, and 0.10 respectively. On these slides, the radixin antibody showed greater optical density (p=0.002) in the High Al group, as compared to the

Standard Al group but greater width (p=0.006) in the Standard Al group. There was no significant difference in area covered by radixin (p=0.32) for the two groups.



Combined stains

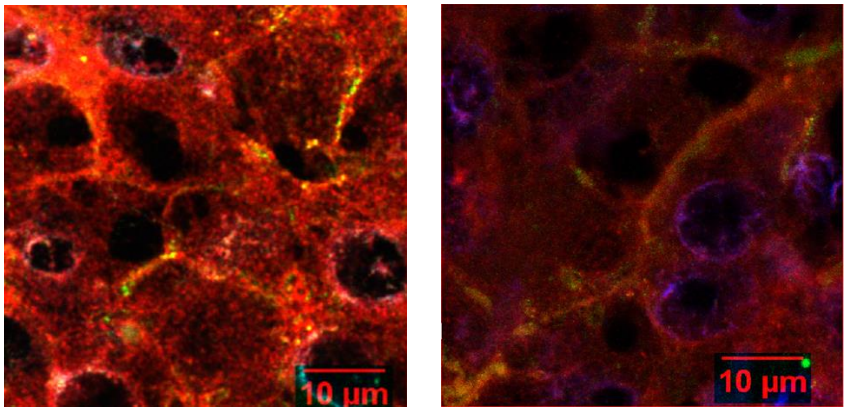


Figure 5.10: Comparison of immunohistochemistry images for Bsep/radixin. No significant difference between groups. Of note: both groups show tight overlap of radixin and Bsep staining.

Bile Acid Assay: The repeated measurement model of analysis revealed no significant difference in the accumulation of total serum bile acids between the two groups (p=0.073). This particular model of analysis was selected because it incorporates correlations within each animal. The Standard Al group total serum bile acid concentration increased by a mean of 3.10µmol/L above baseline, as compared to the High Al group which increased by a mean of 1.99µmol/L. Of note, on day zero before any intervention, the Standard Al group already had a higher mean serum bile acid level, and this trend continued throughout the 3 weeks. Birth weight did not have a significant effect on serum bile acids.

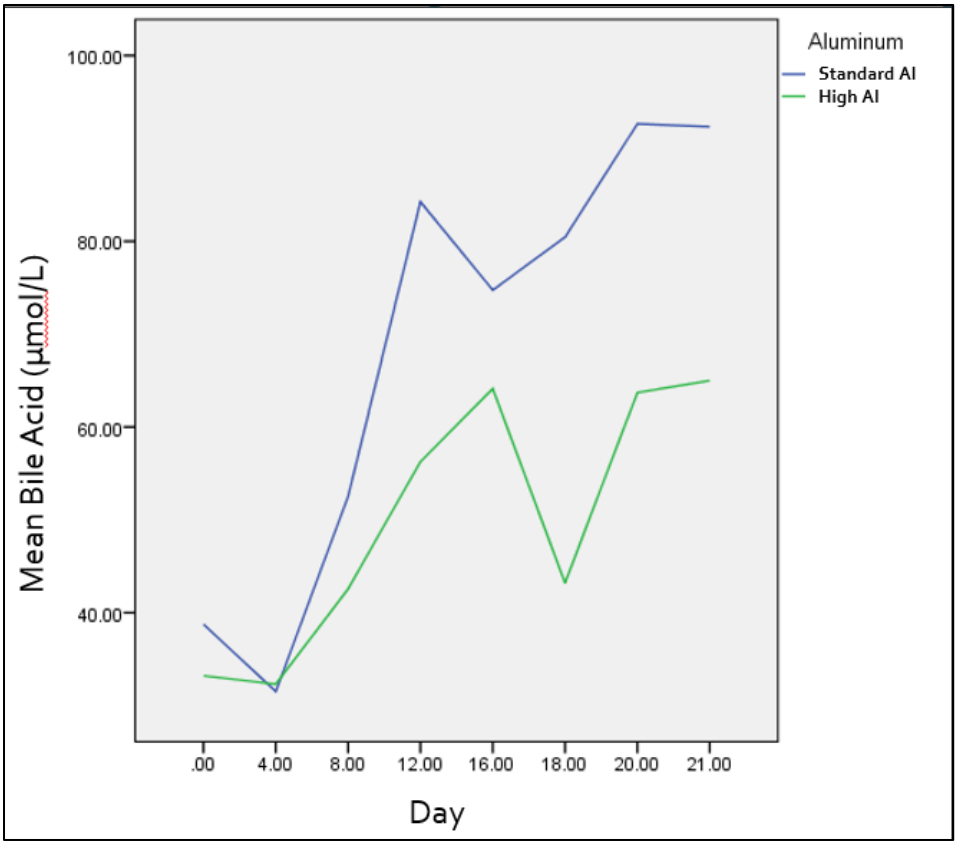


Figure 5.11: Mean serum bile acids. Standard vs High Al

5.6 Discussion:

The goal of this study was to determine if high levels of Al contamination in PN impaired expression and/or cellular positioning of bile acid transporters, thereby potentially contributing to the pathogenesis of PNALD. Further understanding of this complex disease and the involved factors is essential to design effective treatments.

qPCR demonstrated that the mRNA expression of four out of five transporters studied were negatively affected by Al; including Mrp2, Mrp3, Ntcp, and Bsep. However, these results are only statistically significant and may not be clinically relevant.

In our study, we observed statistically significant decreases in Mrp2 mRNA in the High Al group and although we did not find a corresponding difference in protein, this may still be an important finding. There are many possible explanations for the apparent disconnect between the mRNA and protein results. Firstly, transcriptional changes do not always correlate with translational changes and so a decrease in mRNA may not result in a significant change in

protein amount or functioning. The piglet Mrp2 response to inflammation and oxidative stress is relatively unstudied, and just as there are substantial differences in the response of rat Mrp2 versus human MRP2 to identical hepatic insults, it is also possible that the pig Mrp2 also does not downregulate as it does in mice and rats. [Elferink, et al., 2004] Alternatively, a similar pattern of decreased Mrp2 mRNA, but unaltered Mrp2 protein, was also found in a PNALD mouse model by Tazuke et al (2004). The authors theorized that the lack of Mrp2 protein change indicated that their one-week study period was too short. They stated that the down-regulation of Mrp2 mRNA was still an important finding and likely would translate into a decrease in Mrp2 protein in longer trials. [Tazuke, et al., 2004] Given that our model is also relatively short, this may be the explanation for our finding.

If we assume that the Al-induced decrease in Mrp2 mRNA is clinically significant and that it could be eventually translated into a protein deficit, then the mechanism linking Al and Mrp2 could be both inflammation and oxidative stress. Mrp2 has decreased expression in other types of cholestasis and the response appears to be linked to inflammation and oxidative stress. Both inflammatory cytokines and reactive oxygen species can indirectly activate, or inhibit, nuclear transcription factors resulting in decreased Mrp2 expression. [Schmitt, Kubitz, Wettstein, Vom, & Haussinger, 2000; Pascussi, et al., 2003; Gonzalez, et al., 2007] In other oxidative stress models, Mrp2 is internalized in intracellular vesicles, [Kojima, et al., 2003; Kojima, et al., 2008] and these vesicles are then often degraded by lysosomes. Since Al is known to exacerbate oxidative reactions, [Alexandrov, et al., 2005; Percy, et al., 2011; Mailloux, et al., 2011, Gonzalez, et al., 2007] it is possible that this type of intracellular degradation of Mrp2 could occur in our model. For example, in rat models Al exposure caused decreased expression of the Mrp2 protein. [Gonzalez, et al., 2004; Gonzalez, et al., 2007] The authors postulated that the Al-induced reactive oxygen species impaired synthesis of and increased degradation of Mrp2. [Gonzalez, et al., 2007] As we have not examined any markers of oxidative stress in this study, this is mostly conjecture and further work is needed to investigate the mechanisms of Al.

Similar to Mrp2, we observed a decrease in Bsep mRNA in the High Al group, but we do not know if there is a subsequent decrease in protein or if the fold difference is clinically important. If we assume that our observed fold difference is indicative of a significant impairment of Bsep, it is plausible that either Al-induced oxidative stress or inflammatory factors

could be involved. Other PNALD models have demonstrated a decrease in Bsep mRNA in response to similar factors, but we have not examined them in this study. [Li, et al., 2012; Geier, et al., 2005; Elferink, et al., 2004; Hartmann, et al., 2002] Bsep normally cycles between an intracellular pool and the cellular membrane depending on the need of the hepatocyte, and is stabilized by cytoskeletal proteins. [Arrese & Ananthanarayanan, 2004; Schmitt, et al., 2001; Tazuke, et al., 2004; Anwer, 2004] In situations of hyperosmolarity [Schmitt, et al., 2001] and oxidative stress, [Perez, et al., 2006] Bsep is preferentially moved intracellularly. This internalization is conducted by the interactions of protein kinase-C, high levels of calcium, and the integrity of actin cytoskeleton. [Perez, et al., 2006] Additionally, Bsep is controlled by the nuclear factor FXR (which also effects Mrp2), [Kullak-Ublick, et al., 2004; Jung, et al., 2002] and when cytokines such as IL-1β and TNF-α suppress FXR, Bsep is downregulated in response. [Geier, et al., 2005; Elferink, et al., 2004] Whether the internalization and down-regulation of Bsep is a compensatory response of the hepatocyte, or a sign of hepatocyte failure, is still unclear. [Vanwijngaerden, et al., 2011]

In the High Al group, Ntcp mRNA was also decreased and again we do not know the clinical significance or the resulting protein expression. Although we did not investigate inflammatory factors, it is possible that the decrease in mRNA is the first sign of an Al-induced inflammatory cascade negatively affecting Ntcp. [Percy, et al., 2011; Mailloux, et al., 2011; Gui & Wang, et al., 2011; Gonzalez, et al., 2007] Many inflammatory models have demonstrated that Ntcp is downregulated through a post-transcriptional response to cytokines. [Andrejko, Raj, Kim, Cereda, & Deutschman, 2008] The chain of events has been mapped from cytokines like IL-6 or TNF-α through a signal transducer, to the nuclear transcription factor HNF-1a or FXR, and finally to decreased expression of Ntcp. [Green, Beier, & Gollan, 1996; Vanwijngaerden, et al, 2011; Andrejko, et al., 2008; Geier, et al., 2005; Cherrington, et al., 2004] The down-regulation of Ntcp is hypothesized as a method both to clear bile acids out of the hepatocyte and to prevent further bile acid influx. [Zollner, et al., 2003; Zollner, et al., 2001]

The mRNA changes of Mrp3 were unexpected, because most models of PNALD and other types of cholestasis have found an up-regulation of Mrp3 as a compensatory mechanism for the loss of Mrp2. [Zelcer, et al., 2006; Kruh, Belinsky, Gallow, & Lee, 2007; Vanwijngaerden, et al., 2011; Cherrington, et al., 2004] Mrp3 transports bile salts back out of the

hepatocyte and into the portal blood stream, but in healthy subjects the bile flow is mostly forward towards the canaliculi and therefore Mrp3 is found in low amounts. [Kruh, et al., 2007; Jenniskens, Langouche, Vanwijngaerden, Mesotten, & Van den Berghe, 2016]

There are a few possible explanations for the down-regulation of Mrp3 mRNA. Firstly, there is a large standard deviation for the Mrp3 mRNA, so not all piglets had a down-regulation and the overall conclusion of Mrp3 may be skewed by the small group size. The up-regulation of Mrp3 may be also species or even strain dependent; a similar phenomenon is observed in mice, where some strains demonstrate up-regulation of Mrp3 and others remain unchanged when given the same stimuli. [Geier, et al., 2005] Previous PN models in preterm piglets have also found that Mrp3 was significantly decreased in PN exposure, regardless of lipid type.[Vlaardingerbroek, et al., 2014] Alternatively, a few studies have shown a down-regulation of Mrp3 in response to certain inflammatory cytokines. For example, interferon-gamma has been shown to downregulate Mrp3 in humans, while TNF-α caused a 60% decrease in Mrp3 mRNA in mice. [Le Vee, Jouan, Moreau, & Fardel, 2011; Hartmann, et al., 2002] Al triggers pro-inflammatory genes and a subsequent rise in cytokines such as TNF-α and interleukin-5, [Alexandrov, et al., 2005; Gonzalez, et al., 2004] and plausibly interferon-gamma. Therefore, Al could potentially downregulate Mrp3. Alternatively, the decrease in Mrp3 mRNA may be due to the normal Mrp3 ontogeny. Both rat and mouse pups are born with almost no expression of Mrp3 and this transporter increases gradually until an adult level is reached after weaning. [Zhu, et al., 2017; Maher, et al., 2005] If the pig has a similar delayed expression, then our observed Mrp3 decrease may be a normal variant of an already limited expression.

Immunohistochemistry suggested a moderate influence of Al on the placement of Mrp2. The staining of the Mrp2 protein was significantly denser in the Standard Al group despite covering the same area, and having the same width, as the High Al group. This may be an early indicator that some Mrp2 proteins are being dispersed intracellularly, even though the overall cellular Mrp2 protein amount is unchanged. Unlike other models of cholestasis however [Kojima, et al., 2003; Kojima, et al., 2008], the co-localization of radixin and Mrp2, as well as radixin and Bsep, appears to be maintained regardless of Al amount. This may again be explained by the early stage of PNALD created in this model. In other studies where Mrp2 and radixin were studied with immunohistochemistry, the co-localization relationship was not

disturbed until the patient was icteric. [Kojima, et al., 2008] Since we observed no difference in total serum bile acids, and there were no other clinical signs of liver failure, none of our subjects would be considered icteric. We were unable to assess whether or not all three of the targets were still on the cell membrane (due to the lack of a membrane marker), and so it is possible that the cellular placement of both radixin and the apical bile acid transporters are intracellular rather than on the membrane. Further studies with a cell membrane marker are required.

Approximately one quarter of infants develop elevated serum bile acids following three weeks of PN. [Arnold, 2004] The piglet model acts as an abbreviated representative of infant development, where one week of piglet development is similar to one month of human infant development. [Book & Bustad, 1974] We had therefore hoped that a three-week study period in the piglet would create liver disease similar to that seen in one to two months of PN exposure in human infants. Despite these expectations, we did not see a significant rise or difference between the groups for total serum bile acids. Likewise, the previous studies of PNALD using the piglet PN model for two weeks also failed to demonstrate a significant difference in serum bile acids. [Alemmari, et al., 2011 and 2012] Our three-week piglet model likely creates an early stage of PNALD, given that the serum bile acids have not increased and serum bile acids are one of the earliest clinical markers of PNALD. [Benjamin, 1981; Touloukin & Seashore, 1975] It is plausible that hepatobiliary damage takes the same amount of time to develop in pigs as in humans, regardless of the overall accelerated growth of the piglet model. The only animal models of PNALD that have demonstrated increased serum bile acids with Al exposure in a few weeks were completed in adult rats [Gonzalez, et al., 2004; Klein, et al., 1988] who have fully developed organ systems, and likely different physiologic responses to Al. Meanwhile, the only piglet model of PN to develop elevated bilirubin and other markers of cholestasis, used a pre-term piglet model.[Vlaardingerbroek, et al., 2014] Perhaps our newborn piglets do not have the degree of immature hepatobiliary system required to develop PNALD quickly. Alternatively, Al alone may not be sufficient to trigger PNALD and perhaps a more complex model is required.

An important factor in this study is the use of Intralipid, an omega-6 based lipid emulsion for all of the subjects. Omega-6 lipids are pro-inflammatory, because they lead to the development of platelet aggregating thromboxanes and immunosuppressing prostaglandins. [Gura, et al., 2008; Cober & Teitelbaum, 2010; Cowan, et al., 2013] They are also likely

involved in pro-oxidant activities because reactive oxygen-species are byproducts of omega-6 metabolism. [Betteridge, 2000] The presence of this pro-inflammatory and pro-oxidant lipid emulsion may mask, or magnify, the effects of Al in our study. Hypothetically, some of the effects attributed to the inflammatory nature of Al may actually be caused by Intralipid.

The weaknesses of this study involve the confounding factor of sepsis and difficulties with antibodies. Firstly, the three-week long study period placed the piglets at increased risk of sepsis. We had an unexpectedly high attrition rate of 6/20 piglets, with the majority of the animals becoming sick in the last week of the study. We tried many strategies to combat sepsis and rule out other potential explanations for the symptoms. When the piglets first displayed symptoms, we decreased the PN rate from 12mL/kg/hour to 10.8 mL/kg/hour in the third week of PN to reduce the nitrogen load. In the critical care setting, liver failure patients accumulate nutrition by-products such as ammonia following protein administration. We theorized that if liver failure was developing in our piglets, they may also be having difficulty clearing ammonia, [Clay & Hainline, 2007] which could present as lethargy and difficulty breathing in pigs (encephalopathy). This variation in rate should not have affected our outcomes because it was applied evenly to both groups. Unfortunately, this strategy also did not affect the incidence of symptomatic piglets. We then concluded that the symptoms were attributable to sepsis and we adopted a quicker response to initial signs of infection, where if a piglet did not respond to antibiotics in 24 hours, it was euthanized. This policy led to a slight variation in study period for our animals, with two piglets euthanized on day 18 instead of 21 (one High Al and one Standard Al) and one piglet only reaching day 17 (High Al). We felt that the variation in time period was more acceptable than the confounding factor of sepsis. The use of prophylactic antibiotics may also have been a confounder because of the negative effects on normal bowel flora and corresponding immune response. However, we felt that the need to control sepsis was more important in a small study like this, and other piglet studies have followed a similar practice. [Vlaardingerbroek, et al., 2014]

Secondly, pig antibodies were difficult to obtain and even those that were custom made were not perfect. This inhibited our ability to properly assess the cellular localization of apical transporters in our immunohistochemistry experiments (due to a lack of ZO-1 antibody), and

precluded our ability to complete Western blot for any of the targets except Mrp2. Further development of porcine antibodies is needed to continue working with the piglet PN model.

5.7 Conclusion:

In conclusion, we have demonstrated that Al in PN has a statistically significant negative effect on the mRNA of bile acid transporters. High Al caused a decrease in mRNA of both apical transporters Mrp2 and Bsep, and the basolateral transporter Ntcp. Although the Mrp2 protein amount was unchanged between the groups, it did have decreased density in the High Al group, indicating that Al may be interfering with Mrp2 cellular localization. Although we do not know if the mRNA changes are clinically significant, in a longer study, they could tanslate to a deficiency in bile acid transporter proteins, causing cholestasis. Further investigations into the interactions of Al and these bile acid transporters are required, especially in other models of PNALD.

6: EXAMINING THE EFFECTS OF ALUMINUM ON BILE ACID TRANSPORTERS USING A PIGLET PARENTERAL NUTRITION MODEL WITH MIXED LIPIDS

6.1 Abstract:

Background/Purpose: Prolonged parenteral nutrition (PN) use in neonates increases the risk of PN associated liver disease (PNALD). The pathophysiology of PNALD is not fully understood, but it is multi-factorial, with both pro-inflammatory lipids and contaminants such as aluminum (Al) contributing. The objective of this study was to assess the impact of Al on bile acid transporters when a less inflammatory lipid emulsion is used.

Materials and Methods: A 2 week randomized control trial was conducted using the Yucatan miniature piglet PN model. Piglets aged 3-6 days were randomly placed into one of three groups. The high aluminum (High Al) group (N=8) received PN with mixed lipids and 63μg/kg/day of Al, while the baseline aluminum (Standard Al) group (N=7) received PN with mixed lipids and only 24μg/kg/day of Al. The negative control (Reference) group (N=4) was sow-fed, with no PN exposure. Serum and liver samples were collected for analysis. We chose five bile acid transporters (Mrp2, Bsep, Ntcp, Mrp3, and Oatp8), a cytoskeletal protein (radixin), and a nuclear receptor (FXR) as targets important in bile flow. The serum was analyzed for inflammatory markers, while the liver samples were examined using qPCR, immunofluorescence confocal microscopy, and Western blotting.

Results: There was a significantly greater rise in the inflammatory marker C-reactive protein (CRP) in the High Al group as compared to the Standard Al group ($p=0.03$). qPCR revealed a statistically significant decrease in Oatp8, Ntcp, and Mrp3 in favour of the lower Al (Standard Al) group ($p<0.05$). Western blot showed no difference in Mrp2 protein amounts between the High and Standard Al groups and similarly, there was very little difference in the placement of Bsep and Mrp2 in the immunohistochemistry studies.

Conclusions: High Al still caused an increased inflammatory response and decreased amount of mRNA for some of the bile acid transporters, despite the presence of a less-inflammatory lipid solution.

Clinical Relevancy Statement:

PNALD is a multi-factorial disease which has shown promising response to a less-inflammatory mixed lipid solution. However, Al in PN may still increase inflammation and have a negative effect on the mRNA of bile acid transporters, even with this mixed lipid solution. If these Al-induced mRNA changes translate into protein deficits, then Al may be an important factor in PNALD and efforts to reduce Al contamination in infant PN are warranted.

6.2 Introduction:

PNALD is a potentially life-threatening complication of a nutritional intervention used almost universally in neonatal units. Despite recognition of this disease for over 40 years, [Peden, Witzleben, & Skelton, 1971] it remains under investigation and no universal prevention or cure exists. [Beath, et al., 1996; Guglielmi, et al., 2008] Considerable advances have been made in the management of PNALD, and the most successful programs are multi-faceted, including sepsis control, cycling PN, [Cowles, et al., 2010] the use of ursodeoxycolic acid [Simic, et al., 2014] and most recently the implementation of less-inflammatory lipids that favour omega-3 over omega-6 fatty acids. [Le, et al., 2009; Fallon, et al., 2010] Two proposed factors in the pathophysiology of PNALD are pro-inflammatory lipids and Al contamination.

Traditionally soybean oils, which supply mostly omega-6 fatty acids, were the only lipids used in neonatal solutions. For example, Intralipid 20% is comprised of 20% soybean oil, 1.2% egg yolk phospholipids, 2.25% glycerin combined in water. [Fresenius Kabi, "Intralipid", 2017] But omega-6 lipids have pro-inflammatory effects [Gura, et al., 2008; Cober & Teitelbaum, 2010; Cowan, et al., 2013] which make the liver vulnerable to cholestasis. Multiple new lipid formulations with smaller amounts of omega-6 lipids, in exchange for higher amounts of omega-3 lipids have been developed and may have a 'hepato-protective' effect. [Park, et al., 2011; Cober & Teitelbaum, 2010; Cowan, et al., 2013; Pichler, et al., 2014] Pure omega-3 lipid solutions, created with highly purified fish oils [Fresenius Kabi, "Omegaven", 2017] have been trialed with positive results in PNALD, although concern about essential fatty acid deficiency exists. [Le, et al., 2009; Fallon, et al., 2010; Gramlich, et al., 2015] One of the most promising solutions is SMOFlipid, (Fresenius Kabi, Bad Homburg, Germany) which is a mix of 30% soybean oil (omega-6), 30% medium chain triglycerides (rapid energy source), 25% olive oil (omega-9), 15% fish oil (omega-3) and supplemented α-tocopherol (200mg/L). [Fresenius Kabi, "SMOFlipid", 2017] Initial studies have shown that this mix not only avoids essential fatty acid

deficiency but also leads to decreased liver enzymes, bilirubin and serum inflammatory markers. [Goulet, et al., 2010; Pichler, et al., 2014; Muhammed, et al., 2012] However, SMOFlipid is not approved for use in infants in many countries and is still under investigation. [American Society of Parenteral and Enteral Nutrition, 2014] In addition, the new omega-3 based formulas do not completely prevent PNALD, and a small percentage of patients in each study still progress to cholestasis. [Park, et al., 2011; Premkumar, et al., 2013; Sant'anna, et al., 2012; Nandivada, et al., 2016]

Al is a persistent contaminant of infant PN, found in almost all components of PN, but in the highest concentrations in calcium gluconate. [Poole, et al., 2010; Wier & Kuhn, 2012] Our recent study found that in a Canadian neonatal intensive care unit, infants received on average three-times the safe limit advised by the Food and Drug Administration (FDA) (<5µg/kg/day). [FDA, 2004] This Al contamination has been linked to bone disorders and adverse neurologic outcomes in infants [Gura, 2010; Courtney-Martin, et al., 2015] and it may play a role in PNALD. [Alemmari, et al., 2012; Arnold, et al., 2003, Klein, et al., 1984] In the previous chapter, Al had a negative effect on the mRNA of important bile acid transporters, such as Mrp2 and Bsep, which may increase the risk of biliary stasis. Similar results have been obtained by others in rat studies. [Gonzalez, et al., 2004] The majority of the hepato-toxic effects of Al are believed to be secondary to the oxidative stress caused by this cation, as it forms a superoxide radical ion and partners with iron to participate in oxidative reactions. [Alexandrov, et al., 2005; Percy, et al., 2011; Gonzalez, et al., 2007] Al also has a less pronounced pro-inflammatory effect. [Alexandrov, et al., 2005; Guo & Wang, 2011] If Al still causes bile acid transporter impairment despite the presence of SMOFlipid, then a multi-faceted treatment must be developed, to address both pro-inflammatory lipids and Al contamination.

6.3 Objectives:

We hypothesize that both Al contamination and pro-inflammatory lipids in PN cause down-regulation and impaired cellular localization of bile acid transporter proteins. Our objectives are to use the Yucatan miniature pig PN model to build upon our findings of the previous piglet PN model. We will observe the effects of High vs Standard Al in a less-inflammatory, mixed lipid PN solution. If our hypothesis is correct, Al will still negatively affect bile acid transporters, despite the protective effects of the mixed lipids. Changes to bile acid

transporters will be determined by immunohistochemistry, polymerase chain reaction and Western blotting techniques.

6.4 Methods:

Animal Work: All animal work and protocols were reviewed and approved by both the University of Saskatchewan Animal Research Ethics Board and the Institutional Animal Care Committee at Memorial University of Newfoundland. Live animal work was conducted at the Memorial University of Newfoundland vivarium. Yucatan miniature piglets, aged 3-6 days, were placed randomly into one of three groups. If at all possible, the piglets were pair-matched with a littermate of similar size and gender, to ensure comparable group demographics. The High Al group (N=8) received PN with 63µg/kg/day of Al, while the Standard Al group (N=7) received otherwise identical PN with 24µg/kg/day of Al. The amount of Al in the latter group is within the range of contamination found in Canadian neonatal PN. [Hall, Arnold, Miller, & Zello, 2016] See Table 6.1 for an explanation of Al content in our PN. As compared to the previous piglet PN study, the important difference for both of these groups is that the omega-6 based Intralipid was replaced with a mixed lipid solution (SMOFlipid, Fresenius Kabi, Bad Homburg, Germany) for all piglets. The Reference group (N=4) consisted of piglets that were sow-fed for the duration of the study. No interventions were performed on this last group of piglets until the termination of the study.

PN Component*	Al content (unit varies)	Al content¶ (µg/kg/day)
Calcium gluconate ($C1_2H_{22}CaO_{14}$)	2.50 µg/g	3.83
Monopotassium phosphate (KH_2PO_4)	<0.50 µg/g	0.01
Dipotassium hydrogen phosphate (K_2HPO_4)	1.00 µg/g	0.38
Potassium acetate ($C_2H_3KO_2$)	0.80 µg/g	0.28
Glucose ($C_6H_{12}O_6$)	0.90 µg/g	19.46

Vitamins Vial 1: vitamins E, D, A, K, B2, C, B6, and B3. Made in lab.	0.014 mg/L	0.02
Vitamins Vial 2: folic acid, vitamins B7 and B12. Made in lab.	<0.005 mg/L	0.00
Trace elements: zinc, copper, manganese, chromium, selenium and iodine. Made in lab.	0.054 mg/L	0.05
	Standard Al group total= Approx 24 µg/kg/day	
Aluminum ($AlCl_3 \cdot H_20$) Added only to High Al group	111.75 µg/g	38.00
	High Al group total=Approx. 63 µg/kg/day	

Table 6.1: Al contamination of piglet PN components

*All PN components except for those made in lab are products from Sigma-Aldrich, Oakville, ON, Canada. ¶Calculated by multiplying the amount of component added per bag of PN x rate x hours. Ex: Calcium gluconate = 2.5µg/g x 6.41g/L= 16.03µg/L of Al (from calcium gluconate) in dextrose/amino acid solution. 16.03µg/L x 0.75L (amount of dextrose/amino acid solution added per PN bag)=12.02µg per bag of PN. 12.02µg / 902 mL (total volume in bag)= 0.013µg/mL x 12mL/kg/hr x 24 hours/day= 3.83µg/kg/day.

To confirm the Al content of our final PN solutions, random samples of PN were collected and analyzed for Al content via inductively coupled plasma optical emission spectrometry (ICP-OES; Model iCAP 6500 Duo Analyzer; Thermo Scientific, Waltham, MA). Three samples of the Standard Al PN were analyzed and revealed a mean Al content of 23.8 +/- 4.3µg/kg/day, while four samples of the High Al PN had a mean Al content of 62.8 +/- 9.3µg/kg/day.

For piglets in the High Al and Standard Al groups, their surgeries and post-operative care were performed in an identical fashion as described in the previous chapter. Please refer to chapter 5 for details.

The piglets in the High Al and Standard Al groups were kept on a strict PN regimen for the 14 days of the study. For both groups, the lipids were provided in a mixed lipid solution (SMOFlipid, (Fresenius Kabi, Bad Homburg, Germany)) at a rate of 1.9mL/hour (9gm/kg/day).

This is the same amount of lipid as was provided in the previous piglet PN study. The other PN components, including the Al contamination, again mirrored that provided in the previous study. In this project, the High Al group still received approximately 2.5 times the amount of Al contamination as the Standard Al group. In total, the piglets received 0.98 MJ/kg/d and 13.3g amino acids/kg/day with non-protein energy supplied approximately 50:50 carbohydrate and fat. The amino acid-dextrose solution was infused at a rate of 10.1 mL/kg/h (242.4 mL/kg/d). Every second day, the piglets were weighed and the PN rate was adjusted. For complete PN recipes, refer to Appendix C.

Serum samples were collected every four days. At the conclusion of the study, the piglets from all three groups were anaesthetized and the liver removed, followed by euthanasia with an intra-cardiac injection of a lethal dose of pentobarbital sodium (Euthanyl, BiMeda-MTC, Cambridge, ON, Canada). The liver was weighed and $1cm^3$ pieces were snap-frozen in liquid nitrogen.

C-Reactive Protein ELISA Assay: A porcine C-reactive protein (CRP) kit (ALPCO, Salem, NH, USA) was employed for a two enzyme-linked immunoassay (ELISA). Serum samples were aliquoted into 96-well plates with an anti-CRP antibody. The sample was washed, leaving only CRP protein bound to the antibody. A new anti-CRP primary antibody was then introduced to the pre-existing antibody-protein complex. This new primary antibody was conjugated to horseradish peroxidase, which allowed a coloured probe to be tagged to it in the next step. Finally, the coloured probe was quantified in a spectrometer at 450nm; the amount of colour produced directly correlated to the initial amount of CRP protein in the sample. All serum samples were run in duplicate and any pair of samples with a variance greater than 10% was discarded (this applied to only one sample).

Quantitative Polymerase Chain Reaction (qPCR): qPCR was used to evaluate the amount of mRNA transcribed for the bile acid transporters Bsep, Mrp2, Mrp3, Ntcp, Oatp8, the cytoskeletal protein radixin, and the nuclear transcription factor FXR. Firstly, mRNA was extracted from the frozen liver samples using a lysing agent (Trizol, Thermo Fisher Scientific, Waltham, MA, USA), mechanical disruption, and repeated filtrations. The RNeasy Mini Kit (Qiagen, Hilden, Germany) was used for this step. The resulting purified mRNA was quantified using a NanoVue spectrophotometer (GE, Boston, MA, USA). An A260/A280 ratio of 1.8 to 2.0

was used as the limit of acceptable mRNA purity. It was then normalized with nuclease free, sterile water to ensure identical amounts of RNA in each sample. Following the normalization step, the VILO Superscript cDNA Synthesis kit (Thermo Fisher Scientific, Waltham, MA, USA) was used to create cDNA from the mRNA template. For this process, diluted mRNA was combined with the VILO reaction and enzyme mix and subjected to heating in a thermocycler. It was incubated at 25ºC for ten minutes, followed by heating at 42ºC for one hour, and the reaction was terminated with 5 minutes of exposure to 85ºC heat. The resulting cDNA was diluted 20 fold and kept on ice. Diluted cDNA was then added to nucleotides and a fluorescent probe (Power Mastermix, Thermo Fisher Scientific, Waltham, MA, USA) along with both forward and reverse primers. See primer list below. This solution was pipetted in 14.5µL replicates (each containing 2µL of diluted cDNA) into a 96 well qPCR plate, sealed and placed in a Real Time cycler (7300 Real Time PCR Systems, Applied Bio Systems, Foster City, CA, USA). The cycler followed a protocol of 30 minutes of incubation at 95ºC, followed by repeated cycling from 95ºC to 60ºC to induce annealing, and synthesis. A dissociation curve was added to the end of each reaction to assess for unwanted primer-dimers and other artifacts. The Ct value was calculated based on the number of cycles required for adequate detection of the probe, allowing for extrapolation of the original amount of mRNA. Primers were designed with and obtained from Integrated DNA Technologies (Coraville, IA, USA). All primers were tested for efficiency before their use and all primer pairs demonstrated efficiency between 90-110%. [Taylor, et al., 2015] Each sample was run in triplicate.

Target	Forward primer 5'-3'	Reverse primer 5'-3'
Mrp2	CAC AGA GAA GAT CGG TGT AGT G	ACA CAA AGG ACT TGA GGT GAG
Bsep	GAT ATC TGA GTT CCG GAT GGT G	TGC CTC AAC CTT TCC ATC TT
Ntcp	CCC TAT GGC AGC ATT GTG ATA	AAG CCG GTG AAA GGC ATA A
Oatp8	CCA CAT GCA ACC CTT GAT AGA	ATC ATG CTG TGT GAC TGG TAT AA
Mrp3	CCC TGC ACA GCC TAG ATA TTC	GGC TAC AGA GCC CTT CAT ATA C

Radixin	CAG GCT ACC TGG CTA ATG ATA G	CTT CCC TCA GCA TTC CTC TAT G
FXR	GCC TGC CAA AGG TGT ACT AA	GGT AGA AAC CCA GGT TGG AAT AA
Cyclophilin A	CAA GAC TGA GTG GTT GGA TGG	GCT CCA TGG CTT CCA CAA TA
HPRT1	GGT CAA GCA GCA TAA TCC AAA G	GGC ATA GCC TAC CAC AAA CT

Table 6.2: Primers for qPCR

Hypoxanthine-guanine phosphoribosyltransferase 1 (HPRT1), a key enzyme in the synthesis of purines, and cyclophilin A, an enzyme important in intracellular signalling and apoptosis, were used as reference genes. These two reference genes were selected based on their low degree of variability across the different samples. Using the geometric mean of these two reference genes, the qPCR results for each pair of piglets were comparing with the Pfaffl method to obtain relative quantitation (explained below in statistical analysis).

Western Blot: Western blotting was used to evaluate the amount of Mrp2 protein in the liver samples. The Western blot protocol described for the previous piglet PN study was followed exactly as previously described. Please refer to Chapter 5 for details.

Immunofluorescence and Confocal Microscopy: Immunofluorescence was used to characterize the position of the apical bile acid transporters Mrp2 and Bsep. They were also individually co-localized with radixin to explore the relationship between this cytoskeletal protein and the two bile acid transporters. The protocol used was identical to the one as described for the previous piglet PN study. Please refer to Chapter 5 for details.

Statistical Analysis

All statistical analysis was carried out using SPSS version 22 (IBM, Armonk, NY) and SAS (SAS Institute, Cary, NC). Basic demographics (ie: weight) was compared using one-way ANOVA and appropriate post-hoc tests. The CRP ELISA results were examined using a repeated measurement model for longitudinal analysis, paired with non-parametric tests for study of data on separate days (ie: all pigs on day 4). In all of these comparisons, a p value <0.05 was considered statistically significant.

For the qPCR comparison of High vs Standard Al groups, the fold difference between each pair of samples was calculated using the Pfaffl equation.[Pfaffl, 2001] (See equation 5.1). Due to the small sample size, (seven or eight animals in each group), the data was not equally distributed. To accurately account for this, a non-parametric Wilcoxon-Signed-Rank test was performed for each bile acid transporter. This non-parametric test was able to determine if the median fold difference was equal to, or greater than, two (a standard cut-off for significant fold difference in qPCR). [University of Montreal, "Information on qPCR results", n.d.] A p-value of <0.05 was used as statistically significant for the Wilcoxon-Signed-Rank test. Given that the data did not have standard distribution, the median was a more reliable value than the mean.

The Ct values for the four Reference group piglets were then merged into a geometric mean and compared to each of the individual Ct values for the piglets in the other two PN groups (again, using the Pfaffl equation). The median and mean fold differences and standard deviation were then calculated for each bile acid transporter: for the High vs Standard Al, for the High Al vs Reference and lastly for the Standard Al vs Reference. The Wilcoxon-Signed-Rank test was repeated for these analyses as well, to determine if the median fold difference was statistically significant (fold difference of ≥ 2, or <0.5, with a p-value of <0.05). [University of Montreal, "Information on qPCR results", n.d.]

The immunohistochemistry results were compared between the three groups using ANOVA and paired T-tests, (comparing High vs Standard Al, followed by High Al vs Normal, and lastly Standard Al vs Normal). A similar process was used to analyze the densitometry measurements from the Western blots for Mrp2. In all of these comparisons, a p value <0.05 was considered statistically significant.

6.5 Results:

Basic Demographics: A repeated measurement model demonstrated that there was significant weight gain every day for each of the three groups. Overall, both of the PN groups gained more weight than the sow-fed group but there was no difference in weight gain between the High and Standard Al groups ($p=0.51$). Gender was also not significant in weight gain ($p=0.15$).

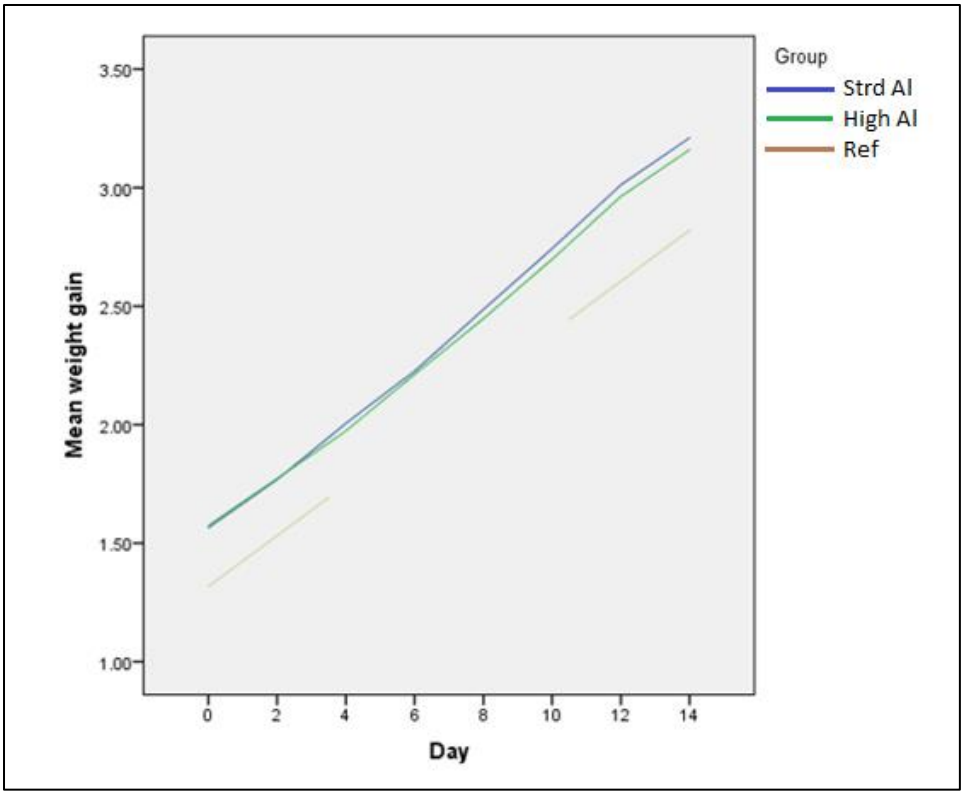


Figure 6.1: Mean weight gain by piglet group

One-way ANOVA demonstrated that there was no significant difference between the three groups for initial piglet weight but there was a difference in final liver weight (p=0.002 respectively). Pairwise analysis using the Bonferroni method demonstrated that the livers for both of the PN groups were on average significantly larger than those of the Reference group. (For High Al vs Reference p=0.006, while for Standard Al vs Reference, p=0.003).

C-reactive Protein Assay: The repeated measurement model showed that CRP in the High Al group increased significantly more over the two weeks, as compared to the Standard Al group (p=0.03). When comparing each day in isolation, there was no difference in serum CRP between the High and Standard Al groups. Similarly, there was no difference between the three groups on day 14 (the only day when the serum CRP of the reference group was measured).

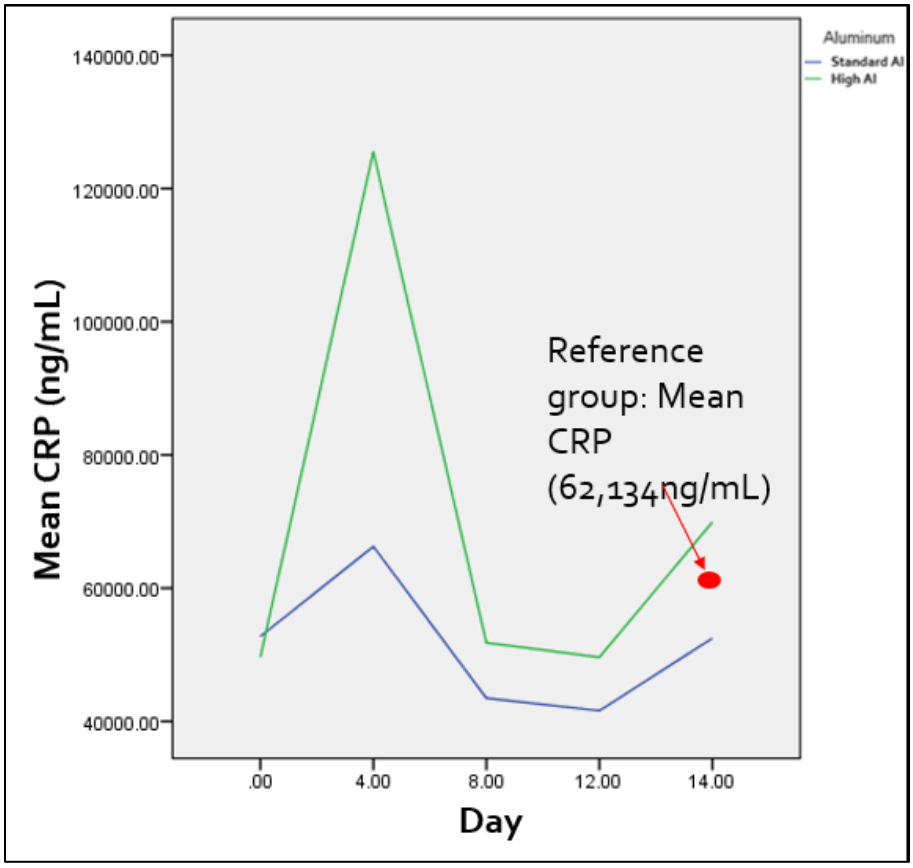


Figure 6.2: Mean serum CRP: High vs Standard Al

RT-qPCR: There was no significant fold difference between the Standard and High Al groups for Mrp2 or Bsep and likewise no significant difference for radixin or FXR. However, all of the baso-lateral bile acid transporters (Oatp8, Ntcp, and Mrp3) displayed a statistically significant fold difference, where there more mRNA in the lower Al (Standard Al) group. Oatp8 had a mean fold difference of 1.2 (SD 1.2), while Ntcp showed a mean difference of 1.6 (SD 0.9). Lastly Mrp3 had a mean fold difference of 1.3 (SD 0.7). When these three baso-lateral transporters were analyzed using the Wilcoxon-Sign-Rank test, the median fold difference was at least equal to 2.0, with a p-value of <0.05. (The Null Hypothesis for the Wilcoxon-Sign-Rank test was that 'the Median fold difference equals 2.0. For Oatp8, Ntcp, and Mrp3, the p values were 0.16, 0.21 and 0.21. As these are all greater than 0.05, the Null Hypothesis for the Wilcoxon-Sign-Rank test must be accepted). This correlates with a statistically significant difference, but may not imply a clinically relevant difference. See Figure 6.3 below.

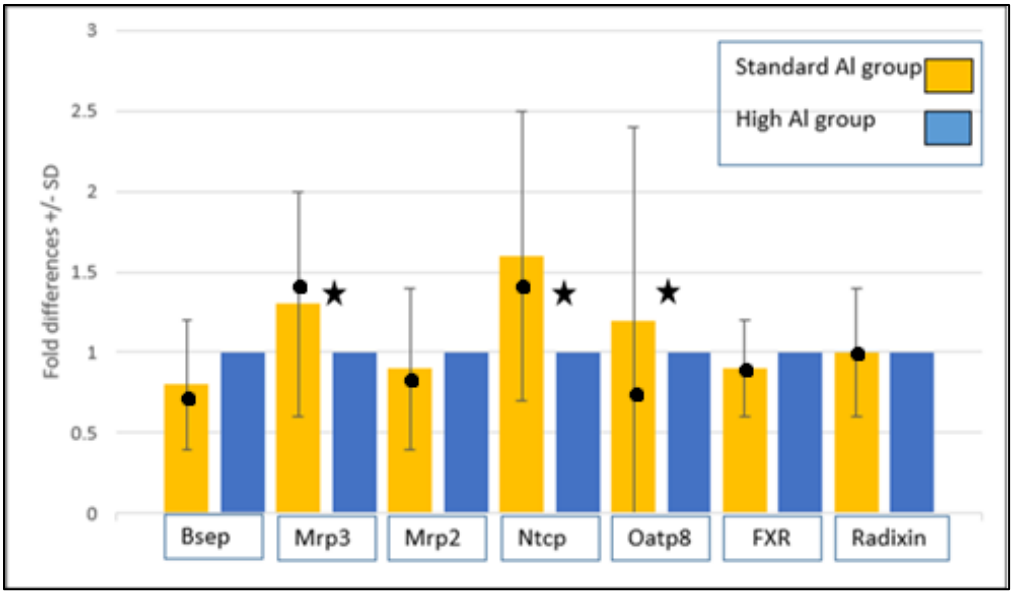


Figure 6.3: Fold differences for qPCR: High vs Standard Al. Mean and median. High Al group arbitrarily set at '1' to demonstrate difference (not an actual value). Bar height at mean fold difference. Black dot set at median value. Star icon indicates statistically significant difference between groups as determined by Wilcoxon-Signed-Rank test.

When the High Al group was compared to the Reference group, most of the targets had more mRNA in the High Al group. This included Mrp2 and Bsep with mean fold differences of 1.9 (SD 0.6) and 2.3 (SD 0.9) along with radixin and Mrp3 (mean fold differences of 1.6 (SD 0.6) and 2.0 (SD 0.9) respectively). The Wilcoxon-Rank-Test correlated with these conclusions and demonstrated a significant difference in the median fold values as well. There was no difference between the two groups when examining Oatp8, Ntcp or FXR. See Figure 6.4.

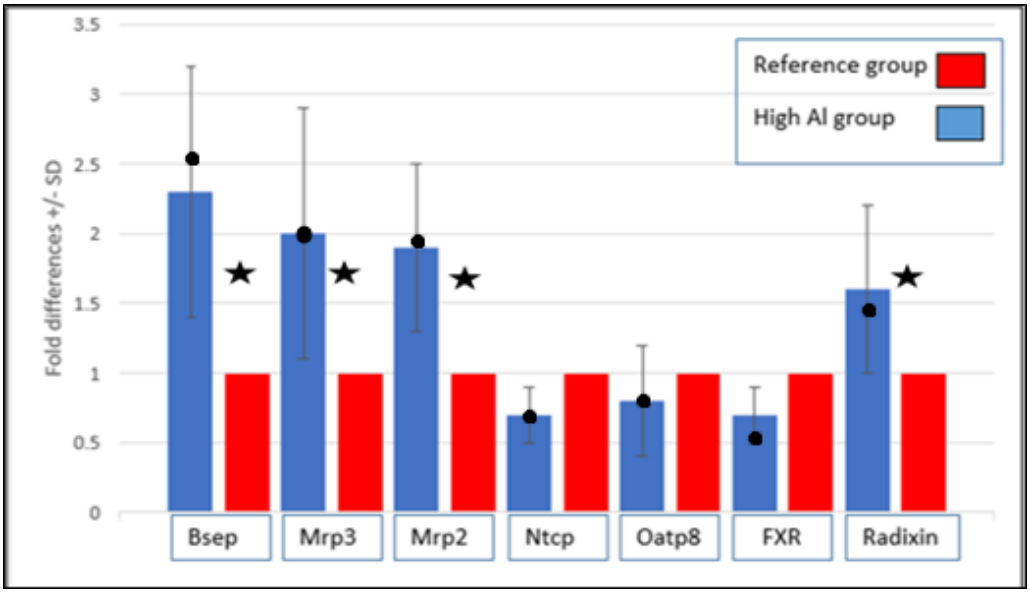


Figure 6.4: Fold differences for qPCR: High Al vs Reference. Mean and median.
Reference group arbitrarily set at '1' to demonstrate difference (not an actual value). Bar height set at mean fold difference. Black dot set at median value. Stars indicate statistically significant difference between groups.

Lastly, the Standard Al and Reference group were compared and again Bsep and Mrp3 had greater amounts of mRNA in the Standard Al group (mean fold differences of 1.6, SD 0.6 and 2.7, SD 1.1 respectively). Ntcp, with a mean fold difference of 1.2 (SD 0.9), also had a significant difference when the variation was accounted for with the Wilcoxon-Signed-Rank test. The remaining targets were not significantly different. See Figure 6.5.

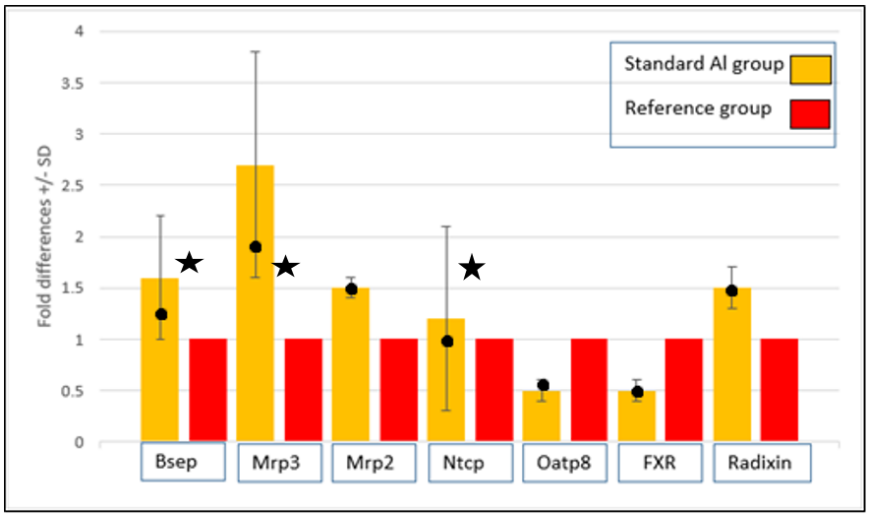


Figure 6.5: Fold differences for qPCR: Standard Al vs Reference. Mean and median.
Reference group arbitrarily set at '1' to demonstrate difference (not actual value). Bar height set at mean value. Black dot at median value. Star indicates statistically significant fold difference between groups.

Western Blot: Similar to the results from the previous piglet PN study, there was no difference among the three groups when compared using ANOVA (p=0.60). The paired T-tests confirmed this lack of difference, for example, when comparing the Standard Al group to the High Al group (p=0.65). For the Standard Al group, the mean protein optical density was 1.12 (SD 0.88), while the High Al group had a mean of 1.45 (SD 1.68). (These are unit-less values because they are generated by the ImageJ program with no outside reference or scale).

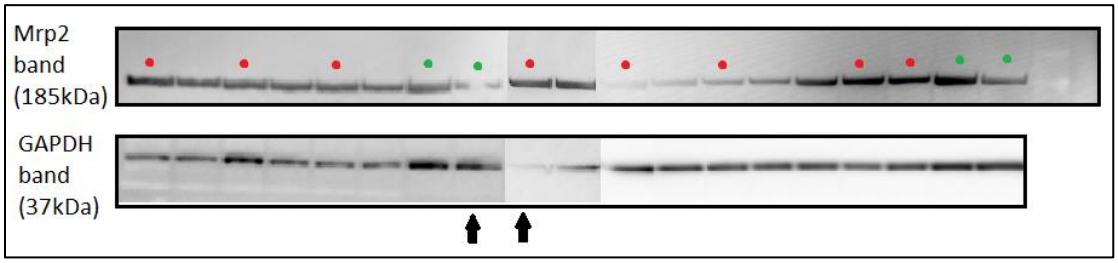


Figure 6.6: Western blots for Mrp2. Red dots indicate High Al group, while green dots indicate Reference group. Two lanes failed protein transfer, indicated by arrows.

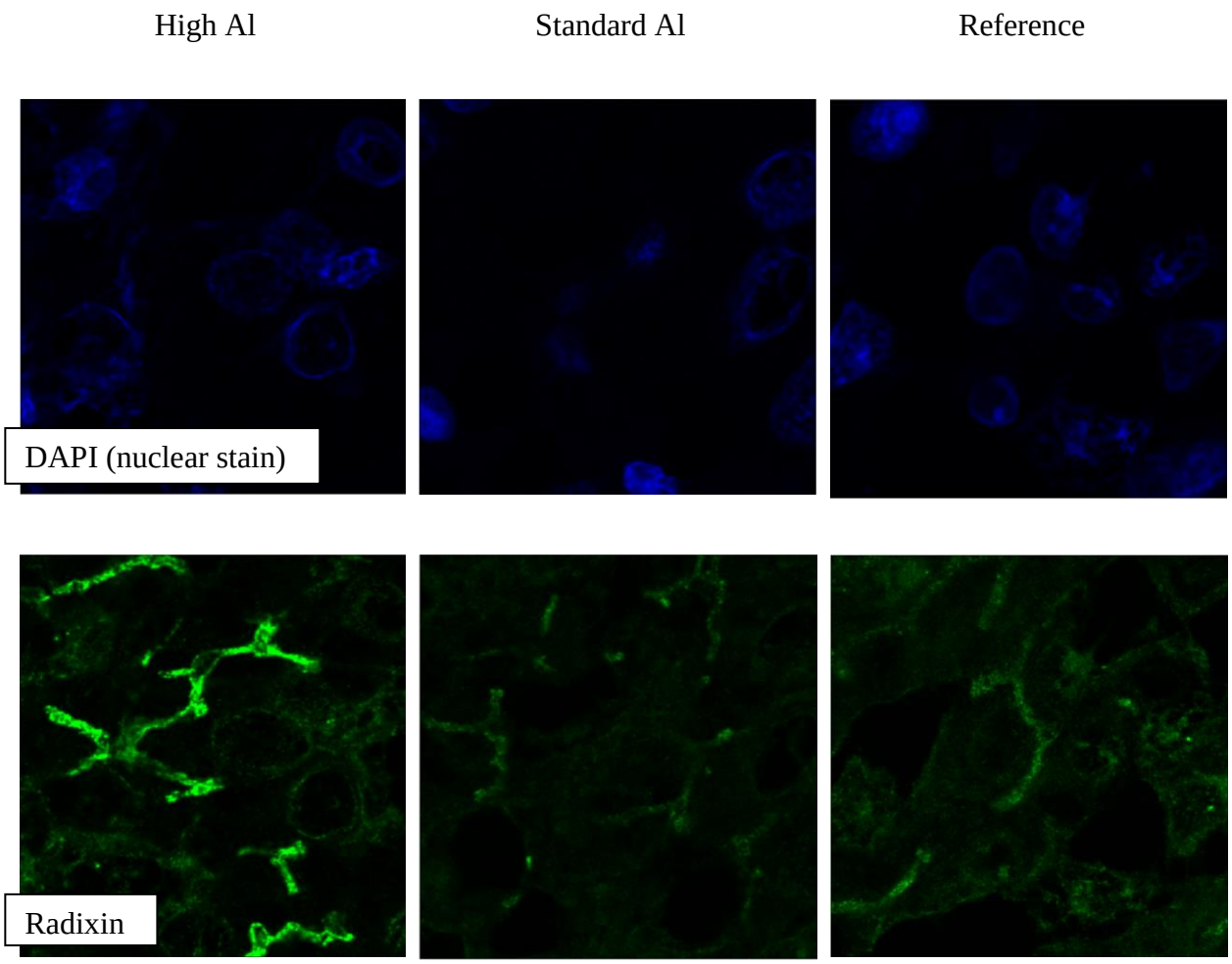
Immunofluorescence and Confocal Microscopy: The co-localization of both apical bile acid transporters Mrp2 and Bsep to radixin was very well preserved in all groups. The signals for Mrp and radixin were perfectly aligned, and likewise for Bsep and radixin. The signals for all three targets were much tighter and cleaner than in the previous piglet PN tissue.

Using ANOVA, all three groups were compared for the Mrp2/radixin slides and there was no difference in the area of staining for either Mrp2 or radixin (p=0.57 and 0.91 respectively). However, in the analysis of density and width for both Mrp2 and radixin, at least one of the groups were significantly different than the other two for each of the measurements (p<0.01 for all four).

When comparing the High and Standard Al groups in the Mrp2/radixin slides, the density of both the Mrp2 and radixin signals was significantly greater in the High Al group (p=<0.01 and <0.01). There was no difference in the area (p=0.43) or width (p=0.06) of the Mrp2 signal. In the same way, there was also no difference in the area (0.80) or the width (0.52) of the radixin signal.

In a comparison of the Standard Al vs Reference groups, the former group had greater width of both Mrp2 (p<0.01) and radixin (p<0.01), as compared to the Reference group. The Reference group also had greater density of both Mrp2 (p<0.01) and radixin (p<0.01).

The findings of the High Al vs Reference group were similar to the comparison of Standard Al to the Reference group. The High Al group had significantly greater width of both Mrp2 ($p<0.01$) and radixin ($p<0.01$), as compared to the Reference group. Likewise, there was also higher density of the Mrp2 signal in the Reference group as compared to High Al ($p<0.01$).



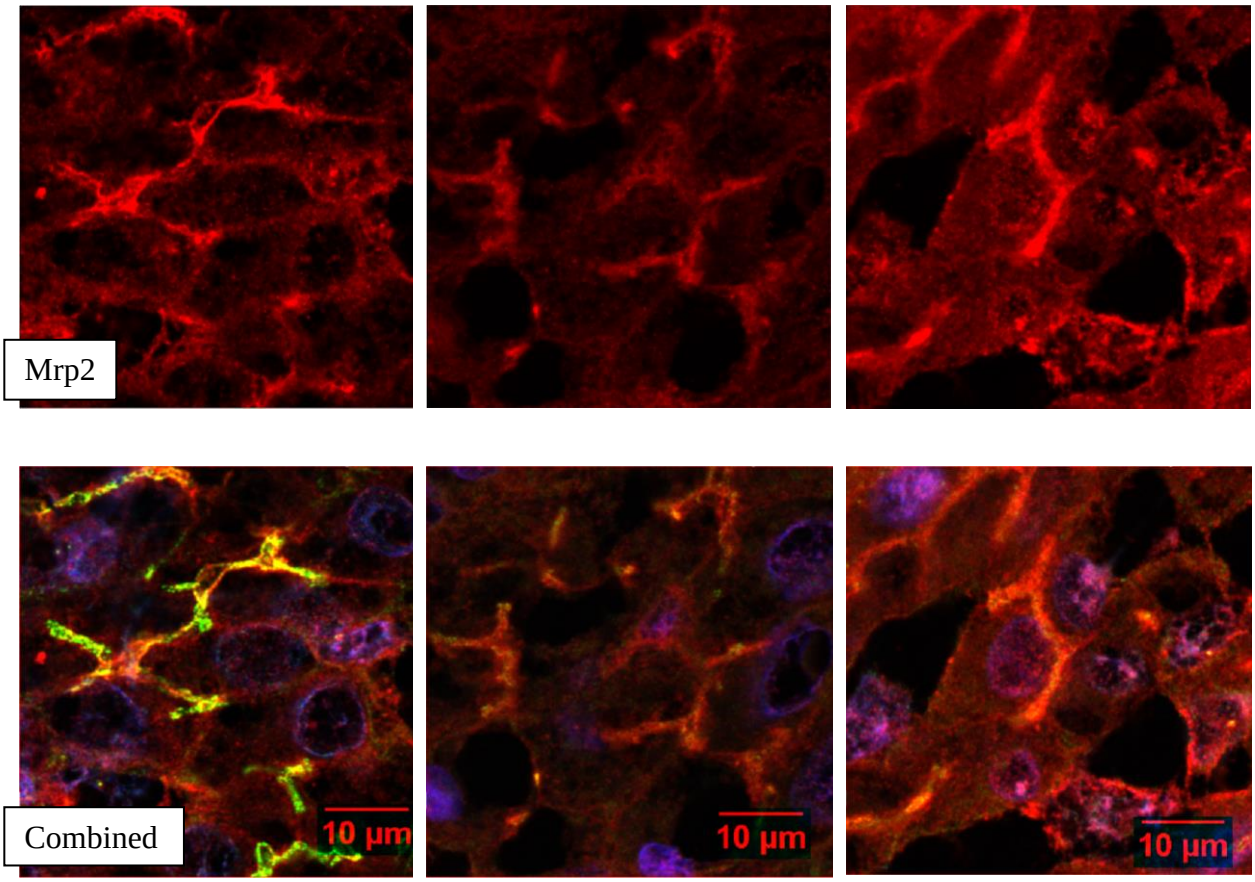


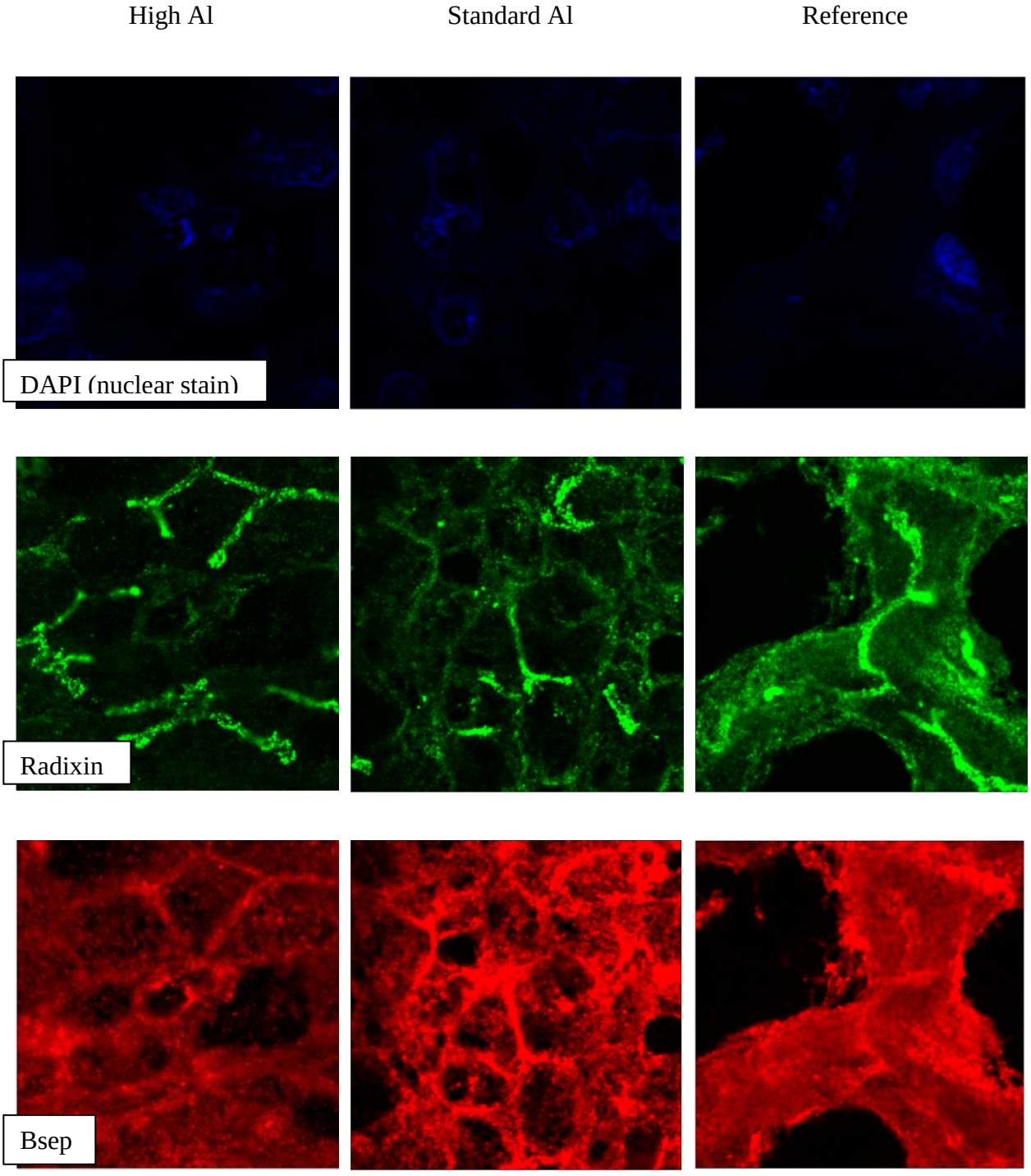
Figure 6.7: Representative IHC images for Mrp2/radixin slides for the three piglet groups

Similar to the Mrp2 results, when the Bsep/radixin slides were analysed using one-way ANOVA, the area of staining for Bsep and for radixin was the same for all three groups ($p=0.63$ and 0.40 respectively). The density and width of staining for both targets however, did vary significantly amongst the groups.

The examination of Bsep/radixin slides in the High vs Standard Al groups showed that almost all measurements for the two targets did not differ significantly, except for the width of Bsep, where the Standard Al group had a greater dispersion ($p=0.03$).

In the comparison of the Standard Al vs Reference group, the findings mirrored the Mrp2 discoveries. There was significantly greater density of Bsep ($p=0.02$) and radixin ($p<0.01$) in those piglets not exposed to PN as opposed to those piglets exposed to the Standard Al PN. When measuring width for both Bsep ($p<0.01$) and radixin ($p<0.01$), the Standard Al group was significantly higher.

Finally, when comparing the High Al group to the Reference group, the density of both Bsep ($p=0.03$) and radixin ($p=0.01$) was again greater in the Reference group. The High Al, meanwhile, had increased width of signal for radixin ($p<0.01$).



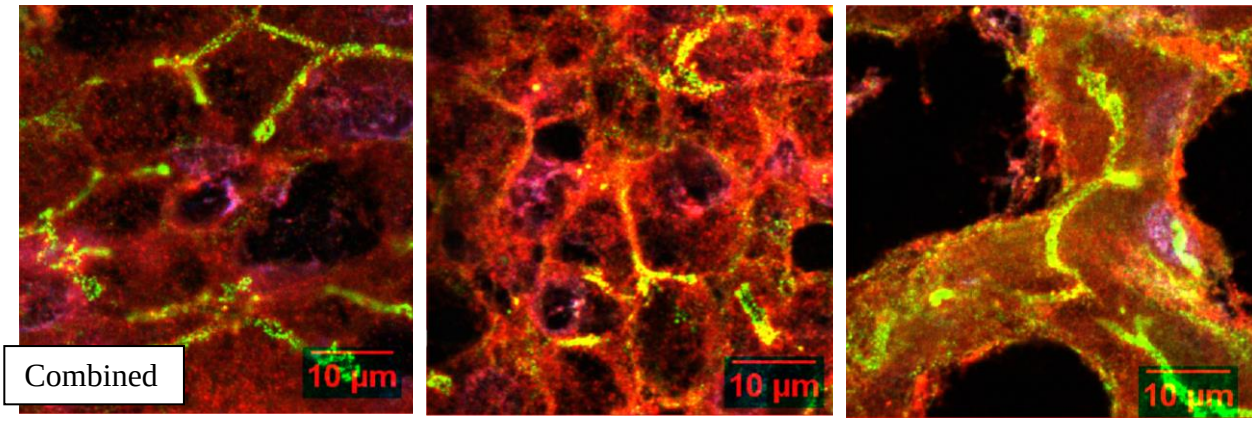


Figure 6.8: Representative IHC images for Bsep/radixin for the three piglet groups

6.6 Discussion:

The goal of this piglet study was to determine if Al in PN could still cause changes to the mRNA of bile acid transporters when a less-inflammatory lipid was used.

The discovery of higher serum CRP in the High Al group, as compared to the Standard Al group, supports the proposed inflammatory mechanism of Al toxicity. CRP is a clinically important acute phase protein that rises within hours of the activation of the inflammatory cascade, peaks within 1-3 days, and rapidly normalizes after the physical insult has resolved. [Koopman, 1996] Most previous work statest that Al toxicity is secondary to oxidative stress reactions, where Al forms superoxide radical ions and inhibits crucial energy pathways in mitochondria. [Alexandrov, et al., 2005; Percy, et al., 2011; Mailloux, et al., 2011; Gonzalez, et al., 2007] However, a few studies of Al exposure in dialysis patients and neuronal cell cultures have found that Al exposure causes a rise in CRP. [Alexandrov, et al., 2005; Guo & Wang, 2011] This suggests that Al may also act through an inflammatory pathway. Inflammation is crucial to the development of PNALD, [Suita, et al., 1999; Lambert & Thomas, 1985; Trauner, et al., 1999; Kosters & Karpen, 2010] and the Al-induced elevation of CRP in our study further strengthens the hypothesis that Al is a key component of PNALD.

When analyzing the qPCR results, the three basolateral transporters Mrp3, Oatp8, and Ntcp, had statistically less mRNA in the High Al group as compared to the Standard Al group. However, this statistical difference may not translate into a clinically relevant difference, so our conclusions are made cautiously and with the caveat that further investigation is required.

There was statistically less mRNA for both Ntcp and Oatp8 in the High Al group as compared to the Standard Al group. Without analyzing the resulting protein, it is impossible to know whether or not this would imply a diminished protein amount or whether this statistically significant change is also clinically significant. However, given the Al-induced rise in CRP, if our observed mRNA deficits were carried through to a protein deficit, the mechanism might be related to inflammation and oxidative stress. Ntcp and Oatp8 are frequently downregulated in other inflammatory models of PNALD. [Green, et al., 1996; Vanwijngaerden, et al., 2011; Geier, et al., 2005; Cherrington, et al., 2004] For example, in rat models the endotoxin lipopolysaccharide downregulated Ntcp by 90%, and even isolated cytokines, such as TNF-α, produced a significant decrease in Ntcp. [Green, et al., 1996] Similar studies have further determined that Ntcp is downregulated as a post-transcriptional response to cytokines. [Andrejko, et al., 2008] These cytokines trigger a series of cellular proteins, until finally nuclear transcription receptors such as HNF-1a or FXR cause the downregulation of Ntcp. [Green, et al., 1996; Vanwijngaerden, et al., 2011; Geier, et al., 2005; Cherrington, et al., 2004] Al could possibly participate in this pathway, as it causes a release of inflammatory cytokines such as TNF-α. [Alexandrov, et al., 2005; Guo & Wang, 2011] Similarly, Oatp8 is susceptible to the down-regulatory effects of the inflammatory cascade. Most of the Oatp transporters are also controlled by FXR, along with other nuclear transcription factors such as the constitutive androstane receptor (CAR). When CAR or FXR are inhibited by pro-inflammatory cytokines they cause downregulation of Oatp transporters. [Pascussi, et al., 2003, Vanwijngaerden, et al., 2011] In fact, many of the same cytokines (IL-6 and TNFα) that initiate an inhibitory series of events for Ntcp have the same effect on many of the Oatp transporters. [Andrejko, et al., 2008; Geier, et al., 2005; Hartmann, et al., 2002] The downregulation of both Ntcp and Oatp8 may be a protective mechanism for the hepatocyte, to limit the amount of toxic bile acids allowed in. [Cherringotn, et al., 2004] The Al-induced decrease in mRNA for these two transporters is important because it occurred despite the presence of the less inflammatory mixed lipid. This change in mRNA could potentially translate into a decreased amount of protein, but further studies are required to determine if the statistical difference is meaningful and if the protein is correspondingly affected.

The downregulation of Mrp3 in the High Al, as compared to the Standard Al group, is interesting because the opposite was expected. Mrp3 is normally found in low amounts in

healthy livers and unlike the other transporters studied in our project, it transports bile acids across the basolateral membrane of the hepatocyte and into the portal circulation. [Kruh, et al., 2007; Jenniskens, et al., 2016] It is commonly upregulated in cholestatic situations as an attempt to clear harmful bile acids from the hepatocyte. [Zelcer, et al., 2006; Vanwijngaerden, et al., 2011; Kruh, et al., 2007; Jenniskens, et al., 2016] One other piglet PN study demonstrated a decrease in Mrp3 but this was observed with all types of lipid in PN and Al was not examined, [Vlaardingerbroek, et al., 2014] so their results do not directly correlate with ours. With only mRNA data, it is difficult to determine the effects of Al on the final protein, but if the mRNA deficit observed here translates into a protein deficit, then the hepatocyte may be vulnerable to cholestasis. In our study, an Al-induced inflammatory cascade may be interfering the with normal Mrp3 response, essentially impairing the ability of the hepatocyte to respond to the mounting bile acids. For example, a few studies have found that high levels of the cytokines interferon-gamma and TNF-α, and especially IL-1β, [Geier, et al., 2005] cause downregulation in Mrp3. [Le Vee, et al., 2011; Hartmann, et al., 2002] Similar cytokines are increased with Al toxicity, [Alexandrov, et al., 2005; Guo & Wang, 2011] making this is a plausible connection. The exact relationship between Mrp3 and cytokines is unclear, especially since there are large differences in the response of Mrp3 between species, and even between strains of the same animal. [Geier, et al., 2005] Alternatively, as outlined in Chapter 5, the observed decrease in Mrp3 may be a normal variation of an already limited piglet Mrp3 expression. [Zhu, et al., 2017; Maher, et al., 2005]

When compared to the Reference group, many of the studied transporters had increased amounts of mRNA in both of the PN groups. These differences could be caused by the many inflammatory factors present in both of the PN groups. Not only are both the High and Standard Al groups exposed to Al, which increases both oxidative stress and inflammation, [Alexandrov, et al., 2005; Percy, et al., 2011; Mailloux, et al., 2011; Gonzalez, et al., 2007] but they are also given SMOFlipid which contains pro-inflammatory omega-6 lipids. [Fresenius Kabi, "SMOFlipid", 2017] This might explain why transporters, such as Bsep had increased amounts of mRNA in the PN groups. Although most inflammatory models of PNALD have demonstrated a decrease in Bsep, [Geier, et al, 2005; Elferink, et al., 2004] there are some models where Bsep responds by increasing. For example, in primary biliary cirrhosis, both the mRNA and protein of Bsep are upregulated. [Arrese & Ananthanarayanan, 2004]

Both of the PN groups had an increased amount of Mrp3 mRNA as compared to the Reference group, and this correlates with many other studies demonstrating upregulation in situations of increased inflammation. [Vanwijngaerden, et al, 2011; Geier, et al, 2005; Cherrington, et al., 2004] This increase of Mrp3 is initiated when FXR is downregulated by a variety of cytokines. By increasing the export of bile acids out of the hepatocyte, Mrp3 may be acting to minimize cholestatic damage. [Jenniskens, et al., 2016; Vanwijngaerden, et al., 2014]

There was no significant difference in Mrp2 protein between any of the three groups, whether comparing the High and Standard Al or the Reference group. This corresponds to the lack of qPCR difference noted in this study. In the previous piglet experiment, with pro-inflammatory lipids, High Al caused statistically significant downregulation of Mrp2 mRNA but no change in Mrp2 protein. It is not surprising therefore, that in our current study with a less inflammatory lipid mix [Klek, et al., 2013; Goulet, et al., 2010], both the mRNA and protein expression for Mrp2 is preserved.

Immunohistochemistry analysis demonstrated that the relationship between the cytoskeletal protein radixin and the apical transporters Mrp2 and Bsep is well preserved regardless of the amount of Al contamination. However, without a cell membrane marker, we cannot determine the cellular localization of these proteins. In the examination of Bsep and radixin by immunohistochemistry, there was minimal difference in either protein when comparing the High vs Standard Al group. Strangely, when comparing the High and Standard Al groups, there was increased density of the Mrp2 and radixin in the High Al group. This is the opposite of the findings in our previous piglet study with the pro-inflammatory lipids. As we do not know the cellular placement of these proteins, it is possible that the Mrp2 is drawn intracellularly (as seen in other models of cholestasis [Kojima, et al., 2003; Kojima et al., 2008]) and the intracellular collection of Mrp2 creates a denser signal. Or perhaps there is increased placement of Mrp2 proteins at the cell membrane as a compensatory response to combat the rising bile acids. A cell membrane marker is required to differentiate the actions of Mrp2.

Not surprisingly, when compared to the healthy piglets with no PN exposure, both the High Al and the Standard Al PN groups demonstrated a dispersed signal with weaker density for radixin, Bsep, and Mrp2. This correlates well with other studies of cholestasis where Mrp2 and radixin lose their co-localization and are dispersed with hepatic insults. [Kojima, et al., 2003;

Kojima et al., 2008; Geier, et al., 2005; Cherrington, et al., 2004; Elferink, et al., 2004; Hartmann, et al., 2002] Similarly, Bsep is susceptible to inflammatory factors and responds with downregulation and intracellular localization. [Schmitt, et al., 2001; Perez, et al., 2006; Geier, et al., 2005; Elferink et al., 2004] As mentioned above, there are too many potential inflammatory factors (including Al) separating the PN from the non-PN piglets to determine which one is responsible for the abnormal cellular localization of radixin and Mrp2.

6.7 Conclusions:

High Al increased serum CRP, which suggests that Al has an inflammatory mechanism in addition to the accepted pro-oxidative one. This link between Al and inflammation is important because of the strong role of inflammation in PNALD. Additionally, despite the presence of the less-inflammatory mixed lipid, Al still caused statistically significant decrease in mRNA for the basolateral transporters. If these mRNA changes are actually clinically relevant and are carried through to the protein level, the bile flow could be hampered.

7: COMPARING AND CONTRASTING THE PIGLET PARENTERAL NUTRITION STUDIES (WITH OMEGA-6 AND MIXED LIPIDS)

The two studies using the piglet PN model were completed two years apart (2013 and 2015) and with some significant methodological differences, but a comparison of the studies provides an opportunity for further examination of the roles of lipid composition and Al contamination in PNALD.

7.1 Methods comparison:

Firstly, both of these studies were carried out using the same species of pig, with both the same lab and standard operating procedures. In both studies, the piglets were the same age and and their group sizes were similar. All of the surgical techniques, the equipment and compounding methods of PN were identical. We also focused on the same bile acid transporters, and conducted very similar analyses, including qPCR, Western blot, IHC and bile acid assays.

The largest difference between the studies was the lipid solution; SMOFlipid (Fresenius Kabi, Bad Homburg, Germany) (a mixed lipid solution) was used for the second study, as compared to Intralipid (Fresenius Kabi, Bad Homburg, Germany) (an omega-6 lipid) for the first study. The omega-3 lipids in the mixed solution are less-inflammatory than the omega-6 lipids and SMOFlipid appears to normalze serum bilirubin for most infants with PNALD. [Goulet, et al., 2010; Pichler, et al., 2014; Muhammed, et al., 2012] However, SMOFlipid is still relatively new and under investigation[Wales, et al., 2014] and we felt it was important to examine in our PNALD model.

A two-week vs a three-week study period was the second most important difference. A three-week duration was selected for the initial study because of literature demonstrating that 25% of infants develop biochemical evidence of PNALD within three weeks [Beale, et al., 1979; Arnold, 2004]. In addition, work by Alemmari et al, using the same piglet PN model had not shown biochemical evidence (ie: elevated total serum bile acids) by two-weeks. [Alemmari, et al., 2011; Alemmari, et al., 2012] However, in our first piglet PN study we discovered that the piglets had a high rate of sepsis when exposed to PN for three weeks. We attempted to control for this because sepsis is a risk factor for PNALD [Suita, et al., 1999; Lambert & Thomas, 1985], but there is still a potential difference in infection rates between the studies.

Statistical analysis

All statistical analysis was carried out using SPSS version 22 (IBM, Armonk, NY) and SAS (SAS Institute, Cary, NC). For the basic demographic data, one-way ANOVA and Kruskal-Wallis, followed by post-hoc tests (where appropriate) were used to compare the initial weights of the piglets. A repeated measure model was then used to analyze weight gain for the five groups. A similar repeated measurement model was also used for the bile acid assay analysis. For all of these tests, $p<0.05$ was significant.

The IHC and the Western blot measurements were analyzed using one-way ANOVA or Kruskal-Wallis and significant results were further explored with post-hoc Bonferroni or Dunnett T3 tests (for parametric vs non-parametric data distributions respectively). A p value of <0.05 was significant.

An attempt was made to compare qPCR results from the two piglet studies; examining Mrp2, Bsep, Mrp3, Ntcp, Oatp8, radixin and FXR. The Pfaffl equation was used again, see Equation 5.1.[Pfaffl, 2001] The geometric mean of primer efficiencies from the mixed lipid and the omega-6 lipid studies were calculated to obtain the *E* values. New Pfaffl calculations were then carried out for all seven targets of interest, using the six different paired comparisons (ie: High Al group from the omega-6 lipid study vs High Al group from the mixed lipid study etc). This type of analysis was unsuccessful, and it produced nonsensical fold differences. This inability to combine the qPCR results is likely because the qPCR plates (and most importantly, the reference genes) are not consistent between the two studies. Without a common reference value or a method of determining the variation in reference genes between plates, it is impossible to accurately combine qPCR values.

7.2 Results:

Basic demographics: Overall, there were no significant differences between the two piglet PN studies, when comparing the initial weights of the piglets. When the starting weights were compared using one-way ANOVA, there was a significant difference among the five groups ($p=0.008$), but it was not large enough to be detected with specific pairwise comparison, using Scheffe's post-hoc analysis. (The smallest p-value for this post-hoc analysis was $p=0.094$).

For the weight gain analysis, both of the Intralipid PN groups were slower to gain weight and significantly smaller than either of the SMOFlipid PN groups (ie: by day four: $p < 0.001$ and $p = 0.034$). But by day eight, only the Standard Al+Intralipid group was significantly smaller than either of the SMOFlipid PN groups ($p = 0.034$) and by day twelve, there was no difference between the weights of the four PN groups.

Immunohistochemistry, Mrp2 and Radixin: Only the Mrp2 area measurements met the requirements to be analyzed using One-way ANOVA and this test revealed that there was no difference between the groups ($p = 0.77$). The remaining five measurements (Mrp2 density, Mrp2 width, radixin area, radixin density and radixin width) were analyzed using the Kruskal-Wallis test and all except radixin area had at least one group that was significantly different from the others. (Radixin area: $p = 0.055$, all others: $p < 0.0001$). See Table 7.1 for the summary of results.

	2013 High Al (+Intralipid)	2015 High Al (+SMOFlipid)
Mrp2 Area	No Difference	
Mrp2 Density	No Difference	
Mrp2 Width	Greater Width ($p = 0.02$)	
Radixin Area	No Difference	
Radixin Density	No Difference	
Radixin Width	Greater Width ($p < 0.001$)	
	2013 Stdrd Al (+Intralipid)	2015 Stdrd Al (+SMOFlipid)
Mrp2 Area	No Difference	
Mrp2 Density	Greater Density ($p < 0.001$)	
Mrp2 Width	No Difference	
Radixin Area	No Difference	
Radixin Density	Greater Density ($p < 0.001$)	
Radixin Width	Greater Width ($p = 0.02$)	
	2013 High Al (+Intralipid)	2015 Stdrd Al (+SMOFlipid)
Mrp2 Area	No Difference	
Mrp2 Density	Greater Density ($p < 0.001$)	
Mrp2 Width	No Difference	
Radixin Area	No Difference	
Radixin Density	Greater Density ($p < 0.001$)	
Radixin Width	Greater Width ($p < 0.001$)	
	2013 Stdrd Al (+Intralipid)	2015 High Al (+SMOFlipid)
Mrp2 Area	No Difference	
Mrp2 Density	No Difference	
Mrp2 Width	Greater Width ($p = 0.03$)	
Radixin Area	No Difference	

Radixin Density	No Difference	
Radixin Width	Greater Width (p=0.002)	

Table 7.1: Mrp2 and radixin: comparison of 2013 vs 2015 IHC measurements

Immunohistochemistry, Bsep and Radixin: Similar to the Mrp2 IHC analysis, only the area of Bsep was normally distributed, allowing for One-Way ANOVA analysis which revealed a significant difference in at least one of the five groups (p=0.02). Post-hoc analysis was therefore conducted. For the other five types of IHC measurements, the Kruskal-Wallis test showed that four of them had at least one significantly different group. (Radixin area had no difference between the groups, with p=0.549). The results for the pairwise comparisons are summarized below.

	2013 High Al (+Intralipid)	2015 High Al (+SMOFlipid)
Bsep Area	No Difference	
Bsep Density		Greater Density (p<0.001)
Bsep Width	No Difference	
Radixin Area	No Difference	
Radixin Density		Greater Density (p<0.001)
Radixin Width	No Difference	
	2013 Stdrd Al (+Intralipid)	2015 Stdrd Al (+SMOFlipid)
Bsep Area		Greater Area (p=0.04)
Bsep Density		Greater Density (p=0.02)
Bsep Width	No Difference	
Radixin Area	No Difference	
Radixin Density		Greater Density (p<0.001)
Radixin Width	No Difference	
	2013 High Al (+Intralipid)	2015 Stdrd Al (+SMOFlipid)
Bsep Area	No Difference	
Bsep Density		Greater Density (p<0.001)
Bsep Width		Greater Width (p=0.006)
Radixin Area	No Difference	
Radixin Density		Greater Density (p=0.013)
Radixin Width	No Difference	
	2013 Stdrd Al (+Intralipid)	2015 High Al (+SMOFlipid)
Bsep Area	No Difference	
Bsep Density		Greater Density (p=0.02)
Bsep Width	No Difference	
Radixin Area	No Difference	
Radixin Density		Greater Density (p<0.001)
Radixin Width	No Difference	

Table 7.2: Bsep and radixin: comparison of 2013 vs 2015 IHC measurements

Western blot: Using the Kruskal-Wallis test, it was determined that there was no significant difference in the density measurements for Mrp2 between the five groups ($p=0.84$).

Bile acid analysis: The repeated measure model demonstrated that both of the Intralipid PN groups had significantly greater rise in total serum bile acids than either of the SMOFlipid PN groups ($p<0.001$ for both).

7.3 Discussion:

Comparing studies completed years apart and with variations in methodology is difficult, but a few findings about lipids and Al in PNALD are notable.

The difference between the initial weights was not statistically significant, although the Intralipid groups were initially slower growers, as compared to the SMOFlipid groups. By the end however, all piglet PN groups weighed the same. This weight fluctuation can be explained by the more stringent weight requirements we used in the later piglet study. In the earlier piglet study, we occasionally used piglets weighing less than one kilogram, but in the later work (with mixed lipids) we did not.

For the Mrp2 and radixin slides, there was a greater width of staining for both radixin and Mrp2 in both Intralipid groups (with either High or Standard Al) as compared to the High Al+SMOFlipid group. Although the findings are relatively inconsistent, the pro-inflammatory, omega-6 Intralipid [Gura, et al., 2008; Cober & Teitelbaum, 2010; Cowan, et al., 2013] may causes some displacement of Mrp2 and the cytoskeleton radixin. A similar broadening of staining patterns has been found in other models of cholestasis as the normal co-localization of Mrp2 and radixin is disrupted. [Kojima, et al., 2008] Surprisingly, there was also greater density of both Mrp2 and radixin staining in both of the Intralipid groups (regardless of Al contamination) when compared to the Standard Al+SMOFlipid group. The opposite was expected, because the Standard Al+SMOFlipid group should benefit from both the hepato-protective lipids and the lower Al contamination. Perhaps, one of the components of SMOFlipid has a negative effect on Mrp2. The effects of a mixed lipid solution on bile acid transporters are unstudied. Overall, it appears that Mrp2 and radixin were more sensitive to the changes in lipid composition than to the alterations in Al in PN.

As for Bsep and radixin, there were also some interesting findings. Regardless of the amount of Al, there was stronger staining for both Bsep and radixin in the groups with SMOFlipid, as opposed to the groups with Intralipid. This can be explained as a response to inflammation. Many other cholestasis models have demonstrated that Bsep is down-regulated and internalized when cytokines suppress upstream regulators of Bsep (such as FXR). [Vanwijngaerden, et al., 2011; Geier, et al., 2005; Elferink, et al., 2004] Radixin meanwhile, does not seem to display a reliable pattern of response in this study. In the Mrp2 slides, radixin was weak in all of the SMOFlipid groups, while it was strong in the same groups for the Bsep slides. This cytoskeletal protein is an important stabilizer of apical transporters and has shown disturbed placement in other models of cholestasis, [Kojima, et al., 2008] but further studies are required to determine its response to PNALD.

There was no difference in the amount of Mrp2 protein for any of the five groups studied and this is consistent with the findings of both of the piglet PN studies. As mentioned in the previous chapters, this may be because our piglets had early PNALD [Tazuke, et al., 2004] or perhaps Mrp2 is not as easily influenced in pigs as it is in rats and humans. [Zelcer, et al., 2006; Oswald, et al., 2001; Alrefai & Gill, 2007]

The total bile acid analysis demonstrated that the SMOFlipid PN piglets did have less of a rise in total bile acids as compared to the Intralipid PN piglets, in keeping with the clinical studies where mixed lipids reversed or delayed the elevation of serum markers of PNALD. [Goulet, et al., 2010; Pichler, et al., 2014; Muhammed, et al., 2012].

7.4 Conclusion:

In summary, this inter-study comparison demonstrates that lipid composition may be an important factor in the cellular localization of bile acid transporters. Bsep appeared to have an adverse reaction to pro-inflammatory lipids, while there was an unexpected negative response of Mrp2 to the mixed lipids. Unlike the individual studies, we did not find an effect of Al, but our comparisons were limited.

8: ALUMINUM ASSOCIATED BILE CANALICULUS CHANGES

8.1 Abstract:

Background/Purpose: Parenteral nutrition (PN) has changed the prognosis of infants with intestinal failure by providing life-sustaining calories in the setting of inadequate absorption of enteral nutrients. However, long-term PN carries the risk of a progressive and potentially life-threatening parenteral nutrition associated liver disease (PNALD). Al is a known contaminant of infant PN, with adverse effects on bone metabolism and neurological development. We hypothesize that Al is also hepato-toxic, especially in the neonate, and may substantially contribute to the multi-factorial PNALD. The objective of this study is to assess the impact of Al on hepatocyte morphology in a piglet model to better understand the pathophysiology of PNALD.

Materials and Methods: A randomized control trial was conducted using a Yucatan miniature piglet PN model. Piglets aged 3-6 days were randomly placed into one of three groups. The High Al group (n=8) received PN with 63µg/kg/day of Al, while the Standard Al group (n=7) received PN with only 24µg/kg/day of Al. In all other aspects, the PN components were the same between the groups. The negative control (Reference) group (n=4) was sow-fed, with no PN exposure. Serum samples for total bile acids were collected throughout the 2-week study period and liver tissue was obtained at the study end. The tissue was gluteradehyde-fixed, epon-embedded, and ultrathin sectioned for viewing under the transmission electron microscope.

Results: The canalicular microvilli were longer in the Standard Al group than in the High Al group ($p=0.01$). We found that both the canalicular area and perimeter were greater in the Standard Al group as compared to the High Al group ($p=0.02$ and 0.02 respectively). There were no significant differences in the structures of the space of Disse between these two groups. As compared to the Reference group, only the High Al group had shorter microvilli in both the canalicular space ($p=0.04$) and in the space of Disse ($p<0.001$). There was no difference in the total serum bile acids between the Standard and High Al groups ($p=0.48$). By day 14, the Reference pigs had a higher total serum bile acid level than either of the Al groups ($p=0.04$).

Conclusions: Al causes structural damage to hepatocytes despite unchanged serum bile acids. High Al in PN was associated with shorter microvilli and smaller canalicular spaces,

which could decrease the excretion area for bile and impair bile flow. These structural defects could translate into an impairment of the bile acid transporters positioned on the microvilli and place the patient at risk of developing PNALD. When compared to healthy controls, only the PN with higher Al contamination is capable of distorting microvilli in both the canalicular space and the space of Disse, within the two-week period of PN, suggesting that there may be a threshold for toxic Al effects. Given these findings, further research is needed to investigate the effects of longer term PN administration in neonates and methods of reducing Al contamination in PN.

Clinical Relevancy Statement:

High amounts of Al contamination in infant PN cause structural damage to hepatocytes, including shortening of the microvilli and shrinkage of the canalicular space. These changes may impair the ability of the hepatocyte to properly excrete bile and place the infant at risk of PNALD. They may precede the development of elevated serum total bile acids, indicating that Al causes significant hepato-toxicity even before the earliest clinical markers of PNALD. Al must be reduced in PN to prevent these effects.

8.2 Introduction:

Al is the third most common element on earth, second only to oxygen and silicon and the most abundant metal in the Earth's crust. [Mujika, et al., 2011] It exists as a trivalent ion and is able to form a variety of strong bonds with many biologic molecules. [Mujika, et al., 2011] Surprisingly it has seemingly no role in the biological functioning of plants and animals. [Sedman, 1990] People are regularly exposed to high amounts of Al, usually through oral ingestion, but over 99% of this ingested Al passes harmlessly out through the gastro-intestinal tract. [Sedman, 1990; Poole, et al., 2010; Greger & Baler, 1983] The gastro-intestinal tract provides an excellent barrier to this metal and the small amount that is absorbed through the intestinal wall is then excreted via the kidney. [Gregor & Baler, 1983] When Al is introduced parenterally, however, the body absorption is much higher. Al travels bound to transferrin in the blood stream and can then be deposited in the brain, bones, and liver. [Sedmen, 1990]

Parenteral Al exposure becomes an issue for premature infants on prolonged PN. Infant PN has a large amount of Al contamination, mostly found in the calcium gluconate components. [Poole, Hintz, Mackenzie, & Kerner, 2008; de Oliveira, Bohrer, Garcia, do Nascimento, &

Noremberg, 2010] This Al is readily absorbed parenterally in the infant and often cannot be adequately eliminated due to renal insufficiency prevalent in premature infants. [Koo, et al., 1989; Koo, Kaplan, & Bendon, 1986; Bohrer, et al., 2010] Al accumulates in hepatic lysosomes and other organelles and causes structural damage to the hepatocyte and impairs biliary secretion. [Gonzalez, et al., 2004; Klein, et al., 1988; Demircan, et al., 1998; Bertholf, et al., 1989] It is believed to be hepatotoxic through its pro-oxidative effects. [Alexandrov, et al., 2005, Percy, et al., 2011; Mailloux, et al., 2011] For example, Al forms a superoxide radical ion, and acts with iron to participate in oxidative reactions. [Alexandrov, et al., 2005; Gonzalez, et al., 2007] It also disrupts mitochondrial functioning and triggers apoptosis. [Gonzalez, et al., 2007]

Previous studies by our group demonstrated that Al in a piglet model caused hepatic structural changes such as blunting of bile canalicular microvilli, a decreased number of microvilli in the bile canaliculi, and increased inflammatory cells. [Alemmari, et al., 2012] In that study, the piglets were administered PN with an omega-6 based lipid, which is pro-inflammatory. [Gura, et al., 2008; Cober & Teitelbaum, 2010; Cowan, et al., 2013] This may have been a contributing factor to the documented liver disease. To determine if Al causes structural damage regardless of the type of lipid involved, we have repeated a similar piglet study using a mixed lipid solution. SMOFlipid (Fresenius Kabi, Bad Homburg, Germany) is a combination of soybean lipids (omega-6 fatty acids), medium chain triglycerides, olive oil and fish oil lipids (omega-3 fatty acids). [Klek, et al., 2013; Goulet, et al., 2010] The majority of infants with PNALD who were switched to SMOFlipid had reversal of their serum hyperbilirubinemia and other markers of PNALD. [Goulet, et al., 2010; Pichler, et al., 2014] It is believed this is because SMOFlipid is less inflammatory. [Klek, et al., 2013; Goulet, et al., 2010]

8.3 Objectives:

We hypothesize that the hepato-protective nature of the mixed lipid solution (SMOFlipid) will not completely protect the hepatocyte and Al-induced structural changes will develop despite a more favourable lipid solution. Our objectives are to use a Yucatan miniature piglet PN model to observe the effects of High vs Standard Al contamination in PN on hepatocyte ultrastructure. We will use total serum bile acids as an early marker of PNALD and transmission electron microscopy to evaluate for changes in the hepatocyte features.

8.4 Methods:

Animal Work: Yucatan miniature piglets, aged 3-6 days, were placed randomly into one of three groups. If at all possible, the piglets were pair-matched with a littermate of similar size, to ensure comparable group demographics. The High Al group (N=8) received PN with 63μg/kg/day of Al, while the Standard Al group (N=7) received otherwise identical PN with 24μg/kg/day of Al. The amount of Al in the latter group is within the range of contamination found in Canadian neonatal PN. [Hall, et al., 2016] The Reference group (N=4) consisted of piglets that were sow-fed for the duration of the study. No interventions were performed on this last group of piglets until the termination of the study.

The piglets in the High and Standard Al groups were kept on a strict PN regime for the 14 days of the study. For both groups, the lipids were provided in a mixed lipid solution (SMOFlipid) at a rate of 1.9mL/hour (45.6 ml/kg/d). In total, the piglets received 0.98 MJ/kg/d and 13.3g amino acids/kg/day with non-protein energy supplied approximately 50:50 carbohydrate and fat. The amino acid-dextrose solution was infused at a rate of 10.1 mL/kg/h (242.4 mL/kg/d). Every second day, the piglets were weighed and the PN rate was adjusted to maintain adequate nutrient delivery. (For complete PN recipes, refer to Appendix C). Every fourth day, serum samples were collected.

At the conclusion of the study, the piglets from all three groups were anaesthetized and liver samples collected. The details of the animal work are explained in chapters five and six.

Transmission Electron Microscopy: Transmission electron microscopy was used to compare the hepatic ultrastructure among the three piglet groups.

Firstly, 0.5cm^2 pieces of liver were fixed in a glutaraldehyde-paraformaldehyde solution immediately upon collection. The samples were kept in this solution at 4ºC overnight and then washed with sodium 0.1M cacodylate buffer three times (ten minutes each, at room temperature). These liver samples, in the sodium cacodylate buffer were then processed at the Memorial University Electron Microscopy/Flow Cytometry Unit. The samples were post-fixed in 1% osmium tetroxide (in a 0.1M sodium cacodylate buffer) for 30 minutes and then excess osmium tetroxide was removed with two sodium cacodylate washings (ten minutes each). Next, the samples were dehydrated in 70% ethanol for ten minutes, followed by staining with saturated uranyl acetate in 70% ethanol for 30 minutes. The dehydration process was completed by exposing the samples to 95% ethanol for fifteen minutes (twice), and then 100% ethanol for

fifteen minutes (three times). Finally, the samples were embedded in epon/araldite resin, through immersion in graded resin solutions. The first immersion was 100% acetone for fifteen minutes (three times), then a 1:1 mixture of acetone and resin for one hour, followed by pure resin for twenty minutes and lastly fresh resin for an additional hour. As the final step, the resin embedded specimens were placed in Beem capsules (Ted Pella Inc, Redding, CA, USA) and the resin was polymerized overnight.

The resin-embedded liver samples were then sectioned on an ultra-microtome at thicknesses of 70-90nm and mounted on nickel grids. These sections were stained again with uranyl acetate (2% in aqueous solution) for 25 minutes and then washed with distilled water five times. A secondary stain of lead citrate was then applied for ten minutes and rinsed off with five more distilled water washings.

These stained sections were viewed and photographed under an electron microscope with energy dispersive spectrometer capability (Hitachi HT7700, Tokyo, Japan), operating at an accelerating voltage of 80kV. For each piglet, the three images of the clearest bile canaliculi and space of Disse were analyzed using the open access ImageJ program.

A variety of measurements were taken in the evaluation of hepatocyte ultrastructure, focusing on three structures crucial to bile acid synthesis and export: the bile canaliculus, the space of Disse and the mitochondria. The bile canaliculus is bordered by hepatocytes and receives bile acids and other products excreted into it, en route to the biliary ductal system. [Slonim & Pollack, 2006] The space of Disse is the region between a sinusoid and hepatocyte where the plasma flows and is filtered for nutrients and bile acids to be drawn into the hepatocyte. [Slonim & Pollack, 2006] Lastly, the mitochondria are the cellular energy structures that facilitate cell activity. Only the significant findings will be discussed below in the results section.

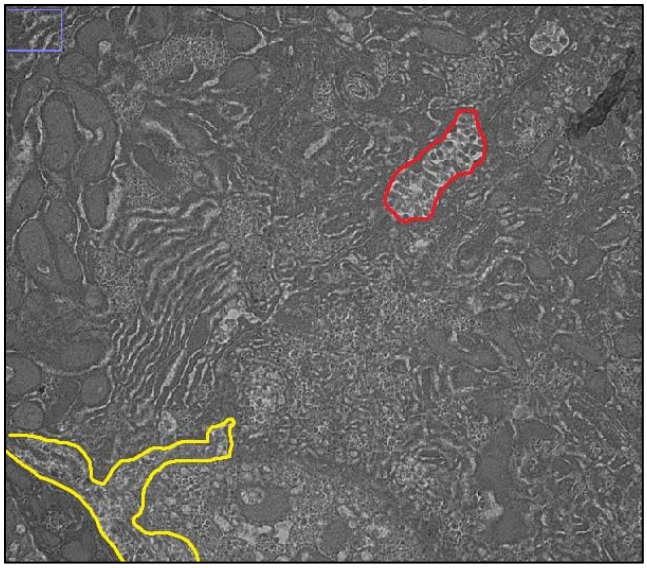


Figure 8.1: Example of TEM mapping. Red line around canalicular space and yellow around space of Disse.

Bile Acid Assay: We used a colorimetric kit (BQKits, San Diego, CA, USA) for the total bile acid assay, following the manufacturer's instructions. In this assay, the oxidation of bile acids produces NADH, which then reacts with nitrotetrazolium blue to form a coloured dye. A spectrophotometer detects the amount of dye formed by measuring absorbance at 540nm and the amount of dye is directly proportional to the quantity of bile acids present in the sample. Each serum sample was tested twice and any sample with variance greater than 10% was re-tested.

Statistical Analysis

All statistical analysis was carried out using SPSS version 22 (IBM, Armonk, NY) and SAS (SAS Institute, Cary, NC). The bile acid assay results were examined using a repeated measurement model for longitudinal analysis, paired with non-parametric tests for study of data on separate days (ie: all pigs on day 4). In these comparisons, a p value <0.05 was considered statistically significant.

The measurements obtained from the transmission electron microscopy was compared between the three groups using ANOVA (and Kruskal-Wallis for non-parametric data sets), followed by paired T-tests, (comparing High vs Standard Al, followed by High Al vs Normal, and lastly Standard Al vs Normal). In all of these comparisons, a p value <0.05 was considered statistically significant.

8.5 Results:

Transmission Electron Microscopy: Initial ANOVA and Kruskal-Wallis analysis of the three groups indicated that there was a significant difference in canalicular area, perimeter and microvilli height ($p=0.001$, $p=0.021$, and $p=0.021$ respectively). When comparing the space of Disse, the same type of analysis indicated a significant difference in average microvillus height ($p=0.024$), while for mitochondrial measurements, there was a difference in the number of hyperdense lesions ($p<0.001$). The pairwise comparisons are outlined below.

In the comparison between the High and Standard Al groups, there were significant differences in the canalicular features. Firstly, both the canalicular area and perimeter were larger in the Standard Al group ($p=0.02$ and $p=0.02$ respectively). Secondly, the microvilli in the Standard Al group were significantly longer than in the High Al group ($p=0.01$). Of note, there was no difference in the number of microvilli per canaliculus between the groups ($p=0.15$).

There was no difference in the space of Disse characteristics between the High and Standard Al groups, in either the width of the space of Disse, nor in its microvilli. Mitochondrial features also did not significantly differ between the two groups.

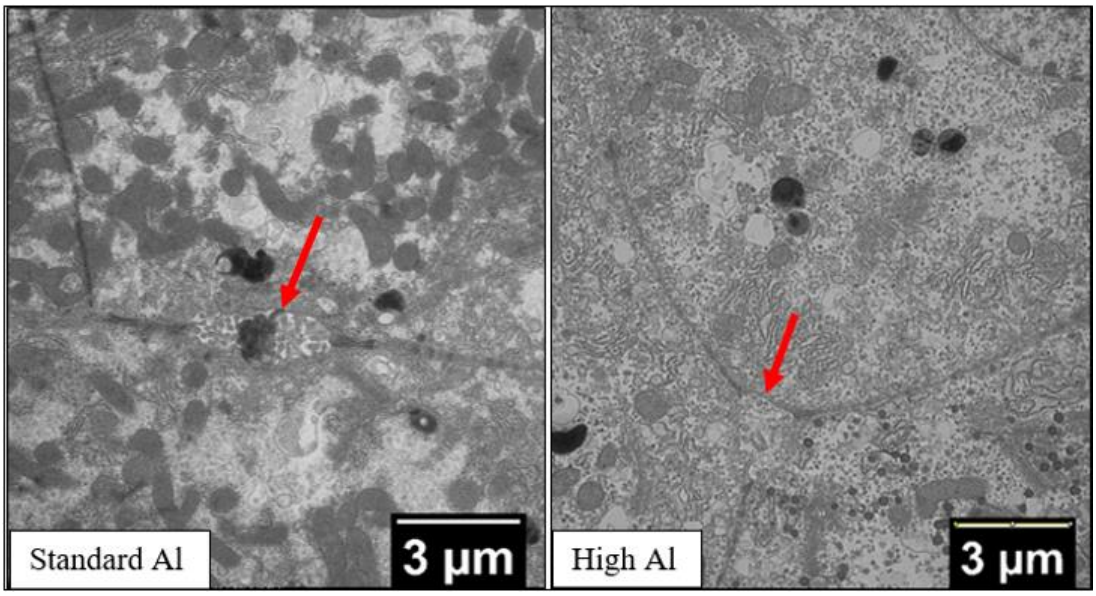


Figure 8.2: Standard vs High Al transmission electron micrographs. The arrow indicates a canalicular space. Note that the microvilli in the Standard Al canaliculi are longer than in the High Al canaliculi.

When comparing the High Al to the Reference group, the average microvillus height in the bile canaliculi was significantly taller in the Reference group ($p=0.04$). No other major differences in the canaliculi structures were found. In the space of Disse, the microvilli were

again significantly taller in those piglets not on PN, as compared to the High Al group ($p<0.001$). Finally, in the examination of mitochondria, significantly more high density marks in the mitochondria were found in the High Al group as compared to the Reference group ($p<0.001$).

With the Standard Al group compared to the Reference group, the canalicular microvilli height was not different ($p=0.72$), but the canalicular area and perimeter were both greater in the Standard Al group ($p<0.001$ and $p=0.02$). There were no significant differences in the size, shape or microvilli of the space of Disse. There was again a significant difference in the hyperdense mitochondrial spots, with a greater number found on average in the Standard Al group as compared to the Reference group.

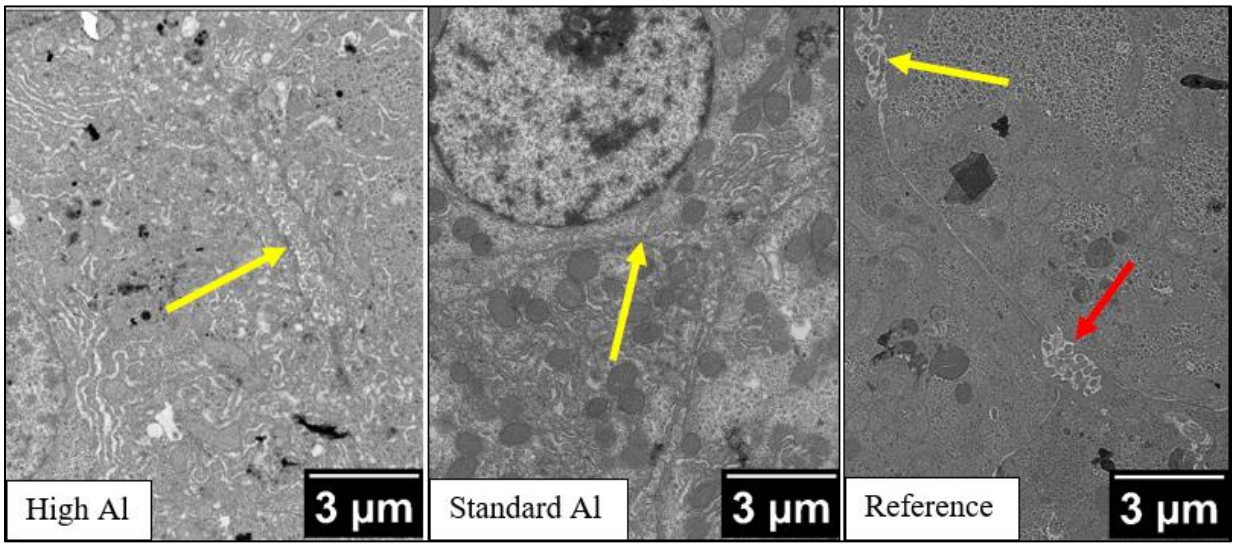


Figure 8.3: Transmission electron micrographs of the three groups. Yellow arrows indicate space of Disse, while the red arrow indicates canalicular space.

Bile acid assay: The total serum bile acids were compared between the Standard Al and High Al groups using a repeated measurement model, and there was no significant difference in the slopes ($p=0.48$) or between the values for each individual day. The day 14 total bile acid values for all three groups were then compared using a Kruskal-Wallis non-parametric test and there was a significant difference. The Reference pigs had a significantly higher serum bile acid level (74µmol/L) than either the Standard or High Al groups at day 14 ($p=0.04$). However, pairwise comparison of only the Standard and High Al group at day 14 still showed no difference between these two groups ($p=1.00$). Serum bile acids were also not related to birth weight ($p=1.00$).

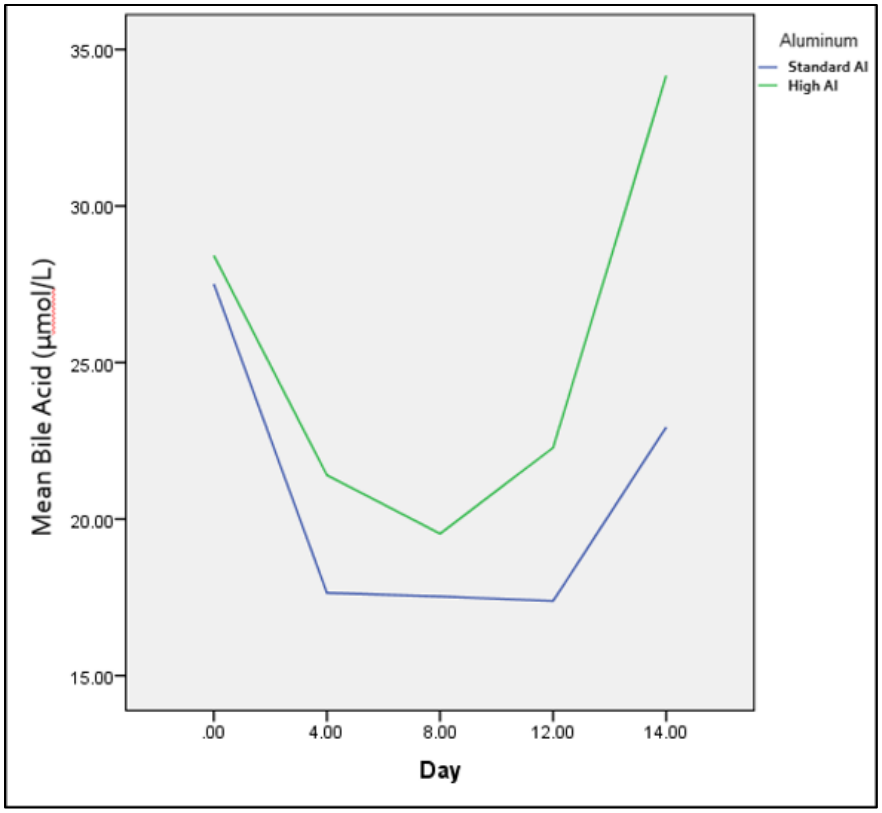


Figure 8.4: Total serum bile acids: High vs Standard Al
The average total serum bile acid value for the Reference group is not included on this graph (day 14 value=74µmol/L).

8.6 Discussion:

Significant changes in the hepatocyte ultrastructure were identified and can be correlated with the Al in PN. The most interesting finding in this study is the shorter canalicular microvilli in the High Al group as compared to the Standard Al group. The same number of microvilli were counted in the two groups and the density of microvilli was identical between the two groups. Similarly blunted microvilli were found in the previous piglet PN model by Alemmari et al as well. [Alemmari, 2012] The shortening of the microvilli may be due to a loss of the protein villin, as is the case in children with biliary atresia. [Phillips, et al., 2003; Jansen & Sturm, 2003] Villin maintains the actin cytoskeleton and is highly prevalent in the microvilli. [Phillips, et al., 2003; Jansen & Sturm, 2003] Actin and the actin-binding proteins form a network that supports the canalicular structure. [Tsukada, Ackerley, & Phillips, 1995] Canalicular microvilli are essential components of bile excretion, and blunting of these structures is found in many types of cholestasis. [Phillips, et al., 2003] The apical bile acid transporters, such as Mrp2 and Bsep, are

located on the bile canaliculi. [Arrese & Ananthanarayanan, 2004; Gonzalez, et al., 2004; Zelcer, et al., 2006] If the canaliculi are blunted due to Al exposure, then there is less surface area for bile acid transporters and their ability to excrete bile could be impaired. Other studies agree that the earliest changes in cholestasis are found around the canaliculi and include shortening, or loss, of microvilli. [Adler, Chung, & Schaffner, 1980]

Of note, there are important differences in hepatocyte structure based on the acinus zone sampled [Boyer, 2013], but we controlled for this discrepancy through random sampling. We collected samples from three different areas of the liver for each piglet and then examined and photographed multiple different ultra-thin sections. We also took our measurements from three different mircographs. All of these randomization steps should ensure that there was equal sampling of acini zones for each piglet group.

When comparing the High Al group to the Standard Al group, the canalicular spaces were also larger in the lower Al group. Previous studies using the piglet PN model showed the opposite effect with a similar dose of Al. In Alemmari et al's work, the higher Al exposure caused a ballooning of canalicular space which could exacerbate bile stasis. [Alemmari, et al., 2012] Ballooning of the canalicular space is frequently seen in different cholestatic scenarios, including in rat models of PNALD, [Adler, et al., 1980; Shu, Li, Zhou, Shi, & Zhang,1991] and is found in inflammatory models as well. [Shu, et al., 1991] The SMOFlipid given to both of our groups may have protected against such an inflammatory development. Alternatively, studies in rat couplet cells found that when hepatocytes are exposed to high osmolarity solutions, both the hepatocytes and the canalicular space shrink. This action is felt to be mediated through aquaporins and tight junctions which become leaky under cholestatic stress. [Jessner, Zsembery, & Graf, 2008] Perhaps the additional Al added to the High Al group creates a higher osmolar environment, and hence a noticeably smaller canalicular space. Either way, the impact of a smaller canalicular spaces is unclear.

The comparison of TEM images between the High and Standard Al groups shows an apparent threshold for Al damage. This difference in hepatotoxicity for high and low Al exposure may be because Al is preferentially excreted via the kidney, but at high doses, biliary excretion becomes more important. [Gonzalez, et al., 2007] Only the PN with the High Al contamination causes blunting of the microvilli in both the space of Disse and in the canalicular

space. Meanwhile, the microvilli in both the canaliculi and the space of Disse of the Standard Al group were the same as a sow-fed piglet. This further strengthens the conclusion that it is the Al in the PN that causes the changes to the microvilli. Both regions of microvilli are important because bile acid transporters function on each. For example, the transporter Ntcp sits on the basolateral membrane of the hepatocyte and moves bile acids into, and out of, the space of Disse. [Alrefai & Gill, 2007] If the microvilli are blunted on both ends of the hepatocyte, then bile flow and synthesis could be greatly inhibited.

Additionally, there were other differences between the sow-fed piglets and the PN groups, such as the Standard Al group developing a larger canalicular area. It is unclear why the Standard Al group would have a larger canalicular area than either the High Al group or the Reference group. This may merely be an artifact attributable to the different genetic predispositions of our small group. For example, the Reference group has only four animals, and a variation in canalicular size in one could significantly skew the results. Finally, regardless of the amount of Al exposure, there were increased amounts of electron dense deposits in the mitochondria of PN piglets. These electron dense deposits are felt to be comprised of calcium, and appear when the cell is facing considerable oxidative stress, such as in an ischemic/reperfusion injury in neurons. [Solenski, diPierro, Trimmer, Kwan, & Helms, 2002; Singh, White, & Bloor, 1980; Yoshimura, et al., 2003] In our study it is unclear if they are directly related to the Al toxicity, but it underscores the presence of oxidative stress in the hepatocyte with prolonged PN exposure.

Neither the High nor Standard Al group developed elevated serum bile acids, nor was there a difference in the total serum bile acids between the two groups. When compared to the Reference piglets, the serum bile acids for both of the PN groups were actually much lower. This is unexpected based on previous animal models of Al and PNALD. For example, in rats Al given parenterally for two weeks caused hyperbilirubinemia. [Klein, et al., 1988] The lack of rise in serum bile acids in our study is most likely due to the early stage of PNALD represented in our model. Although the piglets develop much more rapidly than human infants, (with a two-week old piglet representing a two- month infant) [Book & Bustad, 1974], this does not necessarily mean that the hepatic damage is also expedited. The stage of PNALD in our model may be closer to the early PNALD seen in infants with only 2-3 weeks of PN exposure, where

only one quarter of infants have elevated serum bile acids. [Beale, et al., 1979; Arnold, 2004] Alternatively, our piglets may be too old at the start of the study. For comparison, a previous piglet PN study developed elevated serum bilirubin with two weeks of PN exposure, but the piglets were pre-term.[Vlaardingerbroek, et al., 2014] Perhaps, in a rapidly growing piglet species, our 3-6 day old piglets have livers that are more mature and resistant to cholestasis. Another contributing factor may be that both PN groups were given SMOFlipid, which is a less inflammatory lipid solution, containing the anti-inflammatory α-tocopherol. [Klek, et al., 2013; Goulet, et al., 2010] This lipid solution may have delayed the onset of serum markers of PNALD, an effect that has been observed in other lipid solutions containing omega-3 fatty acids. [Premkumar, et al., 2013; Puder, et al., 2009] The Reference piglets meanwhile, had a much higher average serum bile acid level, presumably because bile acids are excreted in response to oral intake and these piglets were frequently feeding. [Costa, et al., 2010; Guglielmi, et al., 2008; Hofmann, 1995; Lemoy, Westworth, Ardeshir, & Tarara, 2013]

8.7 Conclusion:

Al causes ultrastructural changes in the hepatocyte without and potentially before elevation of serum total bile acids. This is worrisome, because it implies that a rise in serum bile acids is not the first indicator of hepatic damage. Additionally, the presence of a less inflammatory lipid environment does not prevent Al-induced ultrastructural changes. High Al caused blunting of the microvilli in both the canaliculi and space of Disse. Plausibly, with longer exposure, the lower Al PN could also cause similar damage. Microvilli are critical to the normal movement of bile acids and are home to bile acid transporters. Impairment of microvilli could be the first step in the pathogenesis of PNALD. Further research into the long-term effects of Al toxicity is needed, as are methods to decrease the contamination of Al in infant PN.

9: THE EFFECTS OF ALUMINUM AND LIPIDS ON BILE ACID TRANSPORTERS IN A HEPATOCYTE MODEL

9.1 Abstract:

Background/Purpose: Parenteral nutrition associated liver disease (PNALD) is a multi-factorial condition which can lead to cirrhosis and liver failure. The pathophysiology is not fully understood, but many different factors contribute, including pro-inflammatory lipids and contaminants such as aluminum (Al). The objective of this study is to examine the effects of Al and lipids on bile acid transporters, (both alone and in combination), to better understand the pathophysiology of PNALD.

Materials and Methods: This was a trial using sandwich-cultured primary rat hepatocytes. After plating and applying a Matrigel overlay, the hepatocytes were divided into six groups with one negative control and five interventions. The first intervention was Al (12µg/mL), the second was 1% mixed lipid solution (SMOFlipid), and the third was 1% omega-6 lipid solution (Intralipid). For the fourth intervention, the Al (12µg/mL) was mixed with 1% SMOFlipid, and the fifth was a mixture of Al (12µg/mL) and 1% Intralipid. After 60 hours of exposure to the Al and/or lipids, the hepatocytes were collected and the bile acid transporters Mrp2, Bsep, Mrp3, Ntcp, and Oatp2 were evaluated using qPCR, Western blot, and a functional assay.

Results: The qPCR results displayed a wide variety of mRNA changes following the exposure to Al and/or lipids. For Western blot, there was no difference in protein amounts between any of the groups for the Ntcp transporter. However, there was significantly more Oatp2 protein in the isolated Al group as compared to the Al + Intralipid group ($p=0.04$). The cholyl-lysl fluorescein assay, which evaluates Mrp2 functioning, demonstrated that although excretion was impaired in all of the intervention groups, it was the worst in both of the groups containing SMOFlipid.

Conclusions: Al, omega-6 lipids, and mixed lipids all have some degree of negative effect on bile acid transporters both alone and in combination. The effects are varied, but SMOFlipid led to a significant downregulation of mRNA for three of the bile acid transporters and also appears to cause the most impairment of Mrp2 functioning. This is the first evidence that the mixed lipid solution may cause more hepatic damage than previously appreciated.

Additionally, at least for Oatp2, the combination of Al and Intralipid led to significantly less protein than with Intralipid alone. This supports the theory that Al and pro-inflammatory lipids may act synergistically, triggering a greater inflammatory response than either separately.

Clinical Relevancy Statement:

Although SMOFlipid has shown promising results in the reversal of serum markers of PNALD, it may have some negative effects on bile acid transporters. Additionally, Al exacerbates the damage caused by pro-inflammatory lipids and further efforts are needed to reduce the contamination of Al in infant PN.

9.2 Introduction:

Pro-inflammatory lipids play an important role in the development of PNALD. [Gura, et al., 2008; Cober & Teitelbaum, 2010; Cowan, et al., 2013] Until recently, the only lipid emulsion used in infant PN was a solution containing mostly omega-6 lipids (Intralipid (Baxter/Fresenius Kabi, Deerfield, IL,USA)). Omega-6 lipids are labeled as pro-inflammatory because they produce arachidonic acid which subsequently creates platelet aggregating thromboxanes and immunosuppressing prostaglandins. [Le, et al., 2009; Gura, et al., 2008; Cowan, et al., 2013] They also have very little vitamin E to balance these pro-inflammatory effects. [Klek, et al., 2013] Omega-3 lipids, meanwhile are felt to be 'hepato-protective' because they produce anti-coagulating thromboxanes and immune-boosting prostaglandins. [Park, et al., 2011; Cober & Teitelbaum, 2010; Cowan et al., 2013] To address this inflammatory imbalance in infant PN, many new lipid formulations have been developed to replace the omega-6 fatty acids, either partially or completely, with omega-3s and other lipid sources. [Cowan, et al., 2013,100] One of the most popular new formulations is SMOFlipid, (Fresenius Kabi, Bad Homburg, Germany) which is a mix of 30% soybean oil (omega-6), 30% medium chain triglycerides (rapid energy source), 25% olive oil (omega-9), 15% fish oil (omega-3) and supplemented α-tocopherol (200mg/L). [Fresenius Kabi, "SMOFlipid", 2017] This formulation still contains omega-6 lipids to prevent essential fatty acid deficiency [Gramlich, et al., 2015], but also contains omega-3 lipids for their anti-inflammatory benefits. (For comparison, Intralipid has approximately three times more omega-6 lipids [Fresenius Kabi, "Intralipid", 2017]) Multiple recent studies in infants with PNALD have demonstrated significant normalization of bilirubin and other serum markers

of cholestasis using SMOFlipid. [Goulet, et al., 2010; Pichler, et al., 2014; Muhammed, et al., 2012]

Al contamination is another potential factor in the development of PNALD. [Gonzalez, et al., 2004; Klein, et al., 1988; Demircan, et al., 1998; Bertholf, et al., 1989] Although this element is relatively harmless when ingested orally, [Sedman, 1992; Poole, et al., 2010; Greger & Baler, 1983] it builds up to toxic levels in neonates on PN. [Courtney-Martin, et al., 2015] Infant PN contains proportionally high amounts of Al contamination [Poole, et al., 2010; Wier & Kuhn, 2012] and because the parenteral route bypasses the gastrointestinal tract, it must be excreted by the kidneys. [Greger & Baler, 1983] Often, the most premature infants also have inadequate renal functioning. [Koo, et al., 1989; Koo, et al., 1986; Bohrer, et al., 2010] and the Al is accumulated in the bones, brain, and liver. [Sedman, 1992] The work of our group and others have demonstrated that the Al causes mRNA downregulation of bile acid transporters and also significantly alters the hepatic ultrastructure, causing shortening of the canalicular microvilli. [Gonzalez, et al., 2004; Klein, et al., 1988; Demircan, et al., 1998; Bertholf, et al., 1989]

Both Al and omega-6 lipids are believed to negatively affect the liver through complementary pathways. The omega-6 lipids trigger an uncontrolled inflammatory response, with increased production of cytokines and pro-inflammatory mediators. [Betteridge, 2000; Cober & Teitelbaum, 2010; Cowan, et al., 2013; Le, et al., 2009; Klek, et al., 2013] Al, meanwhile is believed to work through a mostly pro-oxidative pathway, where it forms superoxide radical ions, participates in oxidative reactions and interferes with mitochondrial functioning. [Alexandrov, et al., 2005; Percy, et al., 2011; Mailloux, et al., 2011; Gura, et al., 2008] The inflammatory and oxidative stress pathways interact and Al and omega-6 lipids work through both pathways to some degree. [Betteridge, 2000; Cowan, et al., 2013; Alexandrov, et al., 2005; Guo & Wang, 2011] The exposure of both Al and pro-inflammatory lipids may therefore negatively alter hepatocytes to a greater extent than either one of the factors alone.

9.3 Objectives:

We hypothesize that both Al contamination and pro-inflammatory lipids in PN cause down-regulation and impaired functioning of bile acid transporter proteins. By addressing both concerns through the reduction of Al and the switch to a more balanced lipid formulation (SMOFlipid), we theorize that there will be better preservation of bile acid transporters, as

compared to either intervention individually. Our objectives are to use a sandwich-cultured primary rat hepatocyte model to build on our findings from the piglet models. We will isolate the effects of each factor of interest: Al, omega-6 lipids (Intralipid) and mixed lipids (SMOFlipid). We will also determine if the negative effects of Al and lipids are additive. The effects on bile acid transporters will be determined through analysis of mRNA in qPCR for five important bile acid transporters: Mrp2, Mrp3, Bsep, Oatp2, and Ntcp. Two of these transporters (Ntcp and Oatp2) will also be evaluated with Western blot to quantify protein. Finally, the functioning of one of the most important bile acid transporters, Mrp2, will be evaluated with a fluorescent assay.

9.4 Methods:

Cell Culture: Primary Sprague-Dawley rat hepatocytes, designed for sandwich-culture were purchased from Lonza (Allendale, NJ, USA) and kept in liquid nitrogen until use. The hepatocytes were thawed and transferred to TRL/Lonza Thawing and Plating Media (Allendale, NJ, USA) and then centrifuged at 100g for ten minutes. The resulting cell pellet was decanted and re-suspended in TRL/Lonza Animal Maintenance Media (a phenol red-free solution designed for primary hepatocytes, Lonza, NJ, USA). Cell viability was confirmed using the Trypan blue exclusion method and for each vial, a viability of >86% was obtained. The cells were then plated in a collagen-coated 24-well plate with a concentration of 600,000 cells/well and a media volume of 0.5mL/well. The plates were shaken gently every 15 minutes to assist in even cell dispersion until one hour after plating, when the media was exchanged for fresh maintenance media. Care was taken with all aspiration of media from the cells, as the hepatocytes were easily dislodged from their collagen base. The cells were then incubated at 37ºC for 4-6 hours in a 5% CO_2 incubator.

Four to six hours after the initial plating, a collagen matrix overlay was placed on the cells to create the sandwich-culture. The frozen Matrigel (Corning Life Sciences, Corning, NY, USA) was thawed to 4ºC and kept on ice throughout the overlay process. The Matrigel was diluted in maintenance media to obtain a final protein concentration of 0.35mg/mL and this solution was also kept on ice, to prevent it from gelling into a matrix. The maintenance media on the cells was then exchanged for the new media containing the Matrigel and the plate was

returned to the incubator to enable the overlay to form in the heat. The maintenance media was replaced daily for 2 days and on the third day the cells were collected.

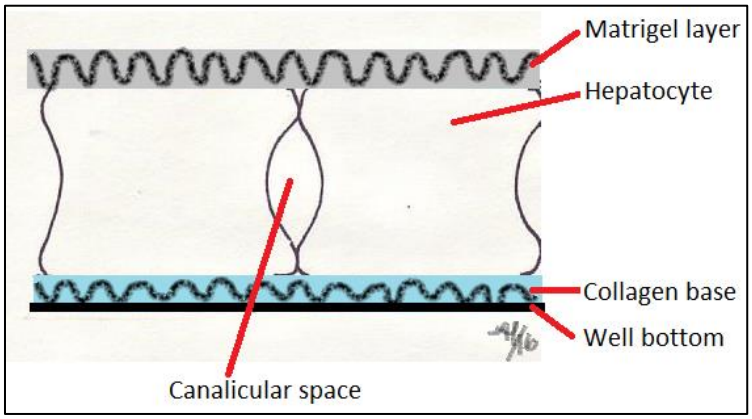


Figure 9.1: Design of sandwich-cultured hepatocytes.

Cell Treatments: This cell model was used to study the impact of Al, omega-6 lipids (Intralipid (Baxter/Fresenius Kabi, Bad Homburg, Germany)) and mixed lipids (SMOFlipid (Fresenius Kabi, Bad Homburg, Germany)) on hepatocytes, both alone and in combination. Three wells in each plate were used as negative controls, where no changes were made to the media. For the next three wells, Al chloride hexahydrate was dissolved in the maintenance media and then sterile filtered, to create an Al solution of 12µgm/mL, a dose that has been proven by Mailloux et al to trigger an inflammatory response in hepatocyte cell cultures. [Mailloux, et al., 2011; Mailloux & Appanna, 2007] The following three wells of cells were exposed to SMOFlipid diluted in maintenance media to create a 1% solution, while Intralipid 20% was also diluted to create a 1% lipid solution for the next three wells. The decision to use 1% lipid solutions was based on the work of Whitfield et al, who demonstrated that this dose not only mimicked the serum lipid level of a child on PN, but also caused changes in cholesterol transporters. [Clayton & Muller, 2000] The fifth set of three wells was exposed to a combination of Al (12µgm/mL) and 1% SMOFlipid and the final set of three wells was given Al (12µgm/mL) and 1% Intralipid. These exposures were continued for approx 60 hours and then the cells were collected and analyzed as described below. The 60-hour time frame was selected after initial experiments demonstrated that the cells began detaching and dying after this time period. According to the manufacturer, the hepatocytes are expected to have normal functioning of transporters within a few hours after overlay of the matrigel.

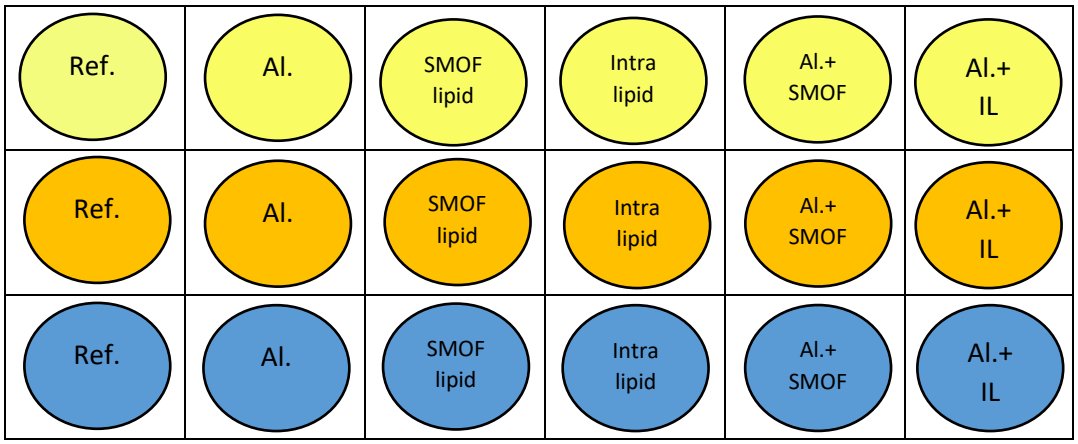


Figure 9.2: Plate layout showing triplicate of each intervention. Yellow wells used for CLF assay. Orange wells used for qPCR. Blue wells used for Western blot. Entire plate was repeated 4x.

Each of the cell treatments was started the day after the overlay was placed on the hepatocytes (ie: approximately 24 hours after original plating). As mentioned above, the maintenance solutions with or without Al and lipids were changed daily. The entire plate experiment was repeated four times.

Quantitative Polymerase Chain Reaction: Bsep, Mrp2, Mrp3, Ntcp, and Oatp2 were examined using qPCR to evaluate the amount of mRNA. Firstly, the PARIS kit (Thermo Fisher Scientific, Waltham, MA, USA) was used to extract mRNA from the cell cultures. The cells were placed on ice to slow down metabolism and then the Cell Disruption Buffer (from the PARIS kit) lyzed the hepatocytes. This lysate was collected, washed and purified through repeated filtrations with the reagents provided by the PARIS kit. The resulting purified mRNA was quantified using a NanoVue spectrophotometer (GE, Boston, MA, USA). An A260/A280 ratio of 1.8 to 2.0 was used as the limit of acceptable mRNA purity. It was then normalized with nuclease free, sterile water to ensure identical amounts of RNA in each sample. Following the normalization step, the VILO Superscript cDNA Synthesis kit (Thermo Fisher Scientific, Waltham, MA, USA) was used to produce cDNA from the mRNA template. For this process, diluted mRNA was combined with the VILO reaction and enzyme mix and subjected to heating in a thermocycler. It was incubated at 25ºC for ten minutes, followed by heating at 42ºC for one hour, and the reaction was terminated with 5 minutes of exposure to 85ºC heat. The resulting cDNA was then added to nucleotides and a fluorescent probe (Power Mastermix, Thermo Fisher Scientific, Waltham, MA, USA) along with both forward and reverse primers. Primers were designed with and obtained from Integrated DNA Technologies (Coralville, IA, USA). See

primer list below. This solution was pipetted in 14.5µL replicates (each containing 2µL of diluted cDNA) into a 96 well PCR plate, sealed and placed in a Real Time cycler (7300 Real Time PCR Systems, Applied Bio Systems, Foster City, CA, USA). The cycler followed a protocol of 30 minutes of incubation at 95ºC, followed by repeated cycling from 95ºC to 60ºC to induce annealing, and synthesis. A dissociation curve was added to the end of each reaction to assess for unwanted primer-dimers and other artifacts. The Ct value was calculated based on the number of cycles required for adequate detection of the probe, allowing for extrapolation of the original amount of mRNA. All primers were tested for efficiency before their use and all primer pairs demonstrated efficiency between 90-110%. [Taylor, et al., 2015] Each sample was run in triplicate.

Target	Forward primer 5'-3'	Reverse primer 5'-3'
Mrp2	CCC AGT CTT CGC TAT CAT CAT C	GCG GGA AGT AGC CAC ATA AA
Bsep	TGA CTG GCA CTG CTA AGA TAT G	GGG TTC CTG AAA TGA GGT TAG T
Ntcp	CGT CTA CAG CAA AGG CAT CTA	TCC CTA TGG TGC AAG GAA TG
Oatp2	CTG ACC ATA ACT CCC ACA GAT AC	GGG AAA GCT GGT CAG GAT ATT
Mrp3	GGC CTG TGC TCA AGA GAT ATA C	CAG CAT ACA GGA GGC AGA TAA A
Beta actin	GGA AAT CGT GCG TGA CAT TAA A	CTC GAA GTC TAG GGC AAC ATA G
GAPDH	GGG AAA CCC ATC ACC ATC TT	ATA CTC AGC ACC AGC ATC AC

Table 9.1: Primers for qPCR

Beta-actin and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were used as the reference genes for qPCR. Beta actin is a structural protein important for cell integrity and GAPDH is glycolytic pathway enzyme. These genes were selected because they are present in high amounts in all cells, which was crucial in a sandwich culture project with extremely low sample volume. In addition, they displayed stability across the different samples. The geometric mean of the reference genes was used for the qPCR calculations and each treatment group was compared to the negative control group. The Pfaffl equation was used for determining fold differences for relative quantitation (explained below in statistical analysis).

Western blot: Protein was extracted from sandwich cultured hepatocytes using the PARIS kit (Thermo Fisher Scientific, Waltham, MA, USA), where cells were lyzed using the Cell Disruption buffer and then mechanically disrupted. The resulting protein pellet was then quantified using a Bradford assay (Thermo Scientific Pierce BCA Protein Assay, Thermo Fisher Scientific, Waltham, MA, USA) and normalized with sterile water. Protein samples were denatured and loaded into a pre-cast graduated 4-12% Bis-Tris polyacrylamide gel with 1xMES buffer solution.(Thermo Fisher Scientific, Waltham, MA, USA) Electrophoresis of samples was conducted at 150V for 30 minutes to separate proteins by size. The separated proteins were then transferred to a 0.2 micron nitrocellulose membrane using a current of 10V for 60 minutes. Transfer of proteins was confirmed with a ponceau stain.

The nitrocellulose membrane was blocked for one hour using 3% skim milk in 1xTBST (tris-buffered saline with tween) for Oatp2 or 3% bovine serum albumin in TBST for Ntcp. It was then washed with TBST and incubated with the primary antibody. For Oatp2, the primary antibody was incubated at room temperature for one hour, while for Ntcp, the primary antibody was incubated at 4ºC overnight. Once the membrane had been exposed to the primary antibody, it was washed with TBS and for both Ntcp and Oatp2, the membrane was incubated with secondary antibody for one hour at room temperature. Finally, it was washed once again and a chemiluminescent solution was added to bind to the horse-radish peroxidase in the secondary antibody. The chemiluminescent signal was then imaged in an AlphaImager Fluor FC2 (Cell Biosciences, Santa Clara, CA, USA).

Once the protein bands had been imaged for either Ntcp or Oatp2, the membrane was washed in a sodium glycine stripping solution for 45 minutes to remove all antibodies. From there, the membrane was washed heavily in TBS (Tris-buffered saline) and re-blocked, this time using 3% bovine serum albumin. After washing off the blocking solution, the primary antibody for GAPDH was placed on the membrane and it was incubated for one hour at room temperature. The membrane was washed once more and then the secondary antibody for GAPDH was incubated for another hour at room temperature. Lastly, the chemiluminescent solution was added to bind to the horse-radish peroxidase in the secondary antibody and the membrane was imaged for the second time in the AlphaImager. This stripping protocol was necessary because the reference protein, GAPDH, was very similar in weight to both of the target proteins (37kDa

for GAPDH vs 38kDa for Ntcp and 74 kDa for Oatp2) and so the membrane could not be cut reliably without damaging either the target or reference protein bands. Similar to the qPCR experiments, GAPDH was used as the reference protein for Western blot, because of its high abundance, and stability across samples.

The primary antibody for Ntcp was a polyclonal rabbit anti-rat (Abcam, Cambridge, UK, Ab131084). It was used in a 1:10,000 dilution with a 9:1 solution of TBST and 3% bovine serum albumin:TBST. The secondary antibody was goat anti-rabbit horse-radish peroxidase conjugated, (Abcam, Cambridge, UK, ab6721) used in a 1:2500 dilution (diluent was a 9:1 solution of TBS and 3% bovine serum albumin: TBST). For Oatp2, we used a polyclonal, rabbit-anti rat, primary antibody in a dilution of 1:1000 (Abcam, Cambridge, UK, ab105124). The secondary antibody was the same as for Ntcp, except that the diluent had 3% skim milk substituted for the bovine serum albumin.

The GAPDH primary antibody was a monoclonal mouse anti-rat antibody (Proteintech, Chicago, IL, USA, 60004-1-Ig), used in a dilution of 1:10,000 with the same diluent as for Ntcp. The secondary antibody was a horse-radish peroxidase conjugated donkey anti-mouse product (Novex/Thermo Fisher, Waltham, MA, USA, A16017) used in a dilution of 1:2000, with the same bovine serum albumin and TBS diluent as for Ntcp.

The digital images obtained from the AlphaImager were inverted and analyzed using the open source program ImageJ. (https://imagej.net/Downloads) Densitometry measurements were conducted on the bands and each band of Ntcp or Oatp2 was normalized to its corresponding GAPDH reference band.

Cholyl-Lysyl-Fluoroscein assay: Cholyl-Lysyl-Fluoroscein (CLF by Corning, Corning, NY, USA) is a fluorescent-labeled bile acid which is excreted from the hepatocyte into the canaliculi by the apical bile acid transporter Mrp2. The presence of this substance in the canaliculi indicates healthy functioning of this bile acid transporter.

On the third day after cell plating, one well from each group, including one well of the negative control group, was selected and the maintenance media was replaced with fresh, pre-warmed, and non-altered TRL/Lonza Maintenance media. The plate was then incubated at 37ºC for 10 minutes. A 5µM solution was created by diluting the CLF in warm maintenance media.

The clean maintenance media in the plate was then replaced with this CLF solution, (0.5mL per well). Incubation was repeated for 15 minutes at 37ºC. The wells were washed three times with ice cold maintenance media to halt cell activity and remove excess solution, and the plate was viewed under a ZOE (Biorad, Hercules, CA, USA) fluorescent microscope.

Statistical Analysis

All statistical analysis was carried out using SPSS version 22 (IBM, Armonk, NY) and SAS (SAS Institute, Cary, NC). For the qPCR data, the fold difference between each cell treatment and the negative control was calculated using the Pfaffl equation (see equation 5.1). [Pfaffl, 2011] The fold differences were calculated for each treatment (ie: lipid/Al) compared to the negative control and then the median fold difference was determined based on the results of the four plates. If a fold difference could not be calculated for at least two plates, it was discarded. Because the replicates of this study are small, and it could be considered a pilot study, the use of non-parametric analysis (ie: median) is more appropriate. The numbers were too small for any other non-parametric test such as Wilcoxon-Signed-Rank test. The median fold difference was therefore used alone and was significant if it was equal to, or greater than two, or less than 0.5 (a standard cut-off for significant fold difference in qPCR). [University of Montreal, "Information on qPCR dates", n.d.] Only significant fold differences will be discussed. Fold differences of greater than two demonstrate an mRNA expression in favour of the negative control group (ie: the treatment under investigation causes downregulation). Whereas, fold differences less than 0.5 represent favourable mRNA expression for the treatment group as compared to the negative control group (ie: upregulation in the lipid or Al group).

For the Western blot, the non-parametric equivalent of the T-test, the Mann-Whitney analysis, was used to evaluate the densitometry measurements and a p value <0.05 was considered statistically significant.

The analysis for the CLF assay was performed subjectively, because although there is technology that can quantify the fluorescence objectively, we did not have access to it for this experiment.

9.5 Results:

RT-qPCR: Effects of the Al and lipid exposures on bile acid mRNA expression were evaluated using qPCR. As the experiment is very small with non-parametric data, only median values are reported.

When examining Mrp2, both SMOFlipid alone and in combination with Al caused a significant increase in Mrp2 mRNA, as compared to the negative control (median fold differences of 0.06 and 0.21 respectively).

Meanwhile, the apical transporter Bsep had a down-regulation of mRNA for isolated SMOFlipid but an up-regulation when SMOFlipid was combined with Al, as compared to the negative control group (fold differences of 6.95 and 0.08). Additionally, the group with both Al and Intralipid had more Bsep mRNA than the negative control group (median fold difference of 0.33).

Oatp2 also had more mRNA for the combination of Al and SMOFlipid as well as for Al and Intralipid (median fold differences of 0.12 and 0.31), as compared to the negative control group. The Oatp2 mRNA was decreased however in the isolated SMOFlipid group, as compared to the negative control group (median fold difference of 4.65).

All isolated exposures of Al, SMOFlipid and Intralipid produced less Ntcp mRNA than was observed in the negative control group. These median differences were 2.10, 4.59, and 2.07, respectively.

Lastly, in the examination of Mrp3, only isolated Intralipid caused any significant response, where there was less mRNA than in the control group, with a median fold difference of 2.29.

	Mrp2 (median fold difference)	Bsep (median fold difference)	Mrp3 (median fold difference)	Ntcp (median fold difference)	Oatp2 (median fold difference)
Al	No difference	No difference	No difference	2.10	No difference
Intralipid	No difference	No difference	2.29	4.59	No difference
SMOFlipid	0.06	6.95	No difference	2.07	4.65
Al+Intralipid	No difference	0.08	No difference	No difference	0.12
Al+SMOFlipid	0.21	0.33	No difference	No difference	0.31

Less mRNA as compared to the negative control group

More mRNA as compared to the negative control group

Table 9.2: qPCR results. All interventions are compared to the negative control group.

Western Blot: The results of the four plates were combined and compared for both Ntcp and Oatp2. Unfortunately, we could not find a viable antibody for Mrp2, Mrp3, or Bsep for use in Western blot.

In the Ntcp Western blots, there was not a statistically significant difference between any of the interventions and the normal, negative control group, using the Mann-Whitney analysis. The isolated treatments of Al, SMOFlipid and Intralipid as compared to the negative control were the same with p values of 0.51, 0.16, and 0.29. When comparing the combined treatments to the negative control, the p values were close but still not significant, with p=0.13 for the Al+SMOFlipid group and p=0.08 for the Al+Intralipid group. Likewise, there was no difference when comparing the isolated Al group to either of the Al and lipid groups, although when comparing the isolated Al to the Al+Intralipid group, there was a borderline p value of 0.08. Of note, there is some variability in some of the GAPDH bands, and this is likely due to inconsistent protein quantity between samples. Although we did normalize the protein samples, we could not evaluate the type of protein normalized and the matrigel protein was likely included in various amounts in the samples. With such small amounts of cells, we could not repeat the Western blot again to verify this discrepancy.

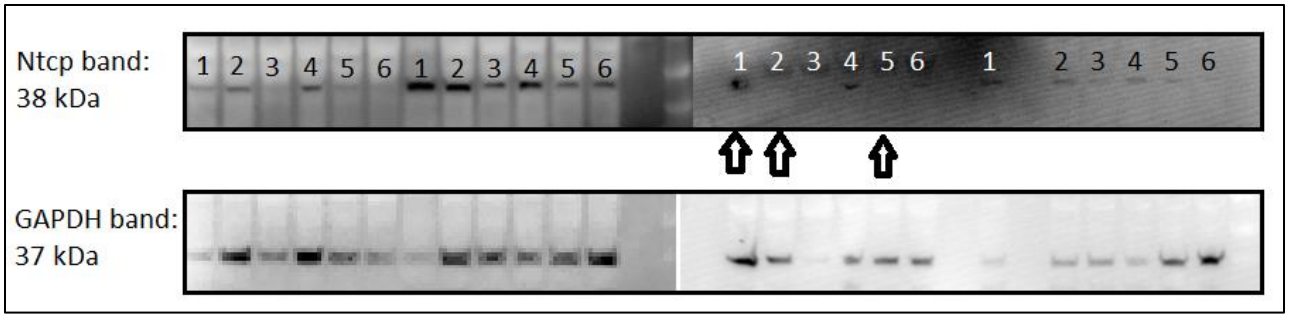


Figure 9.3: Western blots for Ntcp. Lane labels: 1: Reference; 2: Al; 3: SMOFlipid; 4: Intralipid; 5: Al+SMOFlipid; 6: Al+Intralipid. Arrows indicate failed lanes.

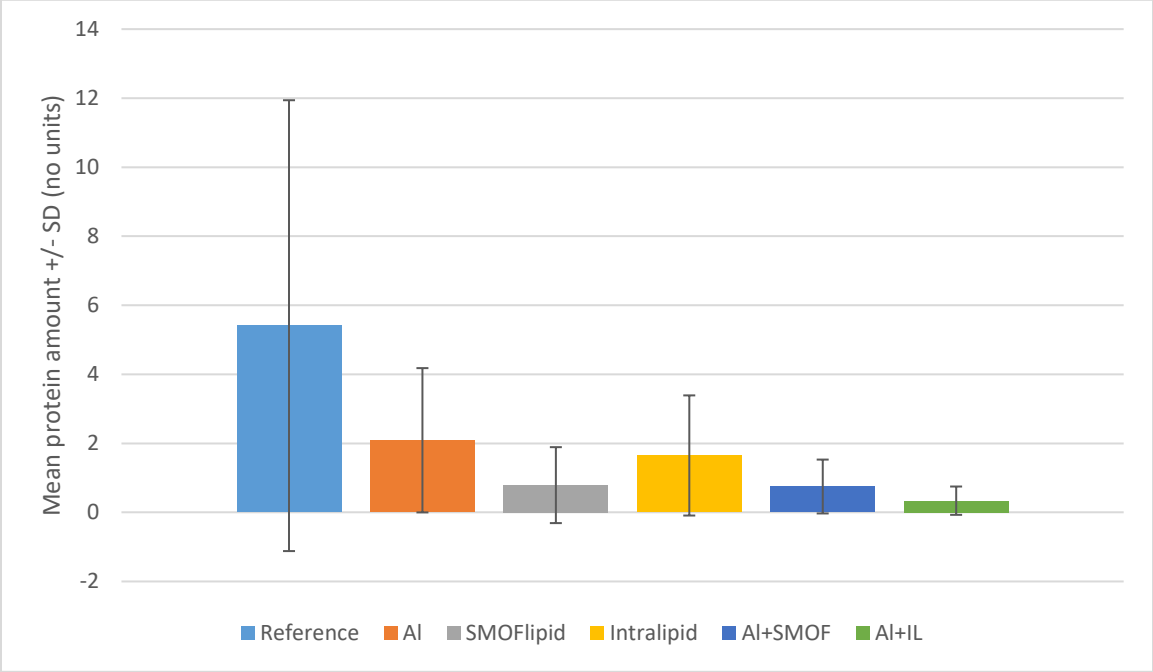


Figure 9.4: Ntcp densitometry results.

The results for Oatp2 again showed no significant difference for any of the individual or combined exposures. The closest any exposure came to significance was the Al+Intralipid group as compared to the negative control group, which had a p-value of 0.08. However, when comparing the isolated Intralipid to the Al+Intralipid group, there was significantly more protein in the isolated Intralipid group (p=0.04). As in the Ntcp experiment, there was still no significant difference when comparing the isolated Al groups to either of the combined groups.

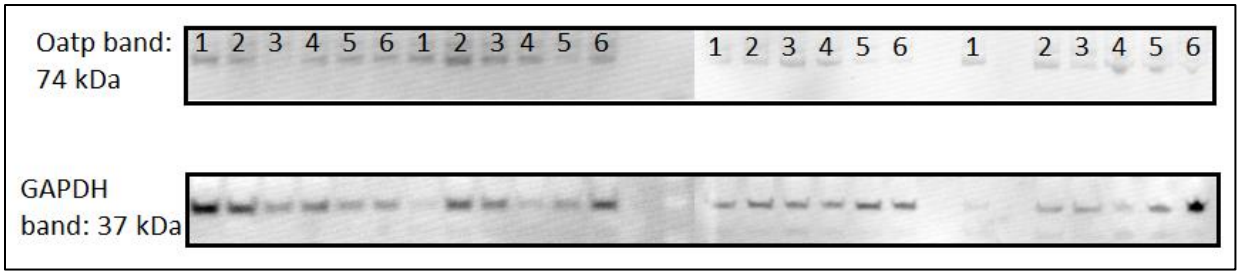


Figure 9.5: Western blots for Oatp2. Lane labels: 1: Reference; 2: Al; 3: SMOFlipid; 4: Intralipid; 5: Al+SMOFlipid; 6: Al+Intralipid.

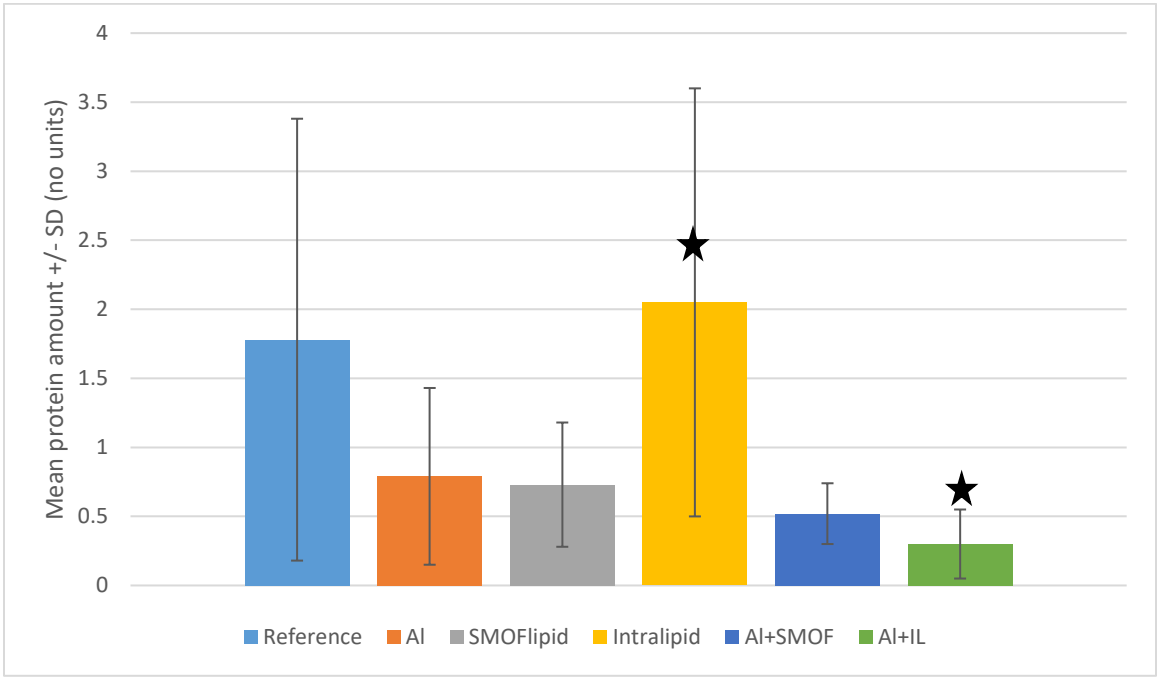


Figure 9.6: Graph of Oatp2 densitometry measurements. Stars indicates two groups with a significant difference between them.

CLF assay: As mentioned above, the interpretation of the CLF assay and the functioning of the Mrp2 transporter is subjective. Overall however, it appears that the CLF bile acid was well secreted in the negative control plate of hepatocytes, and that the isolated Al wells had mildly ballooned canalicular space and fewer areas of functioning excretion. Compared to the normal wells, both of the wells containing SMOFlipid (with or without Al) had virtually no excretion at all, indicating poor functioning of Mrp2. The Intralipid wells did have a few preserved areas of canalicular excretion. Representative images of the CLF assay are presented in Figure 9.7.

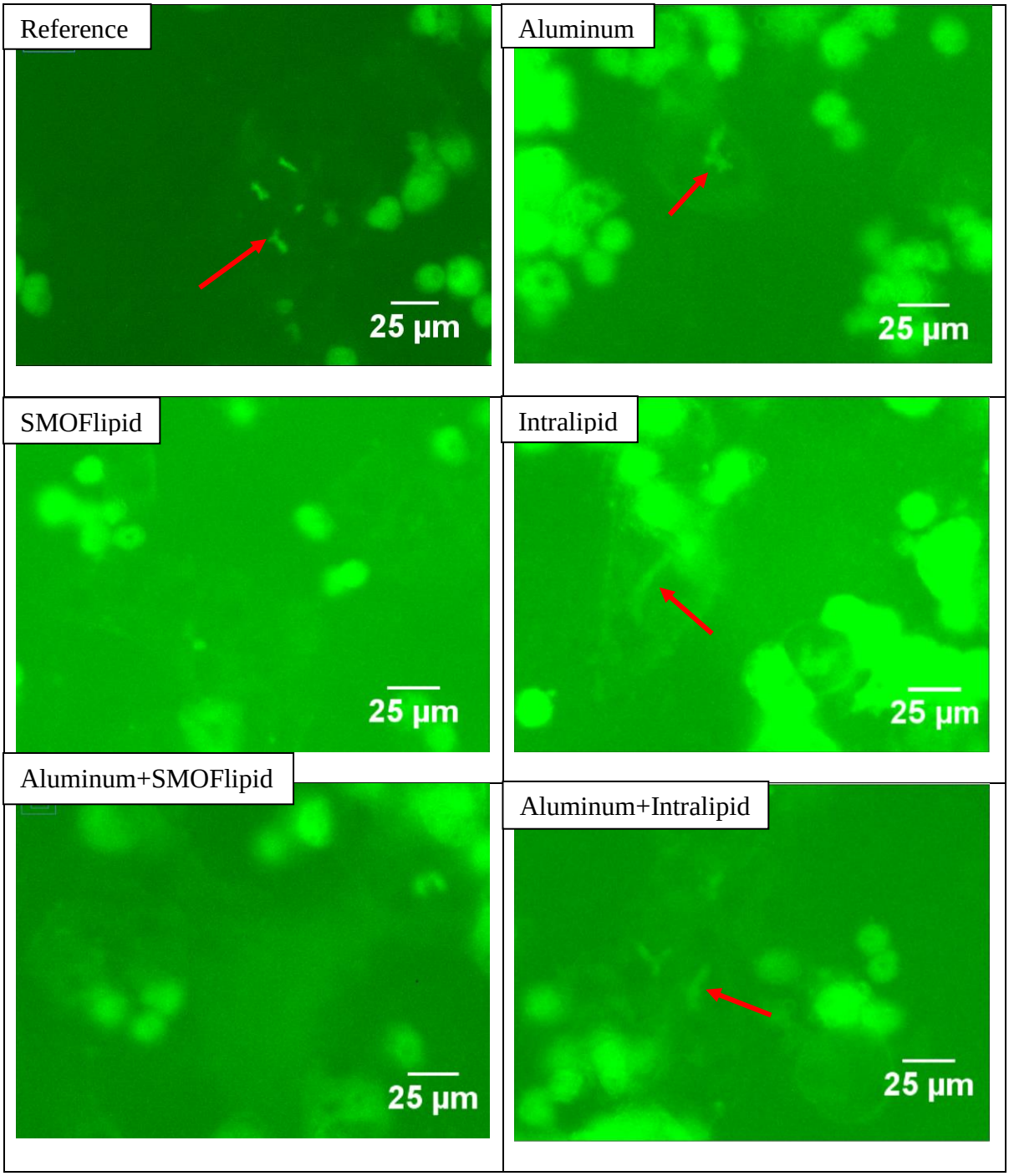


Figure 9.7: Representative images of CLF assay. Red arrows indicate canalicular spaces. Bright green masses are cells that have taken up CLF but are unable to excrete it.

9.6 Discussion:

The goals of this study were to use a simpler model to identify changes in bile acid transporters that could be attributed to Al, lipids, or a combination or both. This is the only one

of our studies that enables a comparison of the effects of two different types of lipids, both with and without Al.

The qPCR results were varied across the exposure groups, but a few findings warrant further investigation. As with all of our previous mRNA work, the end protein result is unknown for all of the transporters (except Ntcp and Oatp2), so our conclusions are mostly speculative. Firstly, the exposure of SMOFlipid alone, which was designed to be less inflammatory than other lipid mixtures, [Goulet, et al., 2010; Picher, et al., 2014; Muhammed, et al., 2012] caused a decrease in mRNA for three bile acid transporters: Bsep, Oatp2, and Ntcp. Perhaps some of the non-omega-6 lipid components in the mixture had an unexpected interaction with bile acid transporters or the small amount of omega-6 lipids in SMOFlipid [Fresensius Kabi, "SMOFlipid", 2017] was somehow sufficient to cause an inflammatory response or directly trigger nuclear receptors such as FXR.[Pascussi, et al., 2003; Vanwijngaerden, et al., 2011; Anderjko, et al., 2008; Geier, et al., 2005; Hartmann, et al., 2002] FXR especially has well defined mechanisms to both directly, and indirectly, decrease the amount of these three transporters, [Kullak-Ublick, et al., 2004; Jung, et al., 2002] so if there is an inflammatory mechanism for SMOFlipid, it may act through this nuclear transcription factor.

Interestingly, isolated Intralipid, which is mostly omega-6 lipids [Fresenius Kabi, "Intralipid", 2017] and perceived to be the most inflammatory lipid solution, [Gura, et al., 2008; Cober & Teitelbaum, 2010; Cowan, et al., 2013] caused a decrease in mRNA for only two transporters: Ntcp and Mrp3. The effect on Ntcp has a logical explanation, as pro-inflammatory cytokines released by Intralipid exposure cause an FXR and HNF-1a -regulated downregulation of Ntcp. [Pascussi, et al., 2003; Anwer, 2004; Perez, et al., 2006; Clay & Hainline, 2007; Green, et al., 1996; Vanwijngaerden, et al., 2001] The implications are unclear for the decrease in Mrp3 mRNA. Usually in situations of increased bile acids and inflammation Mrp3 is upregulated, but the cell culture may not have sufficient bile acids to trigger Mrp3. [Zelcer, et al., 2006; Kruh, et al., 2007; Vanwijngaerden, et al., 2001; Jenniskens, et al., 2016]

Al exposure alone decreased only Ntcp mRNA. Without measuring inflammatory markers, we cannot know the exact reason, but it is possible that the Ntcp mRNA is decreased because of the cytokine-induced inflammatory pathway involving nuclear transcription factors FXR and HNF1-a. [Green, et al., 1996; Vanwijngaerden, et al., 2011; Andrejko, et al., 2008;

Geier, et al., 2005; Cherrington, et al., 2004] In addition to its participation in oxidative reactions, Al also causes an increase in cytokines such as TNF-α [Alexandrov, et al., 2005; Guo & Wang, 2011] and either oxidative stress or this cytokine surge could explain the link between Al and the decrease in Ntcp mRNA.

Meanwhile, the combination of Al and either type of lipid caused an upregulation of mRNA for the transporters Mrp2, Bsep, and Oatp2. Interestingly, when the hepatocytes in our experiment were exposed to either isolated Al or lipid, we observed mostly decreases in mRNA (as compared to the negative control). But when the hepatocytes were exposed to a combination of Al and lipids, the response was either an increase in mRNA or no change. This dichotomy may have a number of explanations. Potentially, the hepatocyte has a different tactic when faced with multiple insults as opposed to a single one. Lipids are hepato-toxic mostly through inflammatory mechanisms, [Gura, et al., 2008; Cober & Teitelbaum, 2010; Cowan, et al., 2013] while the toxicity of Al is mostly due to oxidative stress, with a smaller component of inflammation. [Percy, et al., 2011; Mailloux, et al., 2011; Gonzalez, et al., 2007; Alexandrov, et al., 2005; Guo & Wang, 2011] Although these mechanisms usually supplement each other, [Dinh, et al., 2014] the exact interactions of Al and lipid toxicities are unknown. Regardless of the reason, it appears that very few of the combination groups had the same effect on a transporter as any individual exposure did. Clearly, the interaction between Al and the lipids is more complex than adding the two effects together. These are only pilot studies and further repetitions, including studies with different dosing of Al and lipids may reveal more logical patterns of bile acid transporter change. mRNA findings do not always translate into changes in protein changes, especially in small studies such as this one. Overall, the addition of Al, SMOFlipid, and Intralipid either alone, or in combination, may cause changes to the mRNA of bile acid transporters, but further investigation is required.

Unfortunately, Western blot could be completed only on Ntcp and Oatp2, but the results indicated that the combination of Al and lipids do have a negative impact. In alignment with the qPCR results, all of the treatments led to a decreased amount of Ntcp expression, but none of these changes were significantly less than the normal. The least amount of protein was found in the Intralipid+ Al group where the Ntcp was less than the Reference group with a p value of 0.08. Interestingly, there was also less Ntcp in the Al+Intralipid group than in either the isolated

Intralipid group or the isolated Al group, although neither was statistically significant (p=0.08 for both). This suggests that the combination of the factors is more detrimental to Ntcp than either alone, and that they may have an accumulative effect, but larger studies need to be conducted.

When examining Oatp2 protein, the Al+Intralipid group again had the lowest expression compared the reference group, but was not statistically significant (p=0.08). However, there was significantly less Oatp2 protein in the Al+Intralipid group when compared to the isolated Intralipid group (p=0.04). This implies that although neither Intralipid nor Al alone caused a significant decrease in Oatp2 expression, the combination of factors caused significant impairment.

Finally, the CLF assay showed some surprising results. As expected, the normal group had preserved Mrp2 functioning. Based on the qPCR results, it was difficult to predict the effects of the various lipids and Al on Mrp2 functioning. The Mrp2 mRNA changed very little for any of the groups, except for an increase in mRNA for both the SMOFlipid group and the SMOFlipid+Al group (as compared to the negative control). These two groups are also the only ones that showed significant decrease in Mrp2 functioning in the CLF assay. There was no excretion of the CLF marker in any of the wells with SMOFlipid, either alone or in combination with Al. Perhaps the upregulation of mRNA observed in the qPCR represented the hepatocyte attempting to compensate for an impaired or incorrectly localized transporter. In many other models of cholestasis, Mrp2 is drawn intracellularly [Kojima, et al., 2003 & 2008] and so although we may see a normal amount of mRNA and protein, it is not on the cell surface and therefore non-functional. For an unknown reason, the SMOFlipid interfered more with Mrp2 functioning than any of the other exposures, including isolated Al. If the effects were only due to the pro-inflammatory component of SMOFlipid, then it would be expected that a similar result would be observed in the pure omega-6 Intralipid, but this was not the case. The CLF assay has not been used in this type of solution before, so it is possible that some chemical imbalance of the SMOFlipid impaired the normal uptake of this fluorescent marker and the results are artifacts. Regardless, this finding warrants further investigation into the effects of SMOFlipid.

9.7 Conclusion:

Al, omega-6 lipids, and mixed lipids all cause a variety of effects on bile acid transporters, whether alone or in combination. The effects on mRNA are very diverse, but there

is a difference in the effects of each factor alone as compared to when they are in combination. Western blot for Oatp2 demonstrated that the cumulative effects of Al and Intralipid caused a greater downregulation than either factor alone. Finally, SMOFlipid may cause a displacement or impairment of Mrp2 functioning. Overall, both Al and lipids cause negative changes to bile acid transporters and the effects on bile acid transporters may be synergistic. Larger studies are needed to explore the findings of this pilot project.

10: FINAL DISCUSSION, FUTURE DIRECTIONS, AND CONCLUSIONS

10.1 Main findings:

Our first study examined the prevalence of Al contamination in a NICU and found infants on PN are still at risk of Al-associated toxicities. On average, neonatal PN had almost three times the FDA-recommended limit of Al. Calcium gluconate appeared to be the most likely contributing source, but we could not confirm this because we did not analyze individual components.

Our NICU study was then followed by two piglet PN projects, where we focused on the role of Al and lipids in PNALD. The first of these projects used the Yucatan miniature piglet PN model with an omega-6 lipid source (Intralipid, Fresenius Kabi, Bad Homburg, Germany) and investigated the effects of High (63µg/kg/day) vs Standard (24 µg/kg/day) Al contamination. The qPCR analysis indicated that four out of the five studied bile acid transporters (Mrp3, Bsep, Ntcp and Mrp2) had decreased amounts of mRNA in the High Al group as compared to the Standard Al group. When the Mrp2 protein was analyzed using Western blot, there was no difference between the two groups. The IHC demonstrated that the relationship between radixin and Mrp2, as well as between radixin and Bsep was well preserved regardless of Al contamination. There was, however, a decreased density of the Mrp2 signal in the High Al group, as compared to the Standard Al group and no significant difference in the Bsep staining between the groups. We also found no difference in the total serum bile acids between the two groups. In summary, this study demonstrated that high Al has a negative effect on the mRNA of bile acid transporters in a piglet PN model.

Our second piglet project repeated the High and Standard Al groups, but we exchanged the pro-inflammatory omega-6 lipids for a more hepato-protective mixed lipid emulsion (SMOFlipid, Fresenius Kabi, Bad Homburg, Germany), to determine if the less inflammatory environment would protect against Al-induced changes to bile acid transporters. The High Al group had a greater increase in CRP than the Standard Al group, indicating a potential inflammatory mechanism for Al. We discovered that Al still decreased the mRNA of some of the bile acid transporters, but only the basolateral ones. There was also no change in the Mrp2 protein amount. Lastly, the co-localization between the cytoskeletal protein radixin and the two apical transporters, Mrp2 and Bsep, was still well-preserved in both groups with IHC and

minimal changes were observed between the groups. Overall, High Al appeared to increase inflammation and decrease mRNA for the basolateral bile acid transporters, despite the presence of the less-inflammatory mixed lipid solution.

We then compared these two piglet PN studies and discovered that lipid composition may be an important factor in the cellular localization and density of bile acid transporters. Both of the SMOFlipid groups, regardless of the Al content, had denser Bsep and radixin staining, as compared to either High or Standard Al groups with Intralipid. The results for Mrp2 IHC were not as clear, but it appeared that generally both groups with Intralipid exposure displayed wider but denser Mrp2 staining when compared to groups with SMOFlipid. Similar to Bsep, the Al content did not appear to correlate with the Mrp2 placement. There was also no difference in the Mrp2 Western blot results for any of the groups. Unfortunately, we were unable to compare the qPCR results due to a lack of consistent reference genes.

With the tissue from the second piglet study, we used transmission electron microscopy to evaluate hepatic ultra-structure. Despite the lack of elevation in serum total bile acids, there were significant ultra-structural changes noted, and these changes correlated with the high Al contamination. When comparing the High Al to the Standard Al group (both of which received SMOFlipid), we noted smaller canalicular spaces and shorter microvilli in the group with High Al contamination. The role of Al in ultra-structural impairment was further supported when the High and Standard Al groups were compared to a sow-fed control group and it was observed that only the High Al group had blunting of the microvilli in both the canalicular space and the space of Disse (as compared to the Reference group).

Finally, we attempted to isolate the effects of Al, omega-6, and mixed lipids on bile acid transporters with sandwich-cultured rat hepatocytes. The qPCR results of this study were varied; for example, isolated SMOFlipid exposure caused a significant decrease in mRNA for three of the studied bile acid transporters, while isolated Intralipid caused a decrease for two of the five transporters studied. Of note, almost all of the transporters studied by qPCR had a different reaction when treated with an isolated (Al or lipid) exposure as compared to a combined exposure. For example, Ntcp was down-regulated with each individual exposure, but unaffected with a combined Al and lipid exposure. Western blot for Oatp2 showed that there was less protein in the group with the combination of Al and lipids, as compared to an isolated Al or lipid

treatment. Finally, the CLF assay was conducted to assess Mrp2 functioning and this demonstrated impaired Mrp2 excretion in either of the groups containing SMOFlipid (both with and without Al). Overall, the relationship between Al and lipids appeared to be complex, but for at least Oatp2 the combination of factors was more detrimental than either alone. SMOFlipid also unexpectedly impaired Mrp2 functioning, requiring further investigation.

10.2 Strengths and limitations of study designs:

We chose to explore the relationship between Al, lipids, and bile acid transporters with three different models: a cell culture model, two animal models, and a clinical model. This multi-pronged approach allowed us to determine which findings were generalizable to different populations and which were specific to certain animal/cell models. Results that were repeated in more than one model would be stronger and more likely to be applicable to the human population. It also allowed us to build on our findings sequentially, with later models designed to address weakness in the earlier ones and to isolate specific results.

We started our work with the study of Al in neonatal PN because we wanted to confirm that Al contamination was a concern issue. The results of this study are the easiest to apply to the general neonatal population because it was conducted in a Canadian neonatal intensive care unit, with random samples of used PN and therefore provided data on current PN practices in Canada. The strengths of this study include the use of a sample size which is the same or larger than most other similar studies [Bohrer, et al., 2010; Alvarez, et al., 2007], as well as the use of a standardized method of Al analysis (inductively coupled plasma mass spectrometry) [Saskatchewan Research Council, 2016]. Additionally, we collected all of our samples from a single NICU, which could be a strength or a weakness. As a strength, using a single institution limits the variation in compounding methods between samples because all PN is compounded and processed at the same pharmacy. As a weakness, it could potentially limit the generalizability of the study if other NICUs and hospitals do not follow the same practices as ours. An additional weakness of this study includes the lack of individual component analysis meaning that we were unable to isolate which one was responsible for the Al contamination.

The piglet studies also had numerous strengths and weaknesses. Among the strengths is the selection of the pig as the study animal. The piglet model is the most translatable for infant nutrition research [Book & Bustad, 1974] because piglets and infants have similar physiology,

anatomy and metabolism. [Miller & Ullray, 1987; Baracos, 2004] Additionally, both species are born at a similar stage of development, with immature gastrointestinal and hepatobiliary systems. [Miller & Ullray, 1987] This is particularly important for PNALD, a disease prevalent in premature infants with under-developed hepatobiliary systems. [Touloukian & Seashore, 1975; Shawn, et al., 2012; Beath, et al., 1996; Javid, et al., 2011] Piglets have almost identical nutritional needs as infants, especially in terms of amino acid requirements [Miller & Ullray, 1987] and PN solutions that support piglet growth can be easily modified for infants. [Miller & Ullray, 1987, Baracos, 2004, Book & Bustad, 1974] All of these features make the piglet the ideal choice for infant PN research and increase the applicability of these findings to the human population.

Other strengths include the randomized control design and the inclusion of a negative control group in the second piglet PN study (the sow-fed piglets). Our piglets came from an isolated research herd and we were able to control for other factors that might contribute to PNALD in infant studies (such as pre-existing diseases). We also used multiple different methods of detecting early changes in bile acid transporters and indicators of PNALD, such as serum markers, ultrastructural changes, and alterations in mRNA, and protein amounts, to gather more complete information.

Some of our weaknesses were predictable for piglet work, while others were unexpected. We had difficulty with infection, because the PN piglets did not benefit from the adaptive immunity conferred from sow's milk [Butler & Sincora, 2007], leaving them highly vulnerable to infection. We also discovered a lack of porcine antibodies required for our work. Ideally, we would have completed Western blot on all of the target bile acid transporters, but antibodies were not available. Likewise, the IHC results would have been more useful if we had been able to identify the cellular localization of the bile acid transporters in relation to the cell membrane, using a membrane antibody. Other weaknesses of our study involve the sample size, which is an issue for many animal studies. Due to the expense and space needed for husbandry of large animals, especially with complex treatments like PN, the majority of similar studies use groups of 7 or 8 animals. [Gonzalez, et al., 2004; Alemmari, et al., 2012; Klein, et al., 1988; Demircan, et al., 1998; Gonzalez, et al., 2007] Despite our efforts to ensure comparable group demographics, the group size may skew results. Additionally, our study would have benefited if

both piglet experiments and analysis had been run simultaneously instead of two years apart. This would have enabled more thorough comparison between the omega-6 and mixed lipid trials, especially for qPCR and IHC analysis, (the latter suffered because of long-term storage). Ideally, we would also have included a group of piglets in both studies with no Al in the PN. If we had included a group with Al-free PN, we could have determined if elimination of Al in PN preserves bile acid transporters and prevent the dysfunction found with the High vs Standard Al study. With our current study, the results must be extrapolated with the assumption that bile acid transporters would further benefit from an Al-free PN solution.

Strengths of the hepatocyte study included the simplicity of the study, with less influence of outside confounding factors such as sepsis (as compared to the piglet study). It also had isolated interventions, so that the effects of Al or lipids alone could be observed, along with a negative control. The weaknesses of this study include the small sample size and less direct translation to humans. With only four replicates, it should be viewed as a pilot study, and the findings need to be repeated in larger trials for verification. Additionally, although rats have many of the same bile acid transporters as humans, they do not act identically. For example, rats do not rely on Bsep to the same extent as humans [Alrefai & Gill, 2007], and they have a much lower level of Mrp3 than humans. [Zelcer, et al., 2006] Moreover, due to limited resources, we did not conduct optimization experiments to determine appropriate concentrations of Al and lipids. Instead, we based our dosages on previous literature. Cell culture studies can not completely replicate the complex body system that they have been removed from. Although we selected one of the most accurate cell models (ie: sandwich-cultured system which enables cellular junctions and polarity), the cells are removed from the chemical signals and influence that would affect in-situ hepatocytes. Primary hepatocytes also have a very limited life-span with no ability to replicate, and so any observable findings must occur quickly (within a few days). Clinical evidence of PNALD is not usually apparent until three weeks of PN exposure [Beale, et al., 1979; Arnold, et al., 2003] and thus it is possible that even cellular changes may take longer than a few days to detect.

10.3 New knowledge:

These projects contribute a number of findings to the field of PNALD research. This includes further knowledge about the potential role of Al in PNALD, the response of bile acid

transporters to various insults, the effects of new mixed lipid solutions, and finally the interaction of Al and lipids.

Aluminum: Firstly, our work identified that Al contamination is still an issue in Canadian neonatal PN, with 90% of the samples containing > 5µg/kg/day of Al (FDA limit, 2004). [FDA, 2004; Hall, et al., 2016] Al contamination in infant PN has already been linked to an osteomalacia-type bone disease, potential neurologic impairment, and anemia. [Gura, 2010; Courtney-Martin, et al., 2015] Our work provides evidence that the Al may have a negative effect on bile acid transporters and could contribute to PNALD in infants. In both piglet models, Al content was correlated with a statistically significant decreased amount of mRNA for bile acid transporters. In the model with omega-6 lipids, High Al contamination negatively affected four out of five of the studied bile acid transporters (Mrp2, Bsep, Ntcp and Mrp3), as compared to the Standard Al group. Meanwhile, in the model with the mixed lipids, only the basolateral transporters Ntcp, Mrp3, and Oatp8 were negatively affected by Al. There was also some evidence in the model with omega-6 lipids that High Al caused a weaker Mrp2 signal in the IHC analysis.

We have postulated that the mechanism of Al toxicity may be through both oxidative stress and inflammatory pathways. This is in contrast to the purely oxidative stress mechanism that is usually attributed to Al. It forms a superoxide radical ion, and works synergistically with iron to participate in oxidative reactions. [Alexandrov, et al., 2005; Gonzalez, et al., 2007] Animals exposed to Al accumulate malondialdehyde (a marker of oxidative stress) along with a decrease in the activities of the anti-oxidants glutathione peroxidase, and catalase. [Gonzalez, et al., 2007 and 2004; Guo & Wang, 2011] There are only a few studies suggesting that Al may also have a pro-inflammatory role. For example, dialysis patients with elevated Al had significantly increased CRP (an acute phase inflammatory marker), along with elevated cytokines TNF-α and interleukin-5. [Gou & Wang, 2011] Similarly, in human neuronal studies, Al induced pro-inflammatory genes. [Alexandrov, et al., 2005] Both the oxidative stress and the inflammatory pathways feed off of each other and Al may contribute to both in our studies.

The role of Al as a pro-inflammatory species is supported by the rise of CRP observed in the High Al group of our piglet trial. Of note, this CRP elevation developed despite the presence of the anti-inflammatory mixed lipid solution. [Goulet, et al., 2010; Pichler, et al., 2014;

Muhammed, et al., 2012] In addition, there is a precedent for inflammation to cause many of our observed alterations in bile acid transporters. For example, both Bsep and Ntcp have well-characterized decreases in mRNA and protein in response to cytokines and the inflammatory cascade. [Andrejko, et al., 2008; Geier, et al., 2005; Elferink, et al., 2004] Feasibly, Al may contribute to the inflammation that is known to be a key component of PNALD.

Oxidative stress has not been well studied in PNALD. In our studies, we did not measure any markers of oxidative stress, so we can not conclude that Al increased oxidative stress. However as mentioned above, Al has a well-accepted oxidative stress mechanism in almost all other studies and therefore it likely contributed to oxidative stress in our model as well. To further support this theory, many of our observed decreases in bile acid transporter mRNA could also be explained as a reaction to oxidative stress. Mrp2, in particular has demonstrated a decreased expression in response to oxidative stress. [Gonzalez, et al., 2007 and 2004; Guo & Wang, 2011] Overall, our work supports an inflammatory mechanism for Al toxicity, whereby bile acid transporters are decreased at the mRNA level, but oxidative stress is likely involved as well and warrants further study.

Another important observation from our work is that Al may cause hepatic structural and mRNA changes very early in the PNALD disease process, before traditional clinical markers of disease. In a clinical setting, serum conjugated bilirubin, bile acids, or liver enzymes are the only methods available to detect the onset of PNALD. [Kelly, 1998; Willis, et al., 2010; Suita, et al., 1999] Previously, it was reported that these serum markers preceded any histological changes in the disease. [Benjamin, 1981; Touloukian & Seashore, 1975] In both of our piglet models however, Al caused changes in both the transporter mRNA and in the microvilli structure before elevation of serum markers indicating PNALD. This novel finding in the timeline of PNALD pathogenesis suggests that Al may be involved in the initial events triggering PNALD. It also indicates that by the time serum markers of PNALD are detected, there may already be significant changes to bile acid transporters and the microvilli that support them.

Why particular bile acid transporters are affected in certain models and not in others is unclear. For example, Al affected the mRNA of almost all of the transporters in the omega-6 lipid model, but only the basolateral transporters in the mixed lipid PN model. If Al causes shortening of the canalicular microvilli, (as seen in the TEM trial), it would be reasonable if the

apical transporters on these microvilli were most frequently affected, but it appears that the basolateral transporters are more sensitive to Al. Perhaps the basolateral transporters are more vulnerable because they have more exposure to Al. These transporters line the space of Disse, [Slonim & Pollack, 2006] where plasma and Al from PN would first be encountered by the hepatocyte. In both of the piglet models, the basolateral transporters were downregulated, but only in the omega-6 model were apical transporters negatively affected also. The Al content did not change between models, therefore the difference in affected bile acid transporters may be secondary to the lipid composition. Perhaps the mixed lipids are capable of preserving the apical transporters, but not the basolateral ones. Studies, such as Alemmari et al (2012) have found Al dense deposits in the lysosomes. [Alemmari, et al., 2012] Lysosomes are critical mediators of the inflammatory response, capable of secreting or degrading cytokines and engulfing toxins. [Ge, Li, Gao, & Kao, 2015] The omega-3 lipids in the mixed lipid solution have been shown to modulate many steps in the inflammatory pathway, including inactivating pro-inflammatory nuclear receptors. [Park, et al., 2011; Tillman, 2013] These same receptors also interact with lysosomes, [Ge, Li, Gao, & Kao, 2015] and may trigger them to engulf Al. Thereby, SMOFlipid may be able to prevent Al from reaching the apical bile acid transporters, by indirectly influencing lysosomes. Regardless of the mechanism, it appears that the effects of Al on bile acid transporters are only partially mitigated by mixed lipids. As mentioned before, however, all of these mRNA changes must be interpreted cautiously, as they are only statistically significant and may not be clinically relevant.

Bile acid transporters: This study both confirmed and added new information about the response of bile acid transporters to various stressors.

The apical transporters Bsep and Mrp2 had a statistically significant decreased amount of mRNA when challenged with High Al in an omega-6 lipid PN, which was expected based on their downregulation in other situations of inflammation and cholestasis. [Li, et al., 2012; Schmitt, et al., 2000; Pasciussi, et al., 2003; Geier, et al., 2005; Elferink, et al., 2004; Hartmann, et al., 2002] The Mrp2 response to Al in particular has been previously seen in rat models as well. [Gonzalez, et al., 2004 & 2007] Interestingly, these apical transporters did not have the same reaction when the Al was isolated (as in the hepatocyte work) or when it was combined with SMOFlipid. These are the combined results of two models from two different species, and

at diverse stages of development, so the pooled results must be interpreted cautiously. Additionally, it is assumed that a decrease in mRNA would translate into a decreased amount of protein, increasing the risk of cholestasis, but this is often not the case. Overall though, it appears that Mrp2 and Bsep are negatively affected by Al, but the combined inflammatory lipids and Al cause the greatest decrease of mRNA.

We discovered that the basolateral transporters, especially Ntcp, were very sensitive to Al. In all studies (piglet and hepatocyte), Ntcp had a decreased amount of mRNA with Al exposure and there was correlating data from the Western blot studies in the hepatocytes that this was translated into a decreased protein amount. Ntcp's downregulation in response to inflammatory cytokines has been well described [Green, et al., 1996; Vanwijngaerden, et al., 2011; Andrejko, et al., 2008; Geier, et al., 2005; Cherrington, et al., 2004] and if Al does initiate an inflammatory cascade, this is likely how it would down-regulate Ntcp.

The Oatp transporters meanwhile, appeared to be negatively affected by the combination of Al and SMOFlipid. In the piglet study with mixed lipids, there was a decrease in Oatp8 with High Al as compared to Standard Al, but not in the similar study with omega-6 lipids. Western blot studies in the hepatocyte work also demonstrated that this mRNA downregulation was carried through to the protein expression. Oatp transporters have a very similar reaction to many of the same cytokines as Ntcp and Al may trigger a downregulation in this transporter through an identical inflammatory pathway. [Andrejko, et al., 2008; Geier, et al., 2005; Haartmann, et al., 2002] However, the High Al only caused this downregulation in the presence of SMOFlipid, suggesting that a component in the SMOFlipid may have contributed to the inflammatory pathway. This is supported by the decrease in Oatp2 mRNA observed with isolated SMOFlipid in the hepatocyte study. SMOFlipid has not been studied in relationship to specific bile acid transporters previously, and this requires further research.

Mrp3 had the most unexpected response of any of the transporters. In almost all of the studies, there was a decrease of Mrp3 mRNA. (For example, in response to High Al in the piglet studies, or isolated Intralipid in the hepatocyte studies). Usually Mrp3 is observed in small amounts and upregulates in cholestasis models to compensate for a loss of Mrp2 functioning. [Zelcer, et al., 2006; Kruh, et al., 2007; Vanwijngaerden, et al., 2011; Cherrington, et al., 2004] Our study showed the opposite and it is unclear whether this indicates that Al is interfering with

the normal upregulation, perhaps by increasing specific cytokines known to cause Mrp3 downregulation (ie: interferon-gamma and TNFα [Le Vee, et al., 2011; Hartmann, et al., 2002]). Alternatively, the decrease in Mrp3 may be an artifact of ontogeny. Rat fetuses have no expression of Mrp3 and the expression gradually increases from birth onwards until plateauing after weaning. [Zhu, et al., 2017] Similarly, mouse Mrp3 is low at birth and does not reach adult levels until 3 weeks of age. [Maher, et al., 2005] Previous work by Vlaardingerbroek, et al., also found that Mrp3 was decreased in response to all types of PN, so perhaps other elements in the PN are involved as well. [Vlaardginerbroek, et al., 2014]

Although we had anticipated changes in the cytoskeletal protein radixin based on other models of cholestasis, [Kojima, et al., 2003 & 2008] there was minimal alteration in the mRNA or protein placement in both of the piglet studies. We did observe some changes in the radixin width and density in the IHC trials, but not in a recognizable pattern. It is possible that radixin is effected in later stages of PNALD. This would correlate with the work of Kojima et al (2008), because they observed changes in radixin but all of their subjects had clinically observable icterus, indicative of advanced cholestasis. [Kojima, et al., 2008]

FXR is one of the most important regulators of bile acid transporters, especially in situations of inflammatory stress [Ananthanarayanan, et al., 2001; Zollner & Trauner, 2009; Green, et al., 1996; Vanwijngaerden, et al., 2011; Andrejko, et al., 2008] but we did not observe any significant changes in this nuclear receptor. Of note, we only studied the mRNA of FXR and it is possible that there might be changes in the protein without mRNA alterations, but there is very little evidence for this. The actions of FXR are influenced by pro-inflammatory and oxidative stress in many other models [Ananthanarayanan, et al., 2001; Zollner & Trauner, 2009; Jung, et al., 2002], but very few studies show that this nuclear receptor is actually down-regulated and neither did we.

Lipids: The inflammatory differences between omega-3 and omega-6 lipids have been thoroughly explored by other researchers. [Le, et al., 2009; Fallon, et al., 2010] However, there is limited pre-existing work on the effects of specific lipids on bile acid transporters and our work contributes new knowledge in this area. From our piglet studies, it appears that lipids may influence the cellular localization and density of transporters (as observed in IHC). Bsep appeared to have a stronger staining for all of the SMOFlipid groups in the piglet PN studies.

This suggests that the Bsep protein cell localization benefits from the less-inflammatory nature of this mixed lipid solution. [Goulet, et al., 2010; Pichler, et al., 2014; Muhammed, et al., 2012]

There were multiple observations suggesting a harmful effect of SMOFlipid on the bile acid transporters Oatp2/8 and Mrp2. In the piglet Mrp2 IHC there was some unexpected displacement of Mrp2 in the SMOFlipid groups, which could not be explained by Al content. Then in the hepatocyte study there was a complete loss of Mrp2 function in both of the SMOFlipid groups. The Oatp results are explained above, but were similar changes were noted in both the piglet and the rat hepatocyte work. SMOFlipid and bile acid transporters have never been studied together before, so our findings are the first to suggest that although SMOFlipid may have beneficial effects such as decreasing inflammation, it may also interfere with specific bile acid transporters.

The reasons for the negative influence of SMOFlipid on these bile acid transporters is unclear. Omega-6 lipids comprise 30% of SMOFlipid solutions and these are pro-inflammatory [Fresenius Kabi, "Smoflipid", 2017] but if the omega-6 component was responsible, then there should be a more significant decrease in mRNA (and function) for Mrp2 and Oatp2/8 observed in the omega-6 (Intralipid) groups and this was not the case. Other components of SMOFlipid, either individually or in combination could explain the negative effect. While the omega-3 and omega-9 components of SMOFlipid are consistently less-inflammatory, [Harvey, Xu, Pavlina, Zaloga, & Siddiqui, 2015; Boisrame-Helms, et al., 2014] the medium chain triglyceride component is more controversial. Medium chain triglycerides are included in the mixed lipid emulsion because they can passively enter the mitochondria and require very little processing by the cell, making them a quick energy source. [Mundi, Salonen, & Bonnes, 2016] However, they also can enhance cell apoptosis and significantly shorten monocyte survival time. [Boisrame-Helms, et al., 2014] Relevant to our study, medium chain triglycerides have the potential to considerably alter cell membrane composition and structure, [Boisrame-Helms, et al., 2014] which could alter bile acid transporters positioned on the cell membrane. As compared to the long-chain triglycerides that comprise other types of lipid emulsions (ie: Intralipid, Fresenius Kabi, Bad Homburg, Germany), medium chain triglycerides can trigger an oxidative stress response in resting neutrophils [Wanten, et al., 1999] and similar oxidative stress situations are known to inhibit Mrp2. [Kojima, et al., 2003; Kojima, et al., 2008] Additionally, medium chain

triglycerides can exacerbate the inflammatory cascade in some sepsis models, possibly by activating pro-inflammatory nuclear receptors. [Boiserame-Helms, et al., 2014] If they caused an increase in inflammatory cytokines, this could also impair both the Oatp transporters and Mrp2. [Schmitt, et al., 2000; Pascussi, et al., 2003; Andrejko, et al., 2008; Geier, et al., 2005] In combination, lipids produce different cellular effects than individually, [Harvey, et al., 2015] therefore it is possible that the omega-6 and medium chain triglycerides together produce an unexpectedly inhibitory effect on the bile acid transporters. Regardless, these proposed mechanisms are purely speculative and further research into SMOFlipid and its components is warranted. The individual components should be isolated and their effects on bile acid transporters studied.

Despite the negative findings mentioned above, we observed many results supporting the expected hepato-protective nature of SMOFlipid. In the piglet PN study with mixed lipids, Al did not cause downregulation of as many bile acid transporters and there were minimal changes in the cellular density and organization of Mrp2 and Bsep secondary to Al (as observed in IHC). Also in the hepatocyte study, the Western blot studies demonstrated that the Intralipid and Al caused significant downregulation of the basolateral bile acid transporters, but the SMOFlipid and Al combination did not. Overall, SMOFlipid does appear to mitigate some of the negative effects of Al, but not all of them.

Aluminum and lipids: Finally, Al and lipids have a complicated relationship. The effects of Al appear to be exacerbated by some lipid combinations, especially omega-6. For example, in rat hepatocyte Western blots for Oatp2, the combination of Al and Intralipid significantly inhibited the bile acid transporter as compared to Intralipid alone. Similarly, in the piglet models Al appeared to cause a decrease in the mRNA for more transporters when paired with an omega-6 as compared to a mixed lipid solution. Other interactions of Al and lipids were less straight forward. It appears that the inflammatory and oxidative effects attributed to both omega-6 lipids and Al are synergistic, but the effects are not consistently additive. The relationship between the two may be clearer with a longer model, and further investigation into inflammatory and oxidative stress markers. SMOFlipid appears to compensate for some aspects of Al-induced toxicity, presumably because of the less-inflammatory nature of this lipid solution. [Goulet, et

al., 2010] Based on our findings, the combination of Al and Intralipid is the most detrimental to bile acid transporters and should be avoided.

10.4 Future directions:

This project has produced many areas for future research. The role of Al in PNALD is not understood and requires more work. Our study is one of the few to suggest that Al may have an inflammatory mechanism in addition to its pro-oxidative nature and both mechanisms should be explored further in this disease setting and others. Animal models, such as the piglet PN model, would allow for measurement of specific cytokines or markers of oxidative stress, which would not only explain more of the pathogenesis of PNALD, but also indicate if there is a role for anti-oxidants or other anti-inflammatories in the treatment. However, if we were to use animal models to explore the mechanisms of Al effects, we would first need to address some of the issues in the piglet model or consider using other animals. For example, the confounding sepsis limited our piglet work and may be less of an issue if we used older animals with established immune systems, or other hardier animal species (ie: rats). If our goal was to examine Al in PNALD specifically, we need to confirm that the animal model actually creates a PNALD state. In our piglet work, we are assuming that the bile acid mRNA changes and hepatic ultrastructural changes are indicative of an early PNALD state, but this may not be accurate. Longer term studies, with more robust monitoring for early indicators of PNALD are required (ie: serum liver enzymes and bilirubin, bile flow analysis, and liver histology). Alternatively, previous PNALD work, using piglets delivered pre-term have shown serum and histological evidence of cholestasis within two weeks, so this could be explored in our model. [Vlaardingerbroek, et al., 2014]

There are also many parts of this project that could not be completed due to resources or time, but which would provide crucial information. For example, if the porcine antibodies could be developed for the remaining bile acid transporters, this would demonstrate whether the changes in mRNA attributed to Al persist into protein down-regulation. Most importantly, functional assays (similar to the CLF assay) should be undertaken to determine if the changes in protein and mRNA actually impact functioning of the bile acid transporters. Many companies offer transporter-specific, quantitative functional assays. For example, the B-CLEAR system by Qualyst Transporter Solutions, (Durham, NC, USA) allows for controlled opening of inter-

hepatic bile pockets (canaliculi), so biliary efflux can be measured. This technology could be paired with cell models designed to have only one type of bile acid transporter or with substrates specific to one particular transporter (ie: taurocholate, specific for Ntcp), so that the function of each bile acid transporter could be objectively measured.

Also, to fully explore the effects of Al in PNALD, future studies must examine the benefits of low Al PN formulations. We assume that the mRNA and protein changes observed in our study could be avoided with the elimination of Al contamination, but this needs to be verified with animal studies. It would also be instrumental to conduct infant-based studies in countries where low-Al formulations are already in place (such as the United Kingdom [UK Medicine and Healthcare Regulatory Agency, 2010]). This could be as simple as conducting a retrospective review of the incidence of PNALD before and after the implementation of plastic vials for calcium gluconate. Or it could be a more intensive study, where we could match neonatal units in countries such as the UK (with plastic calcium gluconate vials) to neonatal units in countries still using glass vials. We could then compare serum Al, liver enzymes, and bile acid levels, in addition to comparing outcomes and incidence of PNALD. We would expect that the infants with low Al PN would have improved hepatic functioning and these results could add impetus to other countries to adopt low Al guidelines.

As a separate area of study, there is room for further work examining the effects of mixed lipid solutions on bile acid transporters. These solutions are relatively new and long-term data is lacking. [Wales, et al., 2014] Current clinical studies indicate that SMOFlipid resolves PNALD in most cases based on serum markers, [Pichler, et al., 2014] but this may not correlate with histological changes. Larger animal or cell studies are needed to investigate our findings regarding SMOFlipid and Mrp2, and Oatp2/8. Long-term follow-up of infants on SMOFlipid is also essential, to document any unexpected adverse hepatic effects.

There are still many unanswered questions about PNALD. Given that we now have evidence of changes in hepatocytes before elevation in serum markers, there is concern that the true extent of hepatic damage may be missed in infants with normal serum bile acids. Animal and clinical studies are needed to determine the natural history of hepatic ultra-structural changes in PNALD, both for infants that eventually wean off of PN and for those that do not. This could entail an animal study where PNALD is confirmed in the animals on PN (with both serum

markers and liver biopsy histology) and then the PN is weaned off and the animals are re-examined after cessation of PN (ie: at 6 months, 1, and 2 years post-PN). Infants who successfully wean off of PN (with and without mixed lipids) could still be followed long-term to determine if there are long-lasting hepatic effects, as determined by incidence of liver disease and serum liver function tests.

Other possible areas of research include the connection between bile acid transporters, nuclear transcription factors and other mediators important in PNALD. This project assumes that a decrease in bile acid transporters increases the risk of PNALD, but this relationship is not established. For example, infants born lacking Mrp2 do not develop PNALD-type cholestasis. [Alrefai & Gill, 2007] Perhaps there is a combination of bile acid transporter that when down-regulated produce PNALD. Or perhaps other factors in the hepatocyte are responsible for the disease and the observed downregulation of bile acid transporters is a by-product. Studies exploring this could be done using animal models, where FXR is turned on or off, using agonists such as Obeticholic acid (OCA), a 6α-ethyl derivative of the natural human BA chenodeoxycholic acid (CDCA). [Ali, Carey, & Lindor, 2015] These animal models could be with and without PN, allowing us to observe whether a PNALD-type cholestasis develops when the bile acid transporters controlled by FXR are inactivated. Equally important, other factors outside of bile acid transporters should be included in future studies, including CYP7A1 protein, and transporters in the ileum. These proteins have been found to be significantly altered in other models of PN, [Vlaardingerbroek, et al., 2014] and they may explain some of the discrepancies in our bile acid transporter results.

10.5 Significance and recommendations:

PN has revolutionized neonatal care and has improved in safety since it was introduced decades ago. However, this supportive treatment can still contribute to infant mortality with PNALD. Premature infants have immature, vulnerable livers and a relatively small insult, such as Al contamination, could potentially cause life-long effects. Our studies provide evidence that Al has a negative effect on bile acid transporters, seemingly very early in the disease and with more of a pro-inflammatory effect than previously appreciated. Even with the use of hepato-protective lipids, Al is still capable of changing bile acid transporters in a potentially deleterious manner.

There are many methods of reducing Al and avoiding Al-induced hepato-toxicity completely. PN products with high Al contamination (such as calcium gluconate) should be labeled with more accurate information about Al. Notably, in Canada there are no rules limiting the amount of aluminum contamination in PN and Canadian manufacturers of PN components are not required to list the aluminum content on their products. [Courtney-Martin, et al., 2015] For the few products that do list Al content, the labeling could be improved. For example, calcium gluconate includes a package insert warning of the risk of Al toxicity, but the label lists the expected Al content at the time of expiry. Al accumulates in a time-dependent manner, [Li, 2005; Bohrer, et al., 2001; Bohrer, et al., 2003; Bohrer, et al., 2009] so using the expiry estimate of Al is not accurate for most PN calculations. [Poole, et al., 2010; Smith, et al., 2007] Instead, the package insert should indicate when the calcium gluconate would be expected to have a dangerous level of Al. This data could easily be extrapolated from existing studies on Al leaching from glass. [Bohrer, et al., 2001; Bohrer, et al., 2003; Bohrer, et al., 2009]

Calcium gluconate could be exchanged for calcium chloride, as the latter compound has considerably less Al contamination, but the increased chloride content could cause acidosis and it precipitates easily with phosphate products, leading to PN compounding issues. [Bishop, et al., 1997; Canada TW, 2006] Alternatively, the current 10% calcium gluconate could be switched to a 5% solution. The 10% solution requires additional heating and processing, which in turn increases the Al leaching from glass. [Bohrer, et al., 2003; Pharmaceutical Partners of Canada Ltd., 2015] If calcium gluconate were produced as a 5% solution, the Al leaching could be decreased and the resulting product would still be useful in PN formulations.

The most direct method to reduce Al would be to change the packaging of calcium gluconate from glass to plastic. Glass containers contribute the majority of PN Al through leaching of the vials into their contents, especially during the heat-based sterilization process. [Bohrer, et al., 2003; Bohrer, et al., 2002] Some countries such as the United Kingdom, [UK Medicines and Healthcare Regulatory Agency, 2010] prohibit the glass storage of calcium gluconate for infant use and recommend that it be stored in polyethylene containers instead. Polyethylene has negligible Al content, even when sterilized [Bohrer, et al., 2003] and recent studies have debunked concerns that it could impair male infant fertility. [Yokel & Unrine, n.d.]

A new calcium gluconate PN product stored in plastic, has also just been introduced to the American market. [Yokel & Unrine, n.d.]

As for the new mixed lipids, they appear to partially protect the liver against Al toxicity, but further research needs to be done to determine the long-term impacts on hepatic functioning. If the changes in bile acid transporters caused by SMOFlipid that we observed in our work are corroborated in other studies, it may be necessary to re-evaluate the formulation. Additionally, normalization of serum markers of PNALD in children on PN with mixed lipids [Piper, et al., 2009; Muhammed, et al., 2012; Goulet, et al., 2010] does not guarantee normal liver histology. [Mercer, et al., 2013] Long-term follow-up of infants with prolonged PN exposure is needed.

The best preservation of bile acid transporters is likely with a balanced lipid solution and minimal Al contamination. Just as PNALD is a multi-faceted disease, the most effective treatment will also be multi-pronged, including improvement in both lipid composition and Al contamination.

11. REFERENCES

Adler, M., Chung, K.W., & Schaffner, F. (1980). Pericanalicular hepatocytic and bile ductular microfilaments in cholestasis in man. *American Journal of Pathology*, 98(3):603-16.

Advenier, E., Landry, C., Colomb, V., Cognon, C., Pradeau, D., … Corriol, O. (2003). Aluminum contamination of parenteral nutrition and aluminum loading in children on long-term parenteral nutrition. *Journal of Pediatric Gastroenterology & Nutrition*, 36(4):448-453.

Alemmari, A., Miller, G.G., Arnold, C.J., & Zello, G.A. (2011). Parenteral aluminum induces liver injury in a newborn piglet model. *Journal of Pediatric Surgery*, 46(5):883-7.

Alemmari, A., Miller, G.G., Bertolo, R.F., Dinesh, C., Brunton, J.A., Arnold, C.J., & Zello, G.A. (2012). Reduced aluminum contamination decreases parenteral nutrition associated liver injury. *Journal of Pediatric Surgery*, 47(5):889-94.

Alexandrov, P.N., Zhao, Y., Pogue, A.I., Tarr, M.A., Kruck, T.P., … Lukiw, W.J. (2005). Synergistic effects of iron and aluminum on stress-related gene expression in primary human neural cells. *Journal of Alzheimers Disease*, 8:117–127.

Ali, A.H., Carey, E.J., & Lindor, K.D. (2015). Recent advances in the development of farsenoid X receptor agonists. Annals of Translational Medicine, 3(1): 5.

Alrefai, W.A., & Gill, R.K. (2007). Bile acid transporters: structure, function, regulation and pathophysiological implications. *Pharmaceutical Research*, 24(10):1803-1823.

Alvarez, L., Rebollido, M., Fernandez-Lorenzo, J.R., Cocho, J.A., & Fraga JM. (2007). Electrothermal atomic absorption spectrometry determination of aluminium in parenteral nutrition and its components. *Journal of Trace Elements in Medicine and Biology*, 21(suppl 1):29-30.

American Academy of Pediatrics Committee on Nutrition. (1986). Aluminum toxicity in infants and children. *Pediatrics*, 78:1150-1154.

American Public Health Association, American Water Works Association, Water Environment Federation. (1999). *Standard methods for the examination of water and wastewater*. Retrieved from http://www.mwa.co.th/download/file_ upload/SMWW_1000-3000.pdf.

American Society of Health-Systems Pharmacists & Wheeler, M. (2015). *Drug shortages: sodium phosphate injection*. Retrieved from http://www/ashp.org/menu/DrugShortages/CurrentShortages/Bulletin.aspx?Source=Current&Type=Rss&Id=770.

Ananthanarayanan, M., Balasubramanian, N., Makishima, M., & Mangelsdorf, D.J. (2001). Human bile salt export pump promoter is transactivated by the farnesoid X receptor/bile acid receptor. *The Journal of Biological Chemistry,* 276(31): 28857–28865.

Andrejko, K.M., Raj, N.R., Kim, P.K., Cereda, M., & Deutschman, C.S. (2008). IL-6 modulates sepsis-induced decreases in transcription of hepatic organic anion and bile acid transporters. *Shock,* 29:490–496.

Anwer, M.S. (2004). Cellular regulation of hepatic bile acid transport in health and cholestasis. *Hepatology*, 39:581–590.

Arnold, C. (2004). Thesis: *Parenteral nutrition associated cholestasis: examination of the role of aluminum contamination*. Saskatoon, Sk: Chris Arnold.

Arnold, C.J., Miller, G.G., & Zello, G.A. (2003). Parenteral nutrition-associated cholestasis in neonates: the role of aluminum. *Nutrition Reviews,* 61(9):306-10.

Arrese, M. & Ananthanarayanan, M. (2004). The bile salt export pump: molecular properties, function and regulation. *Pflugers Arch*, 449:123–131.

A.S.P.E.N. (2014). A.S.P.E.N. clinical guidelines: support of pediatric patients with intestinal failure at risk of parenteral nutrition-associated liver disease. *Journal of Parenteral and Enteral Nutrition,* 38(5):538-57.

ASCN/A.S.P.E.N. Working Group on Standards for Aluminum Content of Parenteral Nutrition Solutions. (1991). Parenteral drug products containing aluminum as an ingredient or a contaminant: Response to Food and Drug Administration notice of intent and request for information. *Journal of Parenteral and Enteral Nutrition,* 15(2).

Balistreri, W.F., Heubi, J.E., & Suchy, F.J. (1983). Immaturity of the enterohepatic circulation in early life: factors predisposing to "physiologic" maldigestion and cholestasis. *Journal of Pediatric Gastroenterology and Nutrition,* 2(2):346-54.

Baracos, V.E. (2004). Animal models of amino acid metabolism: a focus on the intestine. *Journal of Nutrition,* 134(6 Suppl):1656S-1659S.

Basiglio, C.L., Toledo, F.D., Boaglio, A.C., Arriaga, S.M., Ochoa, J.E., … Roma, M.G. (2014). Physiological concentrations of unconjugated bilirubin prevent oxidative stress-induced hepatocanalicular dysfunction and cholestasis. *Archives of Toxicology,* 88(2):501-14.

Beale, E.F., Nelson, R.M., Bucciarelli, R.L., Donnelly, W.H., & Eitzman, D.V. (1979). Intrahepatic cholestasis associated with parenteral nutrition in premature infants. *Pediatrics,* 64(3):342-7.

Beath, S.V., Davies, P., Papadopoulou, A., Kahn, A.R., Buick, R.G., … Booth, I.W. (1996). Parenteral nutrition related cholestasis in postsurgical neonates: Multivariate analysis of risk factors. *Journal of Pediatric Surgery,* 31:604–6.

Benjamin, D.R. (1981). Hepatobiliary dysfunction in infants and children associated with long -term total parenteral nutrition: a clinicopathologic study. *American Journal of Clinical Pathology,*76:276-83.

Bertholf, R.L., Herman, M.M., Savory, J., Carpenter, R.M., Sturgill, B.C., … Wills, M.R. (1989). A long-term intravenous model of aluminum maltol toxicity in rabbits: tissue distribution, hepatic, renal and neuronal cytoskeletal changes associated with systemic exposure. *Toxicology and Applied Pharmacology*, 98:58-74.

Betteridge, D.J. (2000). What is oxidative stress?. *Metabolism: Clinical & Experimental,* 49(2 Suppl 1):3-8.

Beuers, U. (2006). Drug insight: mechanisms and sites of action of ursodeoxycholic acid in cholestasis. *Nature Clinical Practice. Gastroenterology & Hepatology*, 3:318-328.

Bindl, L., Lutjohann, D., Buderus, S., Lentze, M.J., & v Bergmann, K. (2000). High plasma levels of phytosterols in patients on parenteral nutrition: a marker of liver dysfunction. *Journal of Pediatric Gastroenterology & Nutrition,* 31(3):313-6.

Bishop, N.J., Morley, R., Day, J.P., & Lucas, A. (1997). Aluminum neurotoxicity in preterm infants receiving intravenous-feeding solutions. *New England Journal of Medicine,* 336(22):1557-1561.

Blackmer, A.B., Warschausky, S., Siddiqui, S., Welch, K.B., Horn, K., … Teitelbaum, D.H. (2015). Preliminary findings of long-term neurodevelopmental outcomes of infants treated with intravenous fat emulsion reduction for the management of parenteral nutrition-associated cholestasis. *Journal of Parenteral and Enteral Nutrition*,39(1):34-46.

Bohrer, D., do Nascimento, P.C., Binotto, R., & Pomblum, S.C. (2001). Influence of the glass packing on the contamination of pharmaceutical products by aluminium. Part I: salts, glucose, heparin and albumin. *Journal of Trace Elements in Medicine and Biol*ogy, 15:95-101.

Bohrer, D., do Nascimento, P.C., Binotto, R., Becker, E., & Pomblum, S. (2002). Contribution of the raw material to the aluminum contamination in parenterals. *Journal of Parenteral and Enteral Nutrition,* 26(6):382-388.

Bohrer, D., do Nascimento, P.C., Binotto, R., & Becker, E. (2003). Influence of the glass packing on the contamination of pharmaceutical products by aluminium. Part III: Interaction container-chemicals during the heating for sterilisation. *Journal of Trace Elements in Medicine and Biology,* 17(2):107-115.

Bohrer, D., Bortoluzzi, F., do Nascimento, P.C., de Carvalho, L.M., & de Oliveira, S.R. (2009). Low availability of aluminum in formulations for parenteral nutrition containing silicate. *Journal of Parenteral and Enteral Nutrition*, 33(6):694-701.

Bohrer, D., Oliveira, S.M., Garcia, S.C., Nascimento, P.C., & Carvalho, L.M. (2010). Aluminum loading in preterm neonates revisited. *Journal of Pediatric Gastroenterology and Nutrition*, 51(2):237-241.

Boisrame-Helms, J., Delabranche, X., Klymchenko, A., Drai, J., Blond, E., … Meziani, F. (2014). Lipid emulsions differentially affect LPS-induced acute monocytes inflammation: in vitro effects on membrane remodeling and cell viability. *Lipids*, 49(11):1091-9.

Boisrame-Helms, J., Said, A., Burban, M., Delabranche, X., Stiel, L., … Meziani, F. (2014). Medium-chain triglyceride supplementation exacerbates peritonitis-induced septic shock in rats: role on cell membrane remodeling. *Shock*, 42(6):548-53.

Book, S. A. & Bustad, L. K. (1974). The fetal and neonatal pig in biomedical research. *Journal of Animal Science*, 38:977-1002.

Boullata, J.I., Gilbert, K., Sacks, G., Labossiere, R.J., Crill, C., … A.S.P.E.N. (2014). Clinical guidelines: parenteral nutrition ordering, order review, compounding, labeling and dispensing. *Journal of Parenteral and Enteral Nutrition*, 38(3):334-377.

Boyer, J.L. (2013). Bile formation and secretion. *Comprehensive Physiology*, 3(3): 1035-1078.

Bursac, Z., Gauss, C.H., Williams, D.K., & Hosmer, D.W. (2008). Purposeful selection of variables in logistic regression. *Source Code for Biology and Medicine*, 3:17.

Buschmann, K., Poeschl, J., Braach, N., Hudalla, H., Kuss, N., & Frommhold, D. (2015). The olive oil-based clinoleic blocks leukocyte recruitment and improves survival during systemic inflammation: a comparative in vivo study of different parenteral lipid emulsions. *Mediators of Inflammation*, 2015:757059.

Bustin, S.A., Benes, V., Garson, J.A., Hellemans, J., Huggett, J., … Wittwer, C.T. (2009). The MIQE Guidelines: *M*inimum *I*nformation for Publication of *Q*uantitative Real-Time PCR *E*xperiments. *Clinical Chemistry*, 55(4):611-622.

Butler, J.E. & Sinkora, M. (2007). The isolator piglet: a model for studying the development of adaptive immunity. *Immunologic Research*, 39(1-3):33-51.

Calkins, K., Lowe, A., Shew, S.B., Dunn, J.C., Reyen, L., … Venick, R. (2013). Short-term intravenous fish oil and pediatric intestinal failure associated liver disease: 3-year follow -up on liver function and nutrition. *Journal of Pediatric Surgery*, 48(1):228-32.

Canada, T.W. (2005). Aluminum exposure through parenteral nutrition formulations: mathematical versus clinical relevance. *American Journal of Health-System Pharmacy*, 62(3):315-318.

Carter, B.A., Taylor, O.A., Prendergast, D.R., Zimmerman, T.L., Von Furstenberg, R. … Karpen, S.J. (2007). Stigmasterol, a soy lipid-derived phytosterol, is an antagonist of the bile acid nuclear receptor FXR. *Pediatric Research,* 62:301-306.

Chen, H.L., Chen, H.L., Liu, Y.J., Feng, C.H., Wu, C.Y. … Chang, M.H. (2005). Developmental expression of canalicular transporter genes in human liver. *Journal of Hepatology,* 43:472-477.

Cherrington, N.J., Slitt, A.L., Li, N., & Klaassen, C.D. (2004). Lipopolysaccharide-mediated regulation of hepatic transporter mRNA levels in rats. *Drug Metabolism and Disposition,* 32:734–741.

Christensen, R.D., Henry, E., Wiedmeier, S.E., & Burnett, J., Lambert, D.K. (2007). Identifying patients, on the first day of life, at high-risk of developing parenteral nutrition–associated liver disease. *Journal of Perinatology,* 27(5):284-90.

Clay, A.S. & Hainline, B.E. (2007). Hyperammonemia in the ICU. *Chest,* 132:1368–78.

Clayton, P.T., Whitfield, P., & Iyer, K. (1998). The role of phytosterols in the pathogenesis of liver complications of pediatric parenteral nutrition. *Nutrition,* 14:158-64.

Clayton, P., & Muller, D. (2000). Effect of intravenous lipid emulsions on hepatic cholesterol metabolism. *Journal of Pediatric Gastroenterology & Nutrition,* 30(5):538-46.

Cober, M.P., & Teitelbaum, D.H. (2010). Prevention of parenteral nutrition–associated liver disease: lipid minimization. *Current Opinion in Organ Transplant,* 15(3):330-3.

Costa, S., Maggio, L., Sindico, P., Cota, F., De Carolis, M.P., & Romagnoli, C. (2010). Preterm small for gestational age infants are not at higher risk for parenteral nutrition–associated cholestasis. *Journal of Pediatrics,* 156:575-9.

Courtney-Martin, G., Kosar, C., Campbell, A., Avitzur, Y., Wales, P.W., … Chambers, K. (2015). Plasma Aluminum Concentrations in Pediatric Patients Receiving Long-Term Parenteral Nutrition. *Journal of Parenteral & Enteral Nutrition,* 39(5):578-85.

Cowan, E., Nandivada, P., & Puder, M. (2013). Fish oil-based lipid emulsion in the treatment of parenteral nutrition-associated liver disease. *Current Opinion in Pediatrics,* 25(2):193 -200.

Cowles, R.A., Ventura, K.A., Martinez, M., Lobritto, S.J., Harren, P.A., … Jan, D.M. (2010). Reversal of intestinal failure–associated liver disease in infants and children on parenteral nutrition: experience with 93 patients at a referral center for intestinal rehabilitation. *Journal of Pediatric Surgery,* 45(1):84-7.

Dahms, B.B., & Halpin Jr, T.C. (1981). Serial liver biopsies in parenteral nutrition associated cholestasis of early infancy. *Gastroenterology,* 81:136-44.

De Marco, G., Sordino, D., Bruzzese, E., Di Caro, S., Mambretti, D., … Guarino, A. (2006). Early treatment with ursodeoxycholic acid for cholestasis in children on parenteral nutrition because of primary intestinal failure. *Alimentary Pharmacology & Therapeutics,* 24:387-394.

de Meijer, V.E., Le, H.D., Meisel, J.A., Gura, K.M., & Puder, M. (2010). Parenteral fish oil as monotherapy prevents essential fatty acid deficiency in parenteral nutrition-dependent patients. *Journal of Pediatric Gastroenterology and Nutrition,* 50(2):212-8.

de Oliveira, S.R., Bohrer, D., Garcia, S.C., do Nascimento, P.C., & Noremberg, S. (2010). Aluminum content in intravenous solutions for administration to neonates: role of product preparation and administration methods. *Journal of Parenteral and Enteral Nutrition,* 34(3):322-328.

Demircan, M., Ergun, O., Coker, C., Yilmaz, F., Avanoglu, S., & Ozok, G. (1998). Aluminum in total parenteral nutrition solutions produces portal inflammation in rats. *Journal of Pediatric Gastroenterology and Nutrition,* 26:274–278.

Deshpande, G., Simmer, K., Deshmukh, M., Mori, T.A., Croft, K.D., & Kristensen, J. (2014). Fish oil (SMOFlipid) and olive oil lipid (Clinoleic) in very preterm neonates. *Journal of Pediatric Gastroenterology & Nutrition,* 58(2):177-82.

Diamond, I.R., Grant, R.C., Pencharz, P.B., de Silva, N., Feldman, B.M., … Wales, P.W. (2016). Preventing the Progression of Intestinal Failure–Associated Liver Disease in Infants Using a Composite Lipid Emulsion: A Pilot Randomized Controlled Trial of SMOFlipid. *Journal of Parenteral and Enteral Nutrition,* (Epub ahead of print: 10.1177/0148607115626921).

Dinh, Q.N., Drummond, G.R., Sobey, C.G., & Chrissobolis, S. (2014). Roles of inflammation, oxidative stress, and vascular dysfunction in hypertension. *Bio-Medical Research International*, ePub: 406960.

Drongowski, R.A., & Coran, A.G. (1989). An analysis of factors contributing to the development of total parenteral nutrition-induced cholestasis. *Journal of Parenteral and Enteral Nutrition,* 13:586–9.

Duro, D., Mitchell, P.D., Kalish, L.A., Martin, C., McCarthy, M., … Duggan, C. (2011). Risk factors for parenteral nutrition-associated liver disease following surgical therapy for necrotizing enterocolitis: A Glaser Pediatric Research Network Study [corrected]. *Journal of Pediatric Gastroenterology & Nutrition,* 52(5):595-600.

El Kasmi, K.C., Anderson, A.L., Devereaux, M.W., Fillon, S.A., Harris, J.K., … Sokol, R.J. (2012). Toll-like receptor 4-dependent Kupffer cell activation and liver injury in a novel mouse model of parenteral nutrition and intestinal injury. *Hepatology*, 55(5):1518-28.

El Kasmi, K.C., Anderson, A.L., Devereaux, M.W., Vue, P.M., Zhang, W., … Sokol, R.J. (2013). Phytosterols promote liver injury and Kupffer cell activation in parenteral nutrition-associated liver disease. *Science Translational Medicine,* 5(206):206ra137.

Elferink, R.O., & Groen, A.K. (2002). Genetic defects in hepatobiliary transport. *Biochimica et Biophysica Acta,* 1586:129–145.

Elferink, M.G.L., Olinga, P., Draaisma, A.L., Merema, M.T., Faber, K.N., … Groothuis, G.M.M. (2004). LPS-induced downregulation of MRP2 and BSEP in human liver is due to a posttranscriptional process. *American Journal Physiology. Gastrointestinal and Liver Physiology,* 287:G1008–G1016

Fallon, E.M., Le, H.D., & Puder, M. (2010). Prevention of parenteral nutrition-associated liver disease: role of omega-3 fish oil. *Current Opinion in Organ Transplantation,* 15(3):334 -40.

Federal Register. (2011). Aluminum in large and small volume parenterals used in total parenteral nutrition. *Fed Regist,* 21:89-90.

Fewtrell, M.S., Edmonds, C.J., Isaacs, E., Bishop, N.J., & Lucas, A. (2011). Aluminium exposure from parenteral nutrition in preterm infants and later health outcomes during childhood and adolescence. *The Proceedings of the Nutrition Society,*70(3):299-304.

Fresenius Kabi. (2012). *SMOFlipid product monograph*. Retrieved from http://fresenius-kabi.ca/en/wp-content/uploads/sites/2/2013/06/SMOFLipid-PM.pdf.

Fresenius Kabi. (2006). *Intralipid 20%, a 20% I.V. fat emulsion, in Excel container*. Product Insert. Retrieved from
http://www.accessdata.fda.gov/drugsatfda_docs/label/2007/017643s072,018449s039lbl.pdf

Fresenius Kabi, (2017). *Omegaven.* Retrieved from http://fresenius -kabi.co.in/products_parenteral_immune_modulation_omegaven.asp

Fresenius Kabi, (2016) *Smoflipid composition.* Retrieved from http://smoflipid.com/smoflipid -composition.html

Fuchs, M., & Sanyal, A.J. (2008). Sepsis and cholestasis. *Clinics in Liver Disease,* 12(1):151-72, ix.

Galicia-Moreno, M., Favari, L., & Muriel, P. (2012). Antifibrotic and antioxidant effects of N -acetylcysteine in an experimental cholestatic model. *European Journal of Gastroenterology & Hepatology,* 24(2):179-85.

Ge, W., Li, D., Gao, Y., & Cao, X. (2015). The Roles of Lysosomes in Inflammation and Autoimmune Diseases. *International Reviews of Immunology,* 34(5):415-31.

Geier, A., Dietrich, C.G., Voigt, S., Ananthanarayanan, M., Lammert, F., … Gartung, C. (2005). Cytokine-dependent regulation of hepatic organic anion transporter gene trans-activators in mouse liver. *American Journal of Physiology. Gastrointestinal and Liver Physiology*, 289:G831–G841

Gissen, P., & Arias, I.M. (2015). Structural and functional hepatocyte polarity and liver disease. *Journal of Hepatology*, 63(4):1023-37.

Gonzalez, M.A., Alvarez Mdel, L., Pisani, G.B., Bernal, C.A., Roma, M.G., & Carillo, M.C. (2007). Involvement of oxidative stress in the impairment in biliary secretory function induced by intraperitoneal administration of aluminum to rats. *Biological Trace Element Research*, 116:329–348.

González, M.A., Roma, M.G., Bernal, C.A., Alvarez, M.L., & Carrillo MC. (2004). Biliary secretory function in rats chronically intoxicated with aluminum. *Toxicological Sciences*, 79:189–195.

Goulet, O., Antebi, H., Wolf, C., Talbotec, C., Alcindor, L.G., … Colomb-Jung, V. (2010). A new intravenous fat emulsion containing soybean oil, medium-chain triglycerides, olive oil, and fish oil: a single-center, double-blind randomized study on efficacy and safety in pediatric patients receiving home parenteral nutrition. *Journal of Parenteral and Enteral Nutrition*, 34(5):485-95.

Gramlich, L., Meddings, L., Alberda, C., Wichansawakun, S., Robbins, S., … Bistrian, B. (2015). Essential Fatty Acid Deficiency in 2015: The Impact of Novel Intravenous Lipid Emulsions. *Journal of Parenteral & Enteral Nutrition*, 39(1 Suppl):61S-6S.

Greger, J.L., & Baler, M.J. (1983) Excretion and retention of low or moderate levels of aluminum by human subjects. *Food and Chemical Toxicology*, 21:473.

Green, R.M., Beier, D., & Gollan, J.L. (1996) Regulation of hepatocyte bile salt transporters by endotoxin and inflammatory cytokines in rodents. *Gastroenterology*, 111:193–198.

Guglielmi, F.W., Regano, N., Mazzuoli, S., Fregnan, S., Leogrande, G., … Francavilla, A. (2008). Cholestasis induced by total parenteral nutrition. *Clinics in Liver Disease*, 12(1):97-110.

Guo, C.H. & Wang, C.L. (2011). Plasma aluminum is a risk factor for oxidative stress and inflammation status in hemodialysis patients. *Clinical Biochemistry*, 44(16):1309-14.

Gura, K.M., Duggan, C.P., Collier, S.B., Jennings, R.W., Folkman, J., … Puder, M. (2006). Reversal of parenteral nutrition-associated liver disease in two infants with short bowel syndrome using parenteral fish oil: implications for future management. *Pediatrics*, 118(1):e197-201.

Gura, K.M., Lee, S., Valim, C., Zhou, J., Kim, S., … Puder, M. (2008). Safety and efficacy of a fish oil-based fat emulsion in the treatment of parenteral nutrition–associated liver disease. *Pediatrics,* 121:e678-e686.

Gura, K.M. (2010). Aluminum contamination in products used in parenteral nutrition: has anything changed? *Nutrition,* 26:585-594.

Hagenbuch, B., & Meier, P.J. (2003). The superfamily of organic anion transporting polypeptides. *Biochimica et Biophysica Acta,* 1609:1–18.

Hall, A.R., Arnold, C.J., Zello, G.A., & Miller, G.G. (2014). Parenteral nutrition associated liver disease: a current review focused on the infant. In: Lelio S, ed. *Parenteral Nutrition: Policies and Procedures, Safe Practices and Potential Complications*. Hauppauge, NY: Nova Science Publishers; 49-84.

Hall, A.R., Arnold, C.J., Miller, G.G., & Zello, G.A. (2016). Infant parenteral nutrition remains a significant risk for aluminum toxicity. *Journal of Parenteral and Enteral Nutrition,* pii: 0148607116638056. [Epub ahead of print].

Halliwell, B. (2007). Biochemistry of oxidative stress. *Biochemical Society Transactions,* 35(5)1147-1150.

Harris, J.K., El Kasmi, K.C., Anderson, A.L., Devereaux, M.W., Fillon, S.A., … Sokol RJ. (2014). Specific microbiome changes in a mouse model of parenteral nutrition associated liver injury and intestinal inflammation. PLoS ONE [Electronic Resource]. 9(10):e110396.

Harrison, C.M., Johnson, K., & McKechnie, E. (2008). Osteopenia of prematurity: a national survey and review of practice. *Acta Paediatrica,* 97:407-413.

Hartmann, G., Cheung, A.K., & Piquette-Miller, M. (2002). Inflammatory cytokines, but not bile acids, regulate expression of murine hepatic anion transporters in endotoxemia. *Journal of Pharmacology and Experimental Therapeutics,* 303, 273–281.

Harvey, K.A., Xu, Z., Pavlina, T.M., Zaloga, G.P., & Siddiqui, R.A. (2015). Omega-9 and inflammation: Modulation of endothelial cell integrity and inflammatory activation by commercial lipid emulsions. *Lipids in Health & Disease,* 14:9.

Heubi, J.E., Wiechmann, D.A., Creutzinger, V., Setchell, K., Squires Jr., R., …Rhodes, P. (2002). Tauroursodeoxycholic acid (TUDCA) in the prevention of total parenteral nutrition–associated liver disease. *Journal of Pediatrics,* 141:237-242.

Hierro, C., Monte, M.J., Lozano, E., Gonzalez-Sanchez, E., Marin, J.J., & Macias, R.I. (2014). Liver metabolic/oxidative stress induces hepatic and extrahepatic changes in the expression of the vitamin C transporters SVCT1 and SVCT2. *European Journal of Nutrition,* 53(2):401-12.

Ho, R.H., Leake, B.F., Roberts, R.L., Lee, W., & Kim, R.B. (2004). Ethnicity-dependent polymorphism in Na+-taurocholate co-transporting polypeptide (SLC10A1) reveals a domain critical for bile acid substrate recognition. *Journal of Biological Chemistry,* 279:7213–7222.

Hofmann, A.F. (1995). Defective biliary secretion during total parenteral nutrition: probable mechanisms and possible solutions. *Journal of Pediatric Gastroenterology & Nutrition,* 20(4):376-90.

Iyer, K.R., Spitz, L., & Clayton, P. (1998). BAPS prize lecture: new insight into mechanisms of parenteral nutrition–associated cholestasis: role of plant sterols. *British Association of Paediatric Surgeons. Journal of Pediatric Surgery,* 33(1):1-6.

Jacquemin, E. (2012). Progressive familial intrahepatic cholestasis. *Clinics & Research in Hepatology & Gastroenterology,* 36 Suppl 1:S26-35.

Jakobsen, M.S., Jorgensen, M.H., Husby, S., Andersen, L., & Jeppesen, P.B. (2015). Low-fat, high-carbohydrate parenteral nutrition (PN) may potentially reverse liver disease in long -term PN-dependent infants. *Digestive Diseases & Sciences,* 60(1):252-9.

Jansen, P.L. & Sturm, E. (2003). Paediatric cholestasis: is villin the villain? *Lancet,* 362(9390):1090-1.

Javid, P.J., Collier, S., Richardson, D., Iglesias, J., Gura, K., … Jaksic, T. (2005). The role of enteral nutrition in the reversal of parenteral nutrition–associated liver dysfunction in infants. *Journal of Pediatric Surgery,* 40:1015-8.

Javid, P.J., Malone, F.R., Dick, A.A., Hsu, E., Sunseri, M., ... Horslen, S.P. (2011). A contemporary analysis of parenteral nutrition-associated liver disease in surgical infants. *Journal of Pediatric Surgery,* 46(10):1913-7.

Jenniskens, M., Langouche, L., Vanwijngaerden, Y.M., Mesotten, D., & Van den Berghe, G. (2016). Cholestatic liver (dys)function during sepsis and other critical illnesses. *Intensive Care Medicine,* 42(1):16-27.

Jessner, W., Zsembery, A., & Graf, J. (2008). Transcellular water transport in hepatobiliary secretion and role of aquaporins in liver. *Wiener Medizinische Wochenschrift* (Vienna Medical Weekly), 158(19-20):565-9.

Josephson, J., Turner, J.M., Field, C.J., Wizzard, P.R., Nation, P.N., … Wales, P.W. (2015). Parenteral Soy Oil and Fish Oil Emulsions: Impact of Dose Restriction on Bile Flow and Brain Size of Parenteral Nutrition-Fed Neonatal Piglets. *Journal of Parenteral and Enteral Nutrition,* 39(6):677-87.

Jung, D., Podvinec, M., Meyer, U.A., Mangelsdorf, D.J., Fried, M., … Kullak-Ublick, G.A. (2002). Human organic anion transporting polypeptide 8 promoter is transactivated by the farnesoid X receptor/bile acid receptor. *Gastroenterology,* 122:1954–1966.

Kelly, D.A. (1998). Liver complications of pediatric parenteral nutrition: epidemiology. *Nutrition,* 14:153-157.

Kikuchi, S., Hata, M., Fukumoto, K., Yamane, Y., Matsui, T., … Tsukita, S. (2002). Radixin deficiency causes conjugated hyperbilirubinemia with loss of Mrp2 from bile canalicular membranes. *Nature Genetics,* 31(3):320-5.

Klein, G.L. (1995). Aluminum in parenteral solutions revisited—again. *American Journal of Clinical Nutrition,* 61:449-456.

Klein, G.L., Alfrey, A.C., Miller, N.L., Sherrard, D.J., Hazlet, T.K., … Coburn J.W. (1982). Aluminum loading during total parenteral nutrition. *American Journal of Clinical Nutrition,* 35(6):1425-1429.

Klein, G., Berquist, W., Ament, M., Coburn, J., Miller, N., & Alfrey, A. (1984). Hepatic aluminum accumulation in children on total parenteral nutrition. *Journal of Pediatric Gastroenterology and Nutrition,* 3:740-743.

Klein, G., Heyman, M., Lee, T., Miller, N., Marathe, G., … Alfrey, A. (1988). Aluminum -associated hepatobiliary dysfunction in rats: relationships to dosage and duration of exposure. *Pediatric Research,* 23:275–278.

Klek, S., Chambrier, C., Singer, P., Rubin, M., Bowling, T., … Shaffer, J. (2013). Four-week parenteral nutrition using a third generation lipid emulsion (SMOFlipid)--a double-blind, randomised, multicentre study in adults. *Clinical Nutrition,* 32(2):224-31.

Kojima, H., Nies, A.T., Konig, J., Hagmann, W., Spring, H., … Keppler, D. (2003). Changes in the expression and localization of hepatocellular transporters and radixin in primary biliary cirrhosis. *Journal of Hepatology,* 39:693–702.

Kojima, H., Sakurai, S., Yoshiji, H., Uemura, M., Yoshikawa, M., & Fukui, H. (2008). The role of radixin in altered localization of canalicular conjugate export pump Mrp2 in cholestatic rat liver. *Hepatology Research,* 38:202-210.

Koo, W.W., Kaplan, L.A., Bendon, R., Succop, P., Tsang, R.C., … Steichen, J.J. (1986). Response to aluminum in parenteral nutrition during infancy. *Journal of Pediatrics,* 109(5):877-883.

Koo, W.W., Kaplan, L.A., Krug-Wispe, S.K., Succop, P., & Bendon, R. (1989). Response of preterm infants to aluminum in parenteral nutrition. *Journal of Parenteral and Enteral Nutrition,* 13(5):516-519.

Koopman, W. (1996). *Arthritis and Allied Conditions: A Textbook on Rheumatology.* Ed: Lea & Febiger; 12th revised edition (January 1993).

Koseesirikul, P., Chotinaruemol, S., & Ukarapol, N. (2012). Incidence and risk factors of parenteral nutrition-associated liver disease in newborn infants. *Pediatrics International,* 54(3):434-6.

Kosters, A., & Karpen, S.J. (2010). The role of inflammation in cholestasis: clinical and basic aspects. *Seminars in Liver Disease,* 30(2):186-94.

Kruger, P.C., Parsons, P.J., Galusha, A.L., Morrissette, M., Recker, R.R., & Howard, L.J. (2014). Excessive aluminum accumulation in the bones of patients on long-term parenteral nutrition: postmortem analysis by electrothermal atomic absorption spectrometry. *Journal of Parenteral and Enteral Nutrition,* 38(6):728-35.

Kruh, G.D., Belinsky, M.G., Gallo, J.M., & Lee, K. (2007). Physiological and pharmacological functions of Mrp2, Mrp3 and Mrp4 as determined from recent studies on gene-disrupted mice. *Cancer and Metastatics Review,* 26(1):5-14.

Kubota, A., Yonekura, T., Hoki, M., Oyanagi, H., Kawahara, H., … Okada, A. (2000). Total parenteral nutrition-associated intrahepatic cholestasis in infants: 25 years' experience. *Journal of Pediatric Surgery,* 35(7):1049-51.

Kullak-Ublick, G.A., Stieger, B., & Meier, P.J. (2004). Enterohepatic bile salt transporters in normal physiology and liver disease. *Gastroenterology,* 126:322–342.

Kurvinen, A., Nissinen, M.J., Gylling, H., Miettinen, T.A., Lampela, H., … Pakarinen, M.P. (2011). Effects of long-term parenteral nutrition on serum lipids, plant sterols, cholesterol metabolism, and liver histology in pediatric intestinal failure. *Journal of Pediatric Gastroenterology & Nutrition,* 53(4):440-6.

Lam, H.S., Tam, Y.H., Poon, T.C., Cheung, H.M., Yu, X., … Ng, P.C. (2014). A double-blind randomised controlled trial of fish oil-based versus soy-based lipid preparations in the treatment of infants with parenteral nutrition-associated cholestasis. *Neonatology,* 105(4):290-6.

Lambert, J.R. & Thomas, S.M. (1985). Metronidazole prevention of serum liver enzyme abnormalities during total parenteral nutrition. *Journal of Parenteral & Enteral Nutrition,* 9(4):501-3.

Le, H.D., Meisel, J.A., de Meijer, V.E., Gura, K.M., & Puder, M. (2009). The essentiality of arachidonic acid and docosahexaenoic acid. *Prostaglandins Leukotrienes and Essential Fatty Acids,* 81(2-3):165-70.

Le Vee, M., Jouan, E., Moreau, A., & Fardel, O. (2011). Regulation of drug transporter mRNA expression by interferon-gamma in primary human hepatocytes. *Fundamental & Clinical Pharmacology*, 25(1):99-103.

Lee, J.H., Chen, H.L., Chen, H.L., Ni, Y.H., Hsu, H.Y., & Chang, M.H. (2006). Neonatal Dubin-Johnson syndrome: long-term follow-up and MRP2 mutations study. *Pediatric Research*, 59(4 Pt 1):584-9.

Lee, S.M., Namgung, R., Park, M.S., Eun, H.S., Kim, N.H., … Lee, C. (2013). Parenteral nutrition associated cholestasis is earlier, more prolonged and severe in small for gestational age compared with appropriate for gestational age very low birth weight infants. *Yonsei Medical Journal*, 54(4):839-44.

Lemoy, M.J., Westworth, D.R., Ardeshir, A., & Tarara, R.P. (2013). Reference intervals for preprandial and postprandial serum bile acid in adult rhesus macaques (Macaca mulatta). *Journal of the American Association for Laboratory Animal Science*, 52(4):444-7.

Li, J., Gong, Y.M., Wu, J., Wu, W.J., & Cai, W. (2012). Anti-tumor necrosis factor-alpha monoclonal antibody alleviates parenteral nutrition-associated liver disease in mice. *Journal of Parenteral & Enteral Nutrition*, 36(2):219-25.

Li, M. (2005). *The Role of Aluminum in Parenteral Nutrition Associated Cholestasis in Infants and Piglets* [thesis]. Saskatoon: University of Saskatchewan.

Maher, J.M., Slitt, A.L., Cherrington, N.J., Cheng, X., & Klaassen, C.D. (2005). Tissue distribution and hepatic and renal ontogeny of the multidrug resistance-associated protein (Mrp) family in mice. *Drug Metabolism & Disposition*, 33(7):947-55.

Mailloux, R.J. & Appanna, V.D. (2007). Aluminum toxicity triggers the nuclear translocation of HIF-1alpha and promotes anaerobiosis in hepatocytes, *Toxicology In Vitro*, 21:16 –24.

Mailloux, R.J., Lemire, J., & Appanna, V.D. (2011). Hepatic response to aluminum toxicity: dyslipidemia and liver diseases. *Experimental Cell Research*, 317,2231–8.

McClure, R.J., & Newell, S.J. (1999). Randomised controlled trial of trophic feeding and gut motility. *Archives of Disease in Childhood. Fetal & Neonatal Ed*, 80(1):F54-8.

Meehan, J.J., & Georgeson, K.E. (1997). Prevention of liver failure in parenteral nutrition –dependent children with short bowel syndrome. *Journal of Pediatric Surgery*,132:473-5.

Meisel, J.A., Le, H.D., de Meijer, V.E., Nose, V., Gura, K.M., … Puder, M. (2011). Comparison of 5 intravenous lipid emulsions and their effects on hepatic steatosis in a murine model. *Journal of Pediatric Surgery*, 46(4):666-73.

Mercer, D.F., Hobson, B.D., Fischer, R.T., Talmon, G.A., Perry, D.A., …Quiros-Tejeira, R.E. (2013). Hepatic fibrosis persists and progresses despite biochemical improvement in

children treated with intravenous fish oil emulsion. *Journal of Pediatric Gastroenterology & Nutrition,* 56(4): 364-369.

Miller, E.R., & Ullray, D.E. (1987). The pig as a model for human nutrition. *Annual Review of Nutrition,* 7:361-382.

Mirtallo, J.M. (2010). Aluminum contamination of parenteral nutrition fluids. *Journal of Parenteral & Enteral Nutrition*, 34(3):346-7.

Monte, M.J., Marin, J.J., Antelo, A., & Vazquez-Tato, J. (2009). Bile acids: chemistry, physiology, and pathophysiology. *World Journal of Gastroenterology*, 15(7):804-16.

Moreno, A., Dominguez, C., & Ballabriga, A. (1994). Aluminum in the neonate related to parenteral nutrition. *Acta Paediatrica*, 83(1):25-29.

Moriya, T., Fukatsu, K., Iwaya, K., Noguchi, M., Murakoshi, S., … Yamamoto, J. (2014). Influence of fish to soybean oil ratio on hepatic mononuclear cell function and survival after intraportal bacterial challenge in parenterally fed mice. *Surgery*, 155(4):711-8.

Muhammed, R., Bremner, R., Protheroe, S., Johnson, T., Holden, C., & Murphy, M.S. (2012). Resolution of parenteral nutrition-associated jaundice on changing from a soybean oil emulsion to a complex mixed-lipid emulsion. *Journal of Pediatric Gastroenterology & Nutrition*, 54(6):797-802.

Mujika, J.I., Ruipérez, F., Infante, I., Ugalde, J.M., Exley, C., & Lopez, X. (2011). Pro-oxidant activity of aluminum: stabilization of the aluminum superoxide radical ion. *The Journal of Physical Chemistry*, 115(24): 6717-23.

Mullick, F.G., Moran, C.A., & Ishak, K.G. (1994). Total parenteral nutrition: a histopathologic analysis of the liver changes in 20 children. *Modern Pathology*, 7:190-4.

Mundi, M.S., Salonen, B.R., & Bonnes, S. (2016). Home Parenteral Nutrition: Fat Emulsions and Potential Complications. *Nutrition in Clinical Practice*, 31(5):629-41.

Mutanen, A., Lohi, J., Heikkila, P., Koivusalo, A.I., Rintala, R.J., & Pakarinen, M.P. (2013). Persistent abnormal liver fibrosis after weaning off parenteral nutrition in pediatric intestinal failure. *Hepatology*, 58(2):729-38.

Muto, M., Lim, D., Soukvilay, A., Field, C., Wizzard, P.R., … Turner, J.M. (2017). Supplemental parenteral vitamin E into conventional soybean lipid emulsion does not prevent parenteral nutrition–associated liver disease in full-term neonatal piglets. *Journal of Parenteral and Enteral Nutrition*, 41(4):575-582.

Nehra, D., Fallon, E.M., Carlson, S.J., Potemkin, A.K., Hevelone, N.D., … Puder, M. (2013). Provision of a soy-based intravenous lipid emulsion at 1 g/kg/d does not prevent cholestasis in neonates. *Journal of Parenteral & Enteral Nutrition*, 37(4):498-505.

Nehra, D., Fallon, E.M., Potemkin, A.K., Voss, S.D., Mitchell, P.D., … Puder, M. (2014). A comparison of 2 intravenous lipid emulsions: interim analysis of a randomized controlled trial. *Journal of Parenteral & Enteral Nutrition,* 38(6):693-701.

Naini, B.V., & Lassman, C.R. (2012). Total parenteral nutrition therapy and liver injury: a histopathologic study with clinical correlation. *Human Pathology,* 43(6):826-33.

Nandivada, P., Chang, M.I., Potemkin, A.K., Carlson, S.J., Cowan, E., … Puder, M. (2015). The natural history of cirrhosis from parenteral nutrition-associated liver disease after resolution of cholestasis with parenteral fish oil therapy. *Annals of Surgery,* 261(1):172-9.

Nandivada, P., Fell, G.L., Gura, K.M., & Puder, M. (2016). Lipid emulsions in the treatment and prevention of parenteral nutrition-associated liver disease in infants and children. *American Journal of Clinical Nutrition,* 103(2):629S-34S.

Nghiem-Rao, T.H., Cassidy, L.D., Polzin, E.M., Calkins, C.M., Arca, M.J., & Goday, P.S. (2013). Risks and benefits of prophylactic cyclic parenteral nutrition in surgical neonates. *Nutrition in Clinical Practice,* 28(6):745-52.

Nghiem-Rao, T.H., Tunc, I., Mavis, A.M., Cao, Y., Polzin, E.M., … Patel, S.B. (2015). Kinetics of phytosterol metabolism in neonates receiving parenteral nutrition. *Pediatric Research,* 78(2):181-9.

Omata, J., Fukatsu, K., Murakoshi, S., Noguchi, M., Miyazaki, H., … Hase, K. (2009). Enteral refeeding rapidly restores PN-induced reduction of hepatic mononuclear cell number through recovery of small intestine and portal vein blood flows. *Journal of Parenteral & Enteral Nutrition,* 33(6):618-25.

Oswald, M., Kullak-Ublick, A., Paumgartner, G., & Beuers, U. (2001). Expression of hepatic transporters OATP-C and MRP2 in primary sclerosing cholangitis. *Liver,* 21:247–253.

Park, K.T., Nespor, C., & Kerner, J. Jr. (2011). The use of Omegaven in treating parenteral nutrition-associated liver disease. *Journal of Perinatology,* 31 Suppl 1:S57-60.

Pascussi, J.M., Dvorak, Z., Gerbal-Chaloin, S., Assenat, E., Maurel, P., & Vilarem, M.J. (2003). Pathophysiological factors affecting CAR gene expression. *Drug Metabolism Reviews,* 35:255-268.

Peden, V.H., Witzleben, C.L., & Skelton, M.A. (1971). Total parenteral nutrition. *Journal of Pediatrics,* 78(1):180-1.

Percy, M.E., Kruck, T.P.A., Pogue, A.I., & Lukiw, W.J. (2011). Towards the prevention of potential aluminum toxic effects and an effective treatment for Alzheimer's disease. *Journal of Inorganic Biochemistry,* 105:1505–12.

Pérez, L.M., Milkiewicz, P., Elias, E., Coleman, R., Sánchez Pozzi, E.J., & Roma, M.G. (2006). Oxidative stress induces internalization of the bile salt export pump, Bsep, and bile salt secretory failure in isolated rat hepatocyte couplets: a role for protein kinase C and prevention by protein kinase A. *Toxicological Sciences*, 91:150–158.

Pfaffl, M.W. (2001). A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Research*, 29(9):e45.

Pharmaceutical Partners of Canada Inc. (2011). *Calcium gluconate injection 10% - important information concerning the presence of aluminum - for health professionals*. Retrieved from http://www.healthycanadians.gc.ca/recall-alert-rappel-avis/index-eng.php

Pharmaceutical Partners of Canada Ltd & Fresensius Kabi. (2015). *Product monograph: calcium gluconate injection, USP 10%.* http://fresenius-kabi.ca/wp -content/uploads/2015/01/EN_WebInsert_CalcGluc.pdf

Phillips, M.J., Azuma, T., Meredith, S.L., Squire, J.A., Ackerley, C.A., … Marsden, P.A. (2003). Abnormalities in villin gene expression and canalicular microvillus structure in progressive cholestatic liver disease of childhood. *Lancet*, 362(9390):1112-9.

Pichler, J., Simchowitz, V., Macdonald, S., & Hill, S. (2014). Comparison of liver function with two new/mixed intravenous lipid emulsions in children with intestinal failure. *European Journal of Clinical Nutrition*, 68(10):1161-7.

Piper, S.N., Schade, I., Beschmann, R.B., Maleck, W.H., Boldt, J., & Rohm, K.D. (2009). Hepatocellular integrity after parenteral nutrition: comparison of a fish-oil-containing lipid emulsion with an olive-soybean oil-based lipid emulsion. *European Journal of Anaesthesiology*, 26:1076-1082.

Pitkänen, O.M. (2004). Parenteral lipids and the preterm infant: between Scylla and Charybdis. *Acta Paediatrica*, 93:1028e30.

Poole, R.L., Hintz, S.R., Mackenzie, N.I., & Kerner, J.A. Jr. (2008). Aluminum exposure from pediatric parenteral nutrition: meeting the new FDA regulation. *Journal of Parenteral and Enteral Nutrition*, 32(3):242-246.

Poole, R.L., Schiff, L., Hintz, S.R., Wong, A., Mackenzie, N., & Kerner, J.A. Jr. (2010). Aluminum content of parenteral nutrition in neonates: measured versus calculated levels. *Journal of Pediatric Gastroenterology and Nutrition*, 50:208-11.

Postuma, R., & Trevenen, C.L. (1979). Liver disease in infants receiving total parenteral nutrition. *Pediatrics*, 63:l 10-115.

Premkumar, M.H., Carter, B.A., Hawthorne, K.M., King, K., & Abrams, S.A. (2013). High rates of resolution of cholestasis in parenteral nutrition-associated liver disease with fish oil -based lipid emulsion monotherapy. *Journal of Pediatrics*, 162(4):793-798.e1.

Puder, M., Valim, C., Meisel, J.A., Le, H.D., de Meijer, V.E., … . Gura, K.M. (2009). Parenteral fish oil improves outcomes in patients with parenteral nutrition-associated liver injury. *Annals of Surgery,* 250:395-402.

Rager, R. & Finegold, M.J. (1975). Cholestasis in immature newborn infants: is parenteral alimentation responsible? *Journal of Pediatrics,* 86(2):264-9.

Rangel, S.J., Calkins, C.M., Cowles, R.A., Barnhart, D.C., Huang, E.Y., … Teitelbaum, D.H. For the 2011 American Pediatric Surgical Association Outcomes and Clinical Trials Committee. (2011). Parenteral nutrition–associated cholestasis: an American Pediatric Surgical Association Outcomes and Clinical Trials Committee systematic review. *Journal of Pediatric Surgery,* Volume 47(1): 225-240.

Robinson, D.T., & Ehrenkranz, R.A. (2008). Parenteral nutrition–associated cholestasis in small for gestational age infants. *Journal of Pediatrics*, 152(1):59-62.

Sangild, P.T., Ney, D.M., Sigalet, D.L., Vegge, A., & Burrin, D. (2014). Animal models of gastrointestinal and liver diseases. Animal models of infant short bowel syndrome: translational relevance and challenges. *American Journal of Physiology*, 307(6), G1147(22).

Sant'Anna, A.M., Altamimi, E., Clause, R.F., Saab, J., Mileski, H., … Sant'Anna, G.M. (2012). Implementation of a multidisciplinary team approach and fish oil emulsion administration in the management of infants with short bowel syndrome and parenteral nutrition -associated liver disease. *Canadian Journal of Gastroenterology,* 26(5):277-80.

Saskatchewan Research Council. (2016). *Environmental Analytical Laboratories*. Retrieved from http://www.src.sk.ca/facilities/pages/environmental-analytical-laboratories.aspx

Savini, S., D'Ascenzo, R., Biagetti, C., Serpentini, G., Pompilio, A., … Carnielli, V.P. (2013). The effect of 5 intravenous lipid emulsions on plasma phytosterols in preterm infants receiving parenteral nutrition: a randomized clinical trial. *American Journal of Clinical Nutrition*, 98(2):312-8.

Schmitt, M., Kubitz, R., Wettstein, M., Vom, D. S., & Ha¨ussinger, D. (2000). Retrieval of the Mrp2 gene encoded conjugate export pump from the canalicular membrane contributes to cholestasis induced by tert-butyl hy-droperoxide and chloro-dinitrobenzene. *The Journal of Biological Chemistry*, 381:487–495.

Schmitt, M., Kubitz, R., Lizun, S., Wettstein, M., & Haussinger, D. (2001). Regulation of the dynamic localization of the rat Bsep gene-encoded bile salt export pump by anisoosmolarity. *Hepatology,* 33:509–518.

Sedman, A. (1992). Aluminum toxicity in childhood. *Pediatric Nephrology*, 6(4):383-393.

Shoda, J., Kano, M., Oda, K., Kamiya, J., Nimura, Y., … Tanaka, N. (2001). The expression levels of plasma membrane transporters in the cholestatic liver of patients undergoing biliary drainage and their association with the impairment of biliary secretory function. *American Journal of Gastroenterology,* 96:3368–3378.

Shu, Z.J., Li, J.S., Zhou, Z.S., Shi, Q.L., & Zhang, T.H. (1991). Histopathologic study of cholestasis induced by total parenteral nutrition or intraperitoneal sepsis in rats. *Journal of Parenteral and Enteral Nutrition,* 15(6):630-6.

Simic, D., Milojevic, I., Bogicevic, D., Milenovic, M., Radlovic, V., … Maksimovic, R. (2014). Preventive effect of ursodeoxycholic acid on parenteral nutrition-associated liver disease in infants. Serbian Archives for Celocultural Chemistry, 142(3-4):184-8.

Singh, S., White, F.C., & Bloor, C.M. (1980). Effect of acute exercise stress in cardiac hypertrophy: I. correlation of regional blood flow and qualitative ultrastructural changes. *Yale Journal of Biology & Medicine,* 53(6):459-70.

Slonim, A.D., Pollack, M.M., Bell, M.J., Berger, J., Carcillo Jr., J.A., … Lynch, R.E. (2006). *Pediatric Critical Care Medicine*. Pennsylvania, USA: Lippincott Williams & Wilkins.

Smith, B.S., Kothari, H., Hayes, B.D., Tataronis, G., Hudlin, M., … Hartman, C. (2007). Effect of additive selection on calculated aluminum content of parenteral nutrient solutions. *American Journal of Health-System Pharmacy*, 64(7):730-739.

Solenski, N.J., diPierro, C.G., Trimmer, P.A., Kwan, A., & Helms, G.A. (2002). Ultrastructural changes of neuronal mitochondria after transient and permanent cerebral ischemia. *Stroke,* 33(3):816-24.

Spagnuolo, M.I., Iorio, R., Vegnente, A., & Guarino, A. (1996). Ursodeoxycholic acid for treatment of cholestasis in children on long-term total parenteral nutrition: a pilot study. *Gastroenterology*, 111(3):716-9.

Speerhas, R.A., & Seidner, D.L. (2007). Measured versus estimated aluminum content of parenteral nutrient solutions. *American Journal of Health-Systems Pharm*acy, 64:740 -746.

Suita, S., Masumoto, K., Yamanouchi, T., Nagano, M., & Nakamura, M. (1999). Complications in neonates with short bowel syndrome and long-term parenteral nutrition. *Journal of Parenteral and Enteral Nutrition,* 23:S106-9.

Taylor, S., Wakem, M., Dijkman, G., Alsarraj, M., & Nguyen, M. (2015). *A Practical Approach to RT-qPCR: Publishing Data That Conform to the MIQE Guidelines.* Retrieved from http://www.bio-rad.com/webroot/web/pdf/lsr/literature/Bulletin_5859.pdf

Tazuke, Y., Kiristioglu, I., Heidelberger, K.P., Eisenbraun, M.D., & Teitelbaum, D.H. (2004). Hepatic P-glycoprotein changes with total parenteral nutrition administration. *Journal of Parenteral and Enteral Nutrition,* 28:1–6.

Teitelbaum, D.H., Tracy, T.F. Jr., Aouthmany, M.M., Llanos, A., Brown, M.B., … Strouse PJ. (2005). Use of cholecystokinin-octapeptide for the prevention of parenteral nutrition -associated cholestasis. *Pediatrics,* 115(5):1332-40.

Tillman, E.M. (2013). Review and clinical update on parenteral nutrition-associated liver disease. *Nutrition in Clinical Practice*, 28(1):30-9.

Tomer, G., Ananthanarayanan, M., Weymann, A., Balasubramanian, N., & Suchy, F.J. (2003). Differential developmental regulation of rat liver canalicular membrane transporters Bsep and Mrp2. *Pediatric Research,* 53:288–294.

Touloukian, R.J., & Seashore, J.H. (1975). Hepatic secretory obstruction with total parenteral nutrition in the infant. *Journal of Pediatric Surgery,* 10(3):353-360.

Trauner, M., Arrese, M., Lee, H., Boyer, J.L., & Karpen, S.J. (1998). Endotoxin downregulates rat hepatic ntcp gene expression via decreased activity of critical transcription factors. *Journal of Clinical Investigation,* 101:2092–2100.

Trauner, M., Fickert, P., & Stauber, R.E. (1999). Inflammation-induced cholestasis. *Journal of Gastroenterology and Hepatology,* 14:946-59.

Tsai, S., Strouse, P.J., Drongowski, R.A., Islam, S., & Teitelbaum, D.H. (2005). Failure of cholecystokinin octapeptide to prevent TPN-associated gallstone disease. *Journal of Pediatric Surgery,* 40(1):263-7.

Tsukada, N., Ackerley, C.A., & Phillips, M.J. (1995). The structure and organization of the bile canalicular cytoskeleton with special reference to actin and actin-binding proteins. *Hepatology*, 21(4):1106-13.

Turner, J.M., Josephson, J., Field, C.J., Wizzard, P.R., Ball, R.O., … Wales, P.W. (2016). Liver disease, systemic inflammation, and growth using a mixed parenteral lipid emulsion, containing soybean oil, fish oil, and medium chain triglycerides, compared with soybean oil in parenteral nutrition-fed neonatal piglets. *Journal of Parenteral and Enteral Nutrition*, 40(7):973-81.

Turner, J.M., Wales, P.W., Nation, P.N., Wizzard, P., Pendlebury, C., …Pencharz, P.B. (2011). Novel neonatal piglet models of surgical short bowel syndrome with intestinal failure. *Journal of Pediatric Gastroenterology and Nutrition*, 52(1):9-16.

University of Montreal: Institute of research in immunology and cancer. (2016). *Information on qPCR Results.* Retrieved from: https://genomique.iric.ca/resources/files/How_to_deal_with_qPCR_results.pdf

UK Medicines and Healthcare Regulatory Agency. (2010). Calcium gluconate injection in small-volume glass containers: new contraindications due to aluminum exposure risk. *MHRA Drug Safety Update*. 4(1):A2.

Vanwijngaerden, Y.M., Wauters, J., Langouche, L., Vander Perre, S., Liddle, C., … Mesotten, D. (2011). Critical illness evokes elevated circulating bile acids related to altered hepatic transporter and nuclear receptor expression. *Hepatology*, 54:1741–1752.

Vanwijngaerden, Y.M., Langouche, L., Derde, S., Liddle, C., Coulter, S., … Mesotten, D. (2014). Impact of parenteral nutrition versus fasting on hepatic bile acid production and transport in a rabbit model of prolonged critical illness. *Shock*, 41:48–54.

Vignal, C., Desreumaux, P., & Body-Malapel, M. (2016). Gut: An underestimated target organ for Aluminum. *Morphologie*, 100(329):75-84.

Vlaardingerbroek, H., Ng, K., Stoll, B., Benight, N., Chacko, S., … Burrin, D. (2014). New generation lipid emulsions prevent PNALD in chronic parenterally fed preterm pigs. *Journal of lipid research*, 55(3): 466-77.

Wanten, G.J., Naber, A.H., Kruimel, J.W., Tool, A.T., Roos, D., & Jansen, J.B. (1999). Influence of structurally different lipid emulsions on human neutrophil oxygen radical production. *European Journal of Clinical Investigation*, 29:357–363.

Wier, H.A., & Kuhn, R.J. (2012). Aluminum toxicity in neonatal parenteral nutrition: what can we do? *Annals of Pharmacotherapy*, 46(1):137-40.

Willis, T.C., Carter, B.A., Rogers, S.P., Hawthorne, K.M., Hicks, P.D., & Abrams, S.A. (2010). High rates of mortality and morbidity occur in infants with parenteral nutrition-associated cholestasis. *Journal of Parenteral and Enteral Nutrition*, 34:32-7.

Wolters, H., Elzinga, B.M., Baller, J.F., Boverhof, R., Schwarz, M., … Kuipers, F. (2002). Effects of bile salt flux variations on the expression of hepatic bile salt transporters in vivo in mice. *Journal of Hepatology*, 37:556–563.

Yokel, R.A. & Unrine, J.M. (2017). Aluminum and phthalates in calcium gluconate; contribution from glass and plastic packaging. *Journal of Pediatric Gastroenterology and Nutrition*, 64(1): 109-114.

Yokel, R.A., Harris, W.R., Spilling, C.D., Abramov, V.P., Lone, J.M., & Kuhn, R.J. (2014). A filtration system that greatly reduces aluminum in calcium gluconate injection, USP used to prepare parenteral nutrition solutions. *Journal of Pediatric Pharmacology and Therapeutics*, 19(3):189-95.

Yoshimura, D., Sakumi, K., Ohno, M., Sakai, Y., Furuichi, M., Iwai, S., & Nakabeppu, Y. (2003). An oxidized purine nucleoside triphosphatase, MTH1, suppresses cell death caused by oxidative stress. *Journal of Biological Chemistry*, 278(39):37965-73.

Zaloga, G.P. (2015). Phytosterols, lipid administration, and liver disease during parenteral nutrition. *Journal of Parenteral & Enteral Nutrition*, 39(1 Suppl):39S-60S.

Zambrano, E., El-Hennawy, M., Ehrenkranz, R.A., Zelterman, D., & Reyes-Mugica, M. (2004). Total parenteral nutrition induced liver pathology: an autopsy series of 24 newborn cases. *Pediatric & Developmental Pathology*, 7(5):425-32.

Zelcer, N., van de Wetering, K., de Waart, R., Scheffer, G.L., Marschall, H.U., & Borst, P. (2006). Mice lacking Mrp3 (Abcc3) have normal bile salt transport, but altered hepatic transport of endogenous glucuronides. *Journal of Hepatol*ogy, 44:768–775.

Zhan, L., Yang, I., Kong, B., Shen, J., Gorczyca, L., … Guo, G.L. (2016). Dysregulation of bile acid homeostasis in parenteral nutrition mouse model. *American Journal of Physiology - Gastrointestinal & Liver Physiology*, 310(2):G93-G102.

Zhu, Q.N., Hou, W.Y., Xu, S.F., Lu, Y.F., & Liu, J. (2017). Ontogeny, aging, and gender-related changes in hepatic multidrug resistant protein genes in rats. *Life Sciences*, 170:108-114.

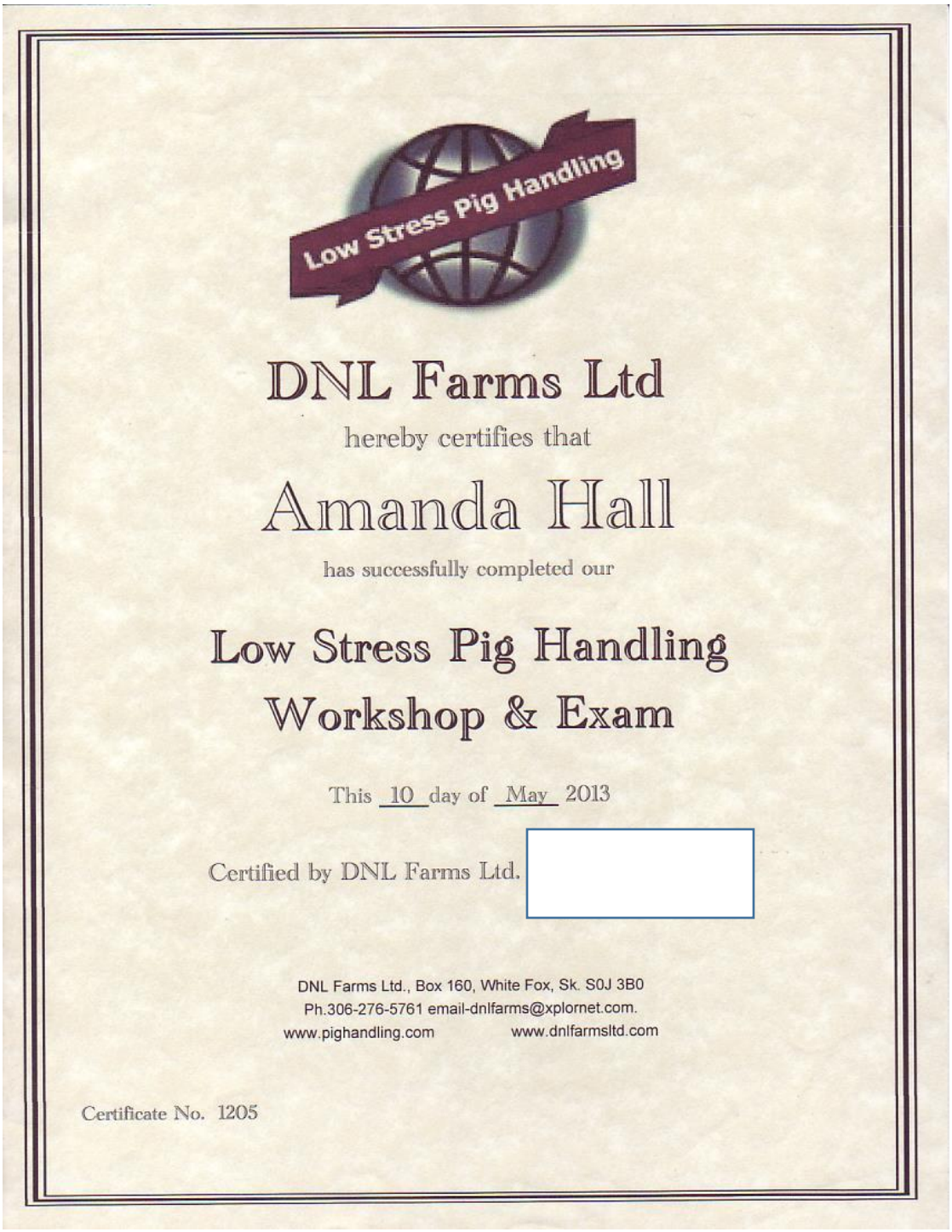
Zollner, G., Fickert, P., Zenz, R., Fuchsbichler, A., Stumptner, C., ... Trauner, M. (2001). Hepatobiliary transporter expression in percutaneous liver biopsies of patients with cholestatic liver diseases. *Hepatology*, 33:633–646.

Zollner, G., Fickert, P., Silbert, D., Fuchsbichler, A., Marschall, H.U., … Trauner, M. (2003). Adaptive changes in hepatobiliary transporter expression in primary biliary cirrhosis. *Journal of Hepatol*ogy, 38:717–727.

Zollner, G. & Trauner, M. (2009). Nuclear receptors as therapeutic targets in cholestatic liver diseases. *British Journal of Pharmacology*, 156:7–27.

APPENDIX A: COURSES

A.1 Swine handling:



Low Stress Pig Handling

DNL Farms Ltd

hereby certifies that

Amanda Hall

has successfully completed our

Low Stress Pig Handling
Workshop & Exam

This 10 day of May 2013

Certified by DNL Farms Ltd.

DNL Farms Ltd., Box 160, White Fox, Sk. S0J 3B0
Ph.306-276-5761 email-dnlfarms@xplornet.com.
www.pighandling.com www.dnlfarmsltd.com

Certificate No. 1205

A.2 Biosafety:

Certificate of Training

This certifies that

Amanda Hall

has successfully completed the following course presented by
Workplace Safety and Environmental Protection:

Biosafety

Instructor: Michelle Heggstrom

UNIVERSITY OF SASKATCHEWAN

30 January 2013

APPENDIX B: PERMISSION TO REPRODUCE ARTICLES/FIGURES

Confirmation Number: 11643067
Order Date: 05/08/2017

If you paid by credit card, your order will be finalized and your card will be charged within 24 hours. If you choose to be invoiced, you can change or cancel your order until the invoice is generated.

Payment Information

Amanda Hall
arb743@mail.usask.ca
+1 (306) 262-3847
Payment Method: CC ending in 8158

Order Details

Parenteral Nutrition : Policies and Procedures, Safe Practices and Potential Complications

Order detail ID:	70416357
Order License Id:	4104520262594
ISBN:	9781633211124
Publication Type:	Book
Publisher:	Nova Science Publishers, Inc.

Permission Status: Granted
Permission type: Republish or display content
Type of use: Thesis/Dissertation
View details

Note: This item will be invoiced or charged separately through CCC's **RightsLink** service. More info **$ 262.70**

Sent: Monday, April 10, 2017 9:55 AM
To: Hall, Amanda
Subject: RE: Revised proofs for JPEN638056

Dear Amanda,

Thank you for getting in touch regarding this question!

Use of the paper, for which you were an author, in your PhD thesis is covered under SAGE's archiving and reuse policies here: https://us.sagepub.com/en-us/nam/journal-author-archiving-policies-and-re-use.

Please be sure to give appropriate credit to JPEN as the original source of the content, as described in this paragraph on the webpage linked to above:

"When posting or reusing your Contribution under this policy, appropriate credit must be given to the SAGE journal where the Contribution has been published, as the original source of the content, as follows: **Author(s), Article Title, Journal Title (Journal Volume Number and Issue Number) pp. xx-xx. Copyright © [year] (Copyright Holder). Reprinted by permission of SAGE Publications.** Additionally, please provide a link to the appropriate DOI for the published version of the Contribution on the SAGE Journals website ([http://journals.sagepub.com)]http://journals.sagepub.com)."

Let me know if you have any further questions!

Best,
Sara

Sara Sarver
Production Editor Manager, Journals
SAGE Publishing
2455 Teller Rd
Thousand Oaks, CA 91320
USA

T: 805.410.7229
F: 805.375.1735

www.sagepublishing.com

Los Angeles | London | New Delhi
Singapore | Washington DC | Melbourne

I gladly give you the permission to use the figure in your thesis.
Good luck o you in your studies and future career
Best regards
Waddah

Waddah A.Alrefai, MD
Associate Professor
Department of Medicine
University of Illinois at Chicago
Research Career Scientist
Jesse Brown VA Medical Center

go for it
leah gramlich

On Fri, Apr 7, 2017 at 10:18 AM, Hall, Amanda <arb743@mail.usask.ca> wrote:
> Dear Dr Gramlich,
>
> I found your article"Essential Fatty Acid Deficiency in 2015: The Impact of
> Novel Intravenous Lipid Emulsions", as published in JPEN very informative.
> Would you give me permission to reproduce your Figure 1: 'Fatty acid
> biosynthesis pathways' as part of my thesis? I am a PhD student at the
> University of Saskatchewan and my thesis examines the roles of aluminum and
> lipids in parenteral nutrition associated liver disease.
>
> Thank you,

APPENDIX C: PN RECIPES

Amino Acid	MW (g/ml)	g/L
Alanine	89.0935	6.12
Arginine	174.2017	3.47
Aspartate	133.1032	3.47
Cysteine (free)	121.1590	0.83
Glutamate	147.1299	6.00
Free-L-Glycine	75.0669	1.76
Histidine	155.1552	1.76
Isoleucine	131.1736	2.64
Leucine	131.1736	5.94
Lysine HCL	146.1882	5.91
Methionine	149.2124	1.10
Phenylalanine	165.1900	3.03
Proline	115.1310	4.73
Serine	105.0930	3.19
Taurine	125.1500	0.28
Typtophan	204.2262	1.21
L-Tyrosine	181.1894	0.43
Valine	117.1469	3.03
Threonine	119.1197	3.03

Trace Elements

	g/L
ZnSO4 7H2O	40.680
CuSO4 5H2O	3.1200
MnSO4 H2P	1.8550
CrCl3 6H2O	0.0512
SeO2	0.0592
NaI	0.0220

Additives

Compound	g/L
Dextrose	90.3
Trihydrate K2HPO4	1.57
Monobasic KH2PO4	1.085
K acetate	1.47
NaCl	2.17
MgSO4	0.78
ZnSO4	0.089
Ca Gluconate (SIGMA)	6.41

Making PN Diet

1) Weigh amino acids required and put in a large beaker. Cover with parafilm and foil until used.
2) Weigh minerals. Each mineral (the potassium sulfates are mixed together) is put in a separate beaker and dissolved in the appropriate amount of HP2C H2O (Pyrogen-free is best)

3) For calcium gluconate: Heat 750ml H2O in a ½ L beaker and gradually add the calcium gluconate to it. You will need to heat to dissolve it.
    - Amount of Pyrogen-Free H2O for each salt:
        - Phosphate -> 25 mL/L
        - K+ Acetate/NaCl -> 10-15 mL/L
        - Sulfates -> 10-15 mL/L
        - Calcium gluconate -> 75 mL/L
    - We make 10L of diet at a time: 10.6 kg on scale.
4) While weighing your amino acids, get 2x 4L beakers and place them on heater (in stirrers). Add 2.5L of Pyrogen-free H2O to each and begin heating. DO NOT let temperature rise above 70° or below 40-50°. Once heated, you can start adding the amino acid mixture. Divide amino acid mix evenly between the two beakers and add gradually.
5) After amino acids are dissolved, add dextrose and minerals in the order that you made them. Ensure each mineral is dissolved completely before adding more minerals to the carboy. Top the carboy up to 10.6 kg (for 102 of diet) with Pyrogen-free H2O.
6) Filter and bag diet
7) To clean carboy, rinse in 6M HCl and Pyrogen-free H2O.