THE EFFECT OF ALUMINUM ON HEPATIC BILIARY TRANSPORTERS AS A CONTRIBUTING FACTOR TO PARENTERAL NUTRITION INDUCED INTRAHEPATIC CHOLESTASIS

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ABSTRACT

Intravenous feeding of patients with essential and balanced nutrition is required when enteral feeding is not tolerated, therefore indicating the need for Total Parenteral Nutrition (TPN). This life-saving therapy is also associated with the increase risk of intrahepatic cholestasis. The incidence of TPN-related hepatobiliary complications is common in both adults and infants on TPN. Previous work in *in vivo* models suggested that one of the potential contributing factors is the aluminum contamination of TPN solutions. The mechanism by which aluminum contributes to the PNAC development, though, was unknown. Aluminum as a risk factor may influence a number of hepatocellular functions to lead to cholestasis but one possible mechanism is the potential for aluminum to cause dysfunction of those transporters responsible in the maintenance of bile flow. To provide some initial information regarding the role of aluminum as a contributing factor to cholestasis and the possible underlying mechanism, cytotoxicity studies were conducted to determine whether aluminum causes direct toxicity of HepG2 cells. Furthermore, the influence of aluminum on the mRNA expression of hepatic biliary transporters (BSEP, MRP2, MATE1, NTCP) and nuclear transcription factor (FXR) in HepG2 cells using real-time RT-PCR analysis was assessed. Since inflammation is a component of cholestasis, these investigations also involved the use of an inflammatory stimulus, lipopolysaccharide (LPS), to determine whether the effects of aluminum were exacerbated by underlying inflammation. My data suggest that for the canalicular hepatic transporters MATE1 and BSEP, aluminum at higher concentration alone as well as with LPS caused increased mRNA expression levels. In addition to this, BSEP mRNA expression was preserved and that of MATE1 was increased on LPS exposure. Given the particular importance of BSEP in the maintenance of bile flow and reported effects of drug-induced inhibition of BSEP to cause hepatic cholestasis, the influence of aluminum on BSEP (and MATE1) protein expression and activity warrant investigation. Further studies may identify that inhibition of BSEP function (and possibly MATE1) by aluminum contamination of total parenteral nutrition formulations may explain, in part, the intrahepatic cholestasis associated with parenteral nutrition.

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This thesis is dedicated to...

...my inspiring father, Rajbir & caring mother, Kuljeet

my loving brother, Karanbir and

my supportive husband, Amijyot

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LIST OF ABBREVIATIONS

Al	Aluminum
ANOVA	Analysis of variance
ASBT	Apical sodium–dependent bile acid transporter
ABC	ATP-Binding Cassette
FBS	Fetal bovine serum
FXR	Farnesoid X receptor
CFTR	Cystic fibrosis transmembrane conductance regulator
CDCA	Chenodeoxycholic acid
DCA	Deoxycholic acid
CA	Cholic acid
MATE	Human multidrug and toxin extrusion
MATE1	Human multidrug and toxin extrusion 1
BSEP	Bile salt excretory pump
MRPs	Multi-drug resistant proteins
MRP2	Multi-drug resistant protein 2
cDNA	Complementary DNA
cMOAT	canalicular Multispecific Organic Anion Transporter
EDTA	Ethylene diamine tetra acetic acid
EMEM	Eagle's Minimal Essential Medium
GLUT	Glucose transporters
HepG2	Human hepatocellular liver carcinoma cell line
IL-1β	Interleukin-1beta
IL-6	Interleukin-6
LPS	Lipopolysaccharide
MCT	Monocarboxylate transporter
MDR	Multidrug resistance
mL	Milliliter
mRNA	Messenger ribonucleic acid
MTT	[3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide]

OCT	Organic cation transporter
PNAC	Parenteral Nutrition Associated Cholestasis
PN	Parenteral Nutrition
NTCP	Na^+ taurocholate co-transporting peptide
OATPs	Organic anion transport polypeptides
RT-PCR	Reverse transcription-polymerase chain reaction
RXR	Retinoid X receptor
SGLT	Sodium dependent secondary active Na^+/glucose transporter
SMCT	Sodium-coupled monocarboxylate transporter
TLR	Toll-like receptors
Tm	Annealing temperature
TNF-α	Tumor Necrosis Factor-α
TPN	Total Parenteral Nutrition
μM	Micromolar

1. Introduction

1.1 Rationale:

Intravenous feeding of patients with essential and balanced nutrition is required when enteral feeding is not tolerated, therefore indicating the need for Total Parenteral Nutrition (TPN). This life-saving therapy is also associated with the increase risk of intrahepatic cholestasis. The incidence of TPN-related hepatobiliary complications is common in both adults (15-85%) and infants (20-90%) on TPN. Parenteral nutrition associated cholestasis is the most common manifestation of liver dysfunction and in infants this accounts for high morbidity and occasional mortality (Kelly, 1998).

The etiology of PNAC is due to multiple factors (Merritt, 1986). A potential contributing factor is the aluminum contamination of TPN solutions. Aluminum related adverse effects on the liver are well documented (Stein, 1978). Various studies have been conducted in Dr Gordon Zello's Laboratory examining the role of aluminum in PNAC. Findings in his laboratory have shown that neonates were more likely to develop cholestasis when on the PN for longer duration (Arnold, 2004). Also there are indications that some component of the parenteral solution might be a contributing factor for PNAC. Li conducted another study with the primary purpose of monitoring the serum aluminum level in correlation with the development of PNAC in the infants requiring PN therapy with gastrointestinal failure (Li, 2005). In another study in Dr Zello's laboratory aluminum loading in neonatal piglets caused hepatic injury. Such evidence suggests a correlation between aluminum levels and PNAC in neonates but the exact mechanism of action of Al in the PNAC development is unknown. Therefore, a step further into this is by what mechanism Aluminum contributes in the development of PNAC is required.

The accumulation of the aluminum in the liver may lead to cholestatic changes (Demircan 1998; Klein, 1998), through mechanisms that remain unclear and ill defined. As cholestasis involves reduced bile flow, alteration in those transporters responsible for the production and maintenance of bile flow as an underlying mechanism of aluminum induced intrahepatic cholestasis is possible. The purpose of my research is to investigate whether aluminum, like other xenobiotics, alters the expression of hepatic biliary transporters as a potential mechanism for cholestasis.

Also, in patients with inflammatory processes or extrahepatic infections inflammation induced cholestasis is a common complication. The potent inducers of inflammation-induced cholestasis are the inflammatory cytokines which are produced in response to different non-infectious and infectious stimuli. The expression, activity and function of various transporters at cellular membranes can be altered by inflammation (Ling et al., 2012). Inflammation results in release of cytokines that can cause changes in biliary transporters expression levels, and in turn, to reductions in bile flow and cholestasis. Inflammation can further lead to repressed activity of nuclear transcriptional regulators that are essential for gene expression of various hepatocyte transporters (Kosters and Karpen, 2010). There are several clinical situations where inflammation is shown to cause or contribute to cholestatic disease. As liver detoxifies a variety of xenobiotics, in acting as the first line of defence against xenobiotics like aluminum, it is possible that the liver can show an interrelated response to inflammation. Therefore, my evaluations will include the determination of a possible additive effect of inflammation on Aluminum induced changes in mRNA expression of hepatic biliary transporters (BSEP, NTCP, MRP2, MATE1) and FXR.

2. Literature Review

2.1 Hepatic Physiology: Liver Structure and Function.

The liver has a wide range of functions including drug biotransformation, detoxification, and synthesis of proteins and formation of enzymes required for digestion. Hepatocytes, which are the predominant cell type in the liver, form a polarized epithelium with distinct basolateral and apical surfaces (Figure 2.1) and hold the machinery for drug metabolism and transport, which is responsible for hepatic drug clearance and formation of bile. The hepatocytes are arranged in plates such that their basal surface faces the blood sinusoids and the apical faces are held together by apical junctional complexes to form the canaliculi (Mirjam and Hoekstra, 1998). The hepatic artery (25% blood supply) and portal vein (75% blood supply) supply blood into hepatic sinusoids. The sinusoids that lie between hepatic plates feed into the central vein which empties blood into the hepatic veins and then into the inferior vena cava. Hepatocytes secrete bile into the canaliculi, which empties into the bile ductules and then into the hepatic ducts.

The functional unit of the liver is the acinus. It is built around a central vein and is composed of many hexagonally arranged hepatic cellular plates of parenchymal cells. The acinus is divided into three zones: the periportal zone (Zone 1), which lies closest to the arterial and portal blood supply; the midzonal zone (Zone 2); and the centrilobular area (Zone 3), which surrounds the central vein. The zonal arrangement of the hepatic acinus is accompanied by a heterogeneous expression of metabolic enzymes and transporters, particularly transporters responsible for the formation of bile (Beath, 2003).

The remarkable diverse functions of the liver are attributed to its structural organisation and these include (1) filtration and storage of blood; (2) bile formation; (3) metabolism of hormones, macronutrients and foreign chemicals; (4) storage of iron and vitamins; and (5) formation of coagulation factors (Erlinger, 1999).

Liver serves as the primary regulatory site for taking up and processing ingested nutrients for distribution to extrahepatic tissues. The liver is of paramount importance in maintaining normal blood glucose levels and cholesterol. In the liver, 80% of cholesterol is converted to bile salts, lipoproteins and phospholipids. The liver is also involved in protein metabolism and is responsible for the production of 90% of plasma proteins (e.g. transferrin, albumin). These proteins not only help develop and maintain plasma osmotic pressure but also serve to transport various drugs, hormones, and minerals (e.g. iron and calcium).

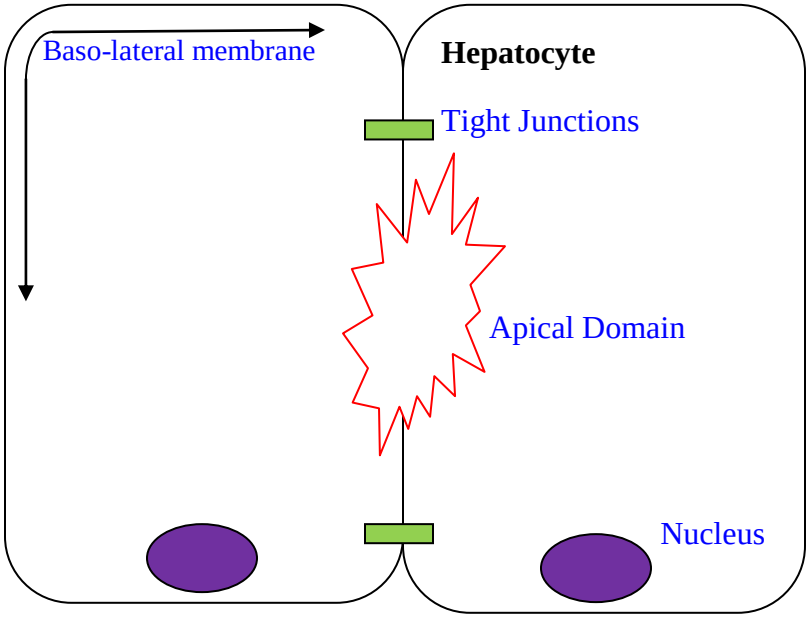

Figure 2.1: Hepatic Polarity.

The apical domain is indicated in red. The basolateral and apical membranes are separated by the tight junctions (filled in green), forming the epithelial sheet. These tight junctions help maintain cellular polarity, causes transcellular vectorial movement of solutes. In addition, it prevents paracellular transport of solutes between adjacent cells.

Furthermore, acute phase proteins like C-reactive protein, ceruloplasmin and other complement factors are also synthesized by liver which play significant roles in the inflammatory response (Chwals, 1995).

2.1.1 Bile secretion and flow:

A major function of liver cells is the synthesis and secretion of bile. Furthermore, hepatocytes are involved in the excretion of xenobiotics and elimination of cholesterol and bilirubin. The production of bile by hepatocytes also aids the digestion and absorption of fat-soluble vitamins and lipids in the intestinal lumen (Scharschmidt, 2003). Bile acids are amphipathic physiological detergents that play essential roles in promoting absorption, excretion and transport of cholesterol, lipids, lipophilic nutrients and other hydrophobic compounds in the liver and intestine (Hofmann, 1999).

Bile synthesis occurs via an osmotic secretory process and the concentration of bile salts and other biliary components in the canalicular lumen act as the driving force (Trauner et al., 1998). Cholesterol is the precursor for bile acid formation, which comes from either the diet or from the breakdown of the fat by the hepatocytes. Cholesterol breakdown yields equal quantities of cholic acid and chenodeoxycholic acids. They are the two primary bile acids in humans and these can be converted to secondary bile acids, deoxycholic acid and lithocholic acid, respectively, by the intestinal bacterial flora (Eloranta and Kullak-Ublick, 2007). These acids conjugate with glycine or taurine forming glyco-conjugated or tauro-conjugated bile acids and forming bile salts of sodium and potassium making them impermeable to plasma membrane and resulting in high concentrations in the bile (Hofmann, 1999). Bile salts have two major actions: First, their detergent action helps emulsify fat, and secondly they help in the absorption of fatty acids, cholesterols and other lipids from the intestinal tract (Guyton, 2005). Bile acids also stimulate bile flow. Bile acids being the major constituent of bile make it unique of all the digestive secretions. Bile acids are excreted into the canaliculi and stored in the gall bladder, from where it is then released into the small intestine and later recycled back via portal vein into the liver (Hofmann, 1999). This continuous process of secretion, absorption followed by resecretion is called enterohepatic circulation (Figure 2.2). This serves as a negative feedback regulation for cholesterol biosynthesis. With increased concentration of bile acids in the liver cells bile acid synthesis decreases and while cholesterol synthesis undergoes a parallel increase (Vlahcevic et al., 1991).

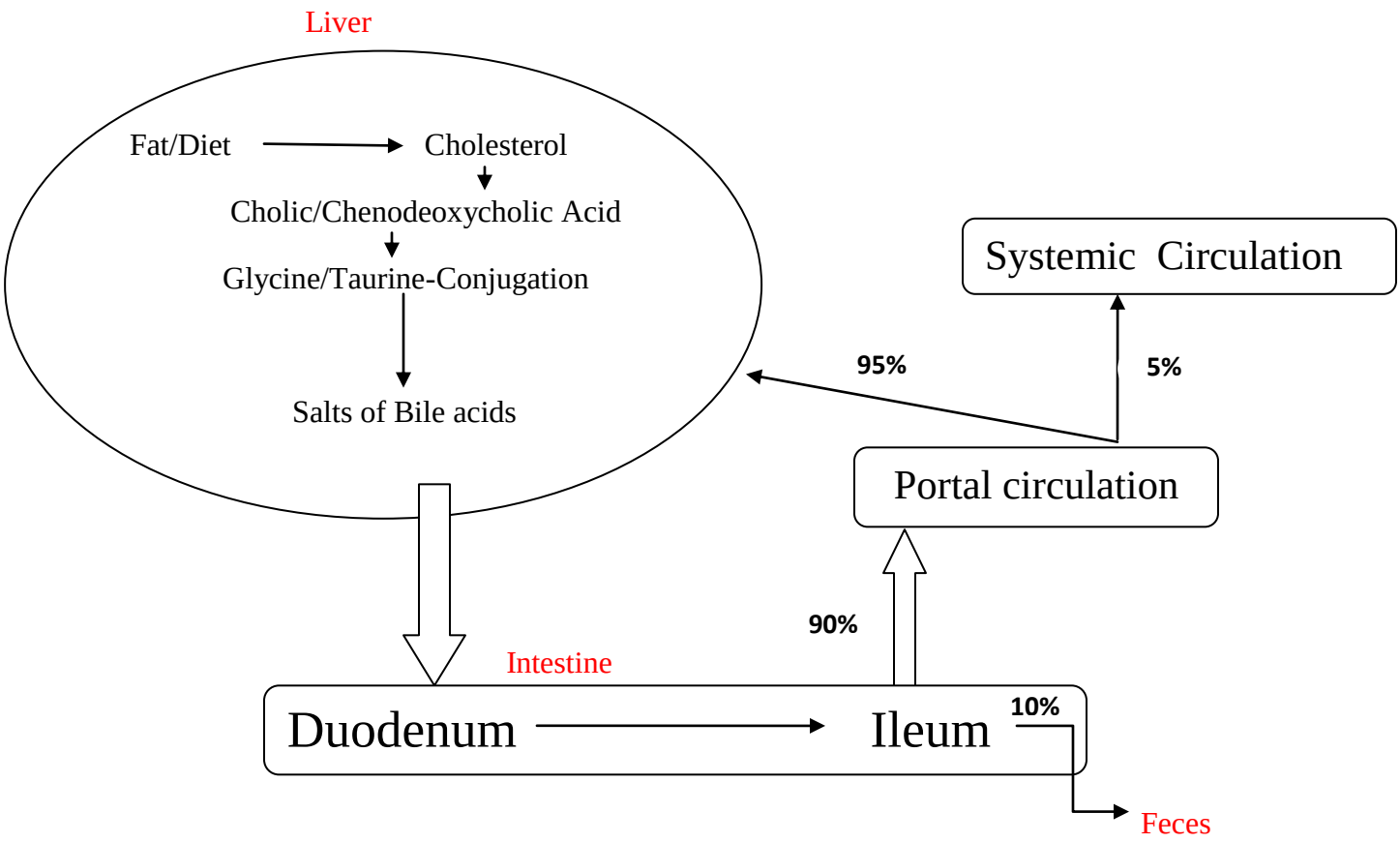

Figure 2.2: Enterohepatic Circulation of Bile Acids.

Bile acid salts are major constituent of bile. Enterohepatic circulation of bile acids involve continuous process of their excretion into the canaliculi, then storage in the gall bladder, from where it is then released into the small intestine and later recycled back via portal vein into the liver. Enterohepatic recirculation is one of the major mechanisms that help to maintain the bile flow.

Three contributing factors to the bile flow are: bile acid dependent bile formation, bile acid independent canalicular secretion and bile acid independent ductal flow (Figure 2.3).

Bile acid-dependent bile formation has three main components: 1) bile acid uptake, 2) intracellular transport and 3) canalicular secretion by the hepatocyte (Kullak-Ublick et al., 2000). Cloning and characterization of various transport proteins involved in the uptake, intracellular transport and secretion of various compounds resulted in important advances in recent years concerning the transport processes and systems involved in the formation of bile (Muller and Jansen, 1997; Trauner et al., 1998; Hoffmaster et al., 2004). The two major transporters that mediate bile acid uptake are Na^+-taurocholate cotransporting polypeptide (NTCP) and organic anion-transporting polypeptide (OATP) (Hagenbuch et al., 1991). The transmembrane Na+ gradient, maintained by the Na^+/K^+-ATPase provides energy for the NTCP. Further, a voltage-dependent carrier and an ATP-dependent transport system mediate the canalicular secretion (Nishida et al., 1999; Trauner et al., 1998). Active secretion by the liver cells of the bile acid provides an osmotic force for canalicular electrolytes and water. Therefore, between the adjacent hepatocytes within the lumen of the bile canaliculus osmotic gradient is created which helps form bile flow. The most important osmotically active solute is the bile acid, generating 50% of the total bile acid-dependent flow (Boyer et al., 1992). Phospholipids also form part of bile acid-dependent flow and is transported by the proteins of multi-drug resistance (MDR) family.

Bile acid-independent bile flow involves canalicular excretion of bicarbonate and glutathione. Glutathione and its conjugates are transported by canalicular multispecific organic anion transporter 1 (cMOAT; also named MRP2) into the canalicular lumen (Oude Elferink et al., 1995). Bilirubin is taken up by the hepatocytes and after conjugation cMOAT or MRP2 helps secrete them into the canalicular lumen (Keppler et al., 1997; Trauner et al., 1998).

Ductular bile flow is stimulated by secretin and consists of bicarbonate-rich solution. Secretin receptors are located on the basolateral membrane and on binding to secretin cAMP production is activated. This then stimulates a chloride channel located on the apical membrane named cystic fibrosis transmembrane conductance regulator (CFTR) (Trauner et al., 1998). This further activates chloride/bicarbonate exchanger on the apical membrane increasing bicarbonate secretion. The apical membrane also has this exchange (Trauner et al., 1998). Bile undergoes modification by the bile ducts and ductules and then is delivered to the intestine (Boyer et al., 1992; Trauner et al., 1998), further leading to enterohepatic circulation as explained in previous

section. The transport system on both apical and baso-lateral membranes participates in the production and maintenance of the bile flow. Any alteration of any of these transporters may cause disruption of bile flow and the development of cholestasis (Lecureur et al., 2000).

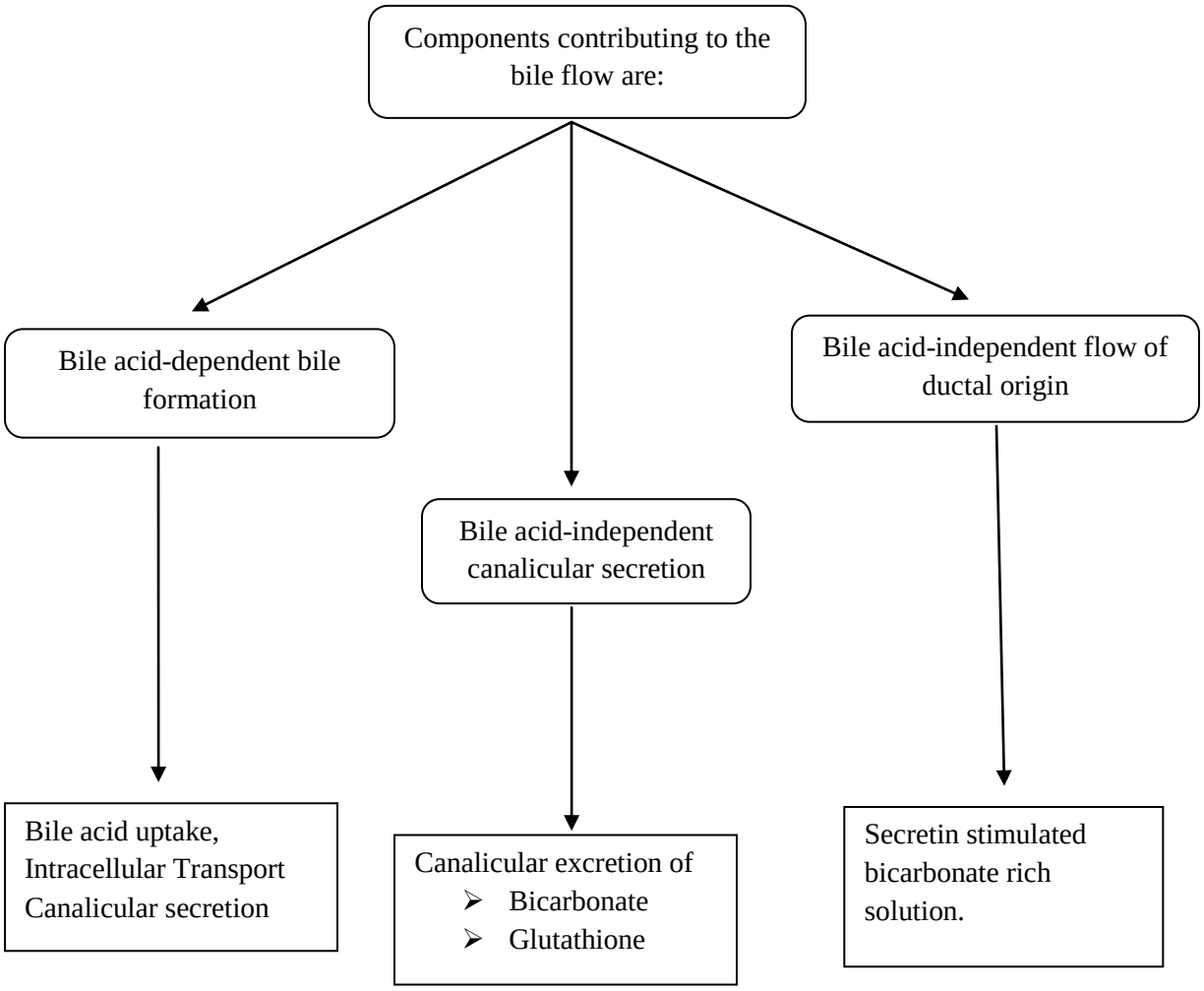

Figure 2.3: Three major contributing factors to Bile flow: Bile acid-dependent bile formation, bile-acid independent canalicular excretion and bile-acid independent flow of ductal origin and the involvement of various ion pumps and transporters located on baso-lateral and apical membranes.

2.2 Cholestasis:

Cholestasis refers to the accumulation of bile in the liver cells and biliary passages. Therefore, cholestasis is a form of a liver injury that occurs because of reduced bile flow from the liver into the duodenum. It can be caused by rapidly developing (acute) or long-term (chronic) interruption in the excretion of bile. The obstruction causes bile salts, the bile pigment, bilirubin, and fats (lipids) to accumulate in the blood stream instead of being eliminated via the biliary system into the gastrointestinal tract. This leads to hyperbilirubinaemia due to the failure of normal amounts of bile to reach the duodenum. Cholestasis can be caused by obstruction within the liver (intrahepatic) or outside the liver (extrahepatic) (Pausch and Gatzen, 2006). Prolonged bile duct obstruction may lead to cirrhosis and secondary fibrosis (Lesur et al., 1993; Afroudakis and Kaplowitz, 1981). At the cellular level in cholestasis bile retained in the liver damages the hepatocytes. But not all bile acids are equally damaging. More hydrophobic bile acids like chenodeoxycholic acid (CDCA) are more injurious than ursodeoxycholic acid, which is more hydrophilic (Araki et al., 2003).

2.2.1 Intrahepatic Cholestasis:

Intrahepatic cholestasis is due to impaired hepatic excretion of bile and may occur from hereditary or acquired disorders. It is characterized by widespread blockage of intrahepatic ducts that impair the body's ability to eliminate bile and the accumulation of the toxic bile acid within hepatocytes which may induce hepatocellular apoptosis (Guglielmi et al., 2008). There could be various causes for intrahepatic cholestasis. Hereditary disorders producing intrahepatic obstruction to biliary excretion is also known as pure cholestasis. These may include Dubin-Johnson syndrome, Rotor syndrome, fibrocystic disease of pancreas, primary biliary cirrhosis, vanishing bile duct syndrome which can be further caused by allograft rejection, graft-versus-host disease, alagill'e syndrome, hodgkin's disease or idiopathic adult ductopenia (Erlinger, 1999). Acquired disorders with intrahepatic excretory defect of bilirubin are largely due to hepatocellular disease and, hence, known as hepatocellular cholestasis. These may include viral hepatitis, alcoholic hepatitis, parenteral nutrition and drug induced cholestasis as from administration of oral contraceptives and chlorpromazine. The space occupying lesions that are due to primary or secondary liver cancer, lymphoma or amyloidosis is also responsible for intrahepatic cholestasis (Erlinger, 1999). Cystic fibrosis also causes obstruction of intrahepatic bile ducts (Erlinger, 1999).

The features of intrahepatic cholestasis include: predominant conjugated hyperbilirubinaemia due to regurgitation of conjugated bilirubin into blood implying impaired biliary excretion, bilirubinuria, elevated levels of serum bile acids, elevated serum alkaline phosphatase levels indicating biliary obstruction and hyperlipidaemia causing increased levels of liver cholesterol (Mohan, 2005).

2.2.2 Extrahepatic Cholestasis:

Extrahepatic cholestasis is caused by mechanical obstruction in large bile ducts outside the liver or within the porta hepatis. The various causes for this include common bile duct stones, inflammatory strictures, carcinoma of the ampulla of Vater, pancreatic carcinoma, pancreatic cyst, biliary atresia, AIDS cholangiopathy, benign bile duct strictures, parasites and portal vein thrombosis (Erlinger, 1999). The features of extrahepatic cholestasis, like in intrahepatic cholestasis, are predominantly conjugated hyperbilirubinaemia, bilirubinuria, elevated levels of serum bile acids causing intense pruritus, high serum alkaline phosphatase and hyperlipidaemia (Mohan, 2005). However, there are certain features which help to distinguish extrahepatic from intrahepatic cholestasis. In extrahepatic cholestasis malabsorption of fat soluble vitamins and steatorrhoea resulting in vitamin K deficiency is an important differentiating characteristic.

2.2.3 Neonatal Cholestasis:

Cholestasis is an important pathological condition and can have a significant effect on morbidity and mortality in both adults and neonates. The incidence is higher in neonates due to underdeveloped hepatic system (Adamkin, 2003). The cholestatic propensity of the neonate is attributed to its immature liver excretory function. In the fetus the hepatic bile flow is dormant and this role is replaced by the placental excretory circulation in the fetus until it completes its intrauterine life (Balistreri, 1985). Stimulation of bile flow occurs after the birth and hepatic functionality undergoes gradual maturation. Various animal studies have shown prenatal presence of NTCP and BSEP but there concentrations at birth are well less than the levels in adults (McKiernan, 2002). Transient disturbances related to bile acid transport are prone to occur in immature hepatocytes and this may cause decrease bile flow, altered cellular bile formation or other clinical cholestatic manifestations (Balistreri, 1985).

2.2.3.1 Predisposing factors to cholestasis in infants:

The various risk factors that predispose newborns to reduced biliary flow include impaired bile acid conjugation, inefficient bile acid uptake, decreased bile secretion and bile acid synthesis deficiencies (Arnold, 2004). Inefficient bile acid uptake is often accompanied by increased levels of bile acids in neonates (Gremse and Balisteri, 1989). There are several manifestations of immature bile acid transport and metabolism and these include increased serum bile acids, enhanced efflux of bile acids, decreased hepatic uptake of bile acids, decreased conjugation, glucuronidation & sulfation, defective quantitative & qualitative synthesis, decrease in bile acid pool size, absence of lobular gradient, decreased bile acid secretion rate and decrease in intraluminal bile acid concentration (Gremse and Balisteri, 1989).

2.3 Parenteral nutrition associated cholestasis:

Parenteral nutrition associated cholestasis is the most common manifestation of liver dysfunction and in infants this accounts for high morbidity and occasional mortality (Kelly, 1998). Cholestasis, cirrhosis and fibrosis may be triggered in these small patients requiring prolonged TPN regimen (Benjamin, 1981). Therefore, there is a serious threat of hepatobiliary complications in younger patients who need prolonged TPN to survive. Parenteral nutrition associated cholestasis (PNAC) encompasses an increase in serum conjugated bilirubin of 2 mg/dl or higher, and a rise in glutamyl transpeptidase, serum transaminase and alkaline phosphatase (Guglielmi et al., 2006).

2.3.1 Total Parenteral Nutrition:

Total parenteral nutrition is intravenous feeding of patients that are unable to tolerate enteral feeding, with essential and balanced nutrition. In neonates, parenteral nutrition is given when they are unable to obtain nutrition via nursing and thus has become a vital component of the care of ill and premature infants. The primary aim of parenteral nutrition (PN) is to provide patients with required calories and protein to prevent malnutrition. A normal diet provides an individual with an adequate mix of carbohydrates, proteins and fat for energy and tissue development. Therefore, parenteral nutrition therapy must also provide patients with these same dietary components. Parenteral Nutrition Solution contains protein in the form of amino acids, carbohydrates in the form of glucose, fat as a lipid emulsion, water, electrolytes, vitamins and trace elements. Though TPN has contributed to improved survival rates of preterm infants and considered as a life saving therapy, it is not without limitations and complications.

The incidence of TPN-related hepatobiliary complications is common in both adults and infants on TPN. The prevalence is reported to be 20 to 90% in paediatric age group, whereas in adults a wide range of about 15 to 85% of various liver dysfunctions has been reported (Luman and Shaffer, 2002). The broad spectrum of pathological, biochemical and clinical manifestations may range from increases in liver enzymes to hepatic steatosis or liver disease that is histologically characterised by intrahepatic cholestasis, reactive bile-duct proliferation, portal fibrosis or periportal inflammation (Guglielmi et al., 2006). End-stage liver disease (ESDL) afflicts 15 to 20% patients receiving TPN for prolonged periods and exhibits high rates of morbidity and mortality (Chan et al., 1999). Another most frequent event is biliary sludge. When on TPN, enteric stimulation is lost, impairing the gallbladder motor function and resulting in sluggish gall-bladder. This further explains the development of gall stones and biliary sludge while on TPN. Messing et al observed in 23 patients on TPN, 50% developed sludge after 4 to 6 weeks on TPN and formation of gallstones in 6 patients (Messing et al., 1983). Total parenteral nutrition is also associated with complications like metabolic imbalance, thromboembolism and liver dysfunction (Taylor et al., 1991; Kelly, 1998).

Histological changes observed in liver biopsies include giant cell transformation, extramedullary haematopoiesis, hepatocellular damage, loss of canalicular microvilli (Erlinger, 1991) and portal fibrosis (Gremse and Balisteri, 1989). In severe cases bile duct proliferation may also occur. Clinically, it is associated with a conjugated hyperbilirubinemia along with increased levels of alkaline phosphatase and serum aminotransferases. The rise in bilirubin is followed by increase in serum bile acid concentrations. Physical findings include jaundice and heptomegaly (Li, 2005).

As the incidence of cholestasis is higher in infants compared to the adults, it suggests that that there is other risk factors that co-exist only in the paediatric population. In neonates due to prematurity and low birth weight, the immunity is impaired and hence they become more susceptible to infections. Various factors predisposing children to PNAC include premature birth, lack of enteral feeding, duration of parenteral nutrition, sepsis, imbalanced nutrients and contamination of PN solutions. The nonnutritional and nutritional components of the parenteral solutions figure as prominent suspects in the list of risk factors (Forchielli, 2003). Various studies identify a strong association between incidence of cholestasis and duration of total parenteral nutrition (Whitington, 1985; Kubota et al., 2000; Kelly, 1998).

The causative factors and the pathogenesis of cholestasis associated with TPN still remain ill-defined. Studies so far suggest that the etiology of the disease is often multifactorial (Merritt, 1986). But the most potential etiology worth attention is the contamination of parenteral nutrition solutions with aluminum.

2.4 Aluminum:

Aluminum is one of the most abundant elements in the earth's crust. The presence of Al in soil, food and drinking water uniformly exposes humans to this element. Over the last few decades, human exposure to Al has been increasing. Al is present mainly in food products and drinking water as a result of water purification processes (Levesque et al., 2000). In adults, food and beverages account for 90-95% total daily intake. Medical treatments and pharmaceutical products also incorporate high levels of Al. Despite its ubiquitous presence in food and water, few biological functions are associated with Al and this element is considered nonessential.

2.4.1 Aluminum Metabolism and Toxicity:

The absorption of aluminum via the gastrointestinal tract is < 1% (Cooper et al, 1984). After absorption, aluminum can either remain free in the blood or is bound to transferrin, the iron-binding plasma protein (Cooper et al., 1984). The transferrin-aluminum complex is then delivered to the liver where it becomes deposited in lipofuscin granules of hepatocytes (Galle et al., 1982), in lysosomes of macrophages (Fiejka et al., 1996) and in the nucleus of hepatocytes (Kushelevsky et al., 1976). This deposition can create ultrastructural lesions in the liver (Galle et al., 1987). A previous study in our laboratory has also detected the early morphological and biochemical changes in the liver following the chronic infusion of high dose aluminum. TEM-EDX (transmission electron microscopy equipped with energy dispersive spectroscopy) demonstrated the presence of aluminum deposits in the lysosomes of the liver cells of piglets (Alemmari et al., 2011). Also, morphological changes included loss of canalicular microvilli and condensation of mitochondria. The ultrastructural changes detected were proportionally increased with the increased duration of aluminum infusion (Alemmari et al., 2011).

Renal elimination is the major route of aluminum excretion (Cooper et al., 1984). In healthy adults, most blood aluminum is cleared in the urine and only a small amount is excreted in bile (Greger and Sutherland, 1997). In premature neonates, the bound aluminum-transferrin complex is nonfilterable and coupled with an underdeveloped renal system and low glomerular

filtration rates aluminum accumulation can occur with repeated exposure to aluminum (Recknagel et al.,1994; Greger and Sutherland, 1997).

Aluminum plays a role in a number of different pathological disorders of mammals. In human medicine, aluminum toxicity was first described in uremic patients on haemodialysis (Berlyne et al., 1970), and later the toxic effects due to high aluminum body loads were noted in several other conditions, including bone disease, encephalopathy, and anemia and also is a contributing factor in Alzheimer's and Parkinson's dementia (Klein, 1995; Koo, 1992; Savory et al., 1996). The toxic effects of aluminum are not limited to humans. Aluminum was shown to inhibit egg laying in hens due to dysfunction of the egg shell gland mucosal ATPase activity mediated by aluminum (Lundholm and Mathson, 1986). Furthermore, the increased levels of aluminum in water are reported in some studies to be associated with the death of birds and fish (Ganrot, 1986; Driscoll, 1985).

Aluminum accumulates significantly in the liver. As liver is involved in the uptake of aluminum and excretion via biliary flux the known effects of aluminum on the liver require discussion. Studies suggest various biochemical changes are associated with aluminum accumulation in the liver and include alterations in oxidant status and release of enzyme markers of hepatic injury due to disruption of hepatocellular integrity and function (Moumen et al., 2001; Wilhelm et al., 1996). High doses of aluminum also cause reductions in bile flow and elevation in serum bile acids (Klein et al., 1988). Dose dependent cytotoxic effects include multifocal hepatocellular degeneration and fibrous tissue proliferation (Roy et al., 1991; Bertholf et al., 1989), as well as increases in MDH (malondialdehyde), a marker of lipid peroxidation in the liver leading to cell damage and reductions in GSH (glutathione) antioxidant levels and subsequent oxidant damage (Turgut et al., 2006).

Also, there is evidence of other heavy metals that accumulate in the liver to contribute to hepatotoxicity. Isolated perfused livers from Sprague-Dawley rats when exposed to cadmium chloride caused acute hepatotoxicity and this was evident from cadmium-induced changes in bile flow, alanine aminotransferase (ALT) leakage and urea synthesis (Lupo et al., 1986). Cadmium also induced lipid peroxidation. Rapid decrease in bile flow followed by complete cholestasis was caused by cadmium in livers perfused from Sprague-Dawley rats. A variety of biochemical and anatomic effects of lead which includes ultra-structural alterations of the spleen and liver have been demonstrated (Hoffmann et al., 1972). Further, lead markedly increases lethality in

LPS-treated rats (Seyle et al., 1966). This observation is attributed to the fact that LPS detoxification in the liver is impaired by the lead and hence causing endotoxicity (Trejo and Di Luzio, 1971). Heavy metals contamination such as mercury, lead, cadmium and arsenic of herbal supplements has also been considered to cause hepatic liver injuries leading to cholestasis (Ramachandran and Kakar, 2009).

2.4.2 Aluminum in Total Parenteral Nutrition Solutions

In 1982, the contaminant aluminum of PN solutions was identified in a protein source, casein hydrolysate, and was found in the blood, urine and bones of patients administered these solutions (Klein et al., 1982). The concentration of aluminum in PN solutions ranged from 112 to 196 µg/L. Later, another study showed higher contamination levels of aluminum in PN, particularly in phosphates, calcium salts and trace elements (Popinska et al., 1999). Guidelines for contaminant aluminum in PN solutions were published by the Food and Drugs Administration (FDA) of U.S.A., where it stated that for small volumes of solution, maximum levels of aluminum should be identified on the label and in large volumes the concentration of aluminum should not exceed 25 µg/L (FDA, 2000).

2.4.3 Aluminum & Parenteral Nutrition Associated Cholestasis (PNAC):

The most implicated constituent in the pathogenesis of PNAC is the contaminant aluminum (Arnold et al., 2003). Contaminated PN solutions, heparin, mineral salts and blood products like albumin are the major causes of parenteral aluminum exposure (Arnold et al., 2003). The greatest contamination is found in calcium and phosphate solutions, vitamin C preparations with trace elements (Greger and Sutherland, 1997; Davis et al., 1999). The average contaminant levels of TPN with aluminum indicate that neonatal patients receive 16.7 µg/kg/day as a mean daily intake (Moreno et al., 1994). It was then shown that toxic levels were approximately reached when premature infants were on a load of 15-30 µg/kg/day of PN solutions (Bishop et al., 1997).

Various studies have been done with regard to aluminum toxicity. Klein et al studied the aluminum accumulation in 5 children of 18-34 months of age and who were on PN for 18 to 33 months (Klein et al., 1984). Klein et al reported elevated liver enzymes and increases in serum, urine and hepatic aluminum concentrations. The histopathological findings included periportal fibrosis, bile duct proliferation and cellular necrosis, which were attributed to the abnormal accumulation of aluminum in the liver. Further, in a prospective study of newborn infants on PN

solutions elevated serum and urine aluminum levels were found in infants on PN relative to controls and ~89% of total aluminum intake was accounted for from PN solution (Moreno et al., 1994).

Various studies have been conducted in Dr Gordon Zello's Laboratory examining the role of aluminum in PNAC. In a retrospective study of infants at the Royal University Hospital to establish PNAC prevalence, neonates were more likely to develope cholestasis when the duration on PN was greater than 18 days (Arnold, 2004). Taking into consideration the duration of PN it was clear that the longer requirement of PN as the nutrition source, higher was the risk of developing PNAC. This further indicates that some component of the parenteral solution might be a contributing factor for PNAC. For an example, toxins like aluminum require a long period of hepatic accumulation before manifestation of harmful effects (Arnold, 2004).

Li conducted another study with the primary purpose of monitoring the serum aluminum level in correlation with the development of PNAC in the infants requiring PN therapy with gastrointestinal failure (Li, 2005). Serum aluminum as well as bilirubin concentrations were determined in 16 infants with some kind of gastrointestinal pathology on various durations of PN therapy. Five out of 16 (31.3%) infants developed PNAC by the end of three weeks.

In another study in Dr Zello's laboratory aluminum loading in neonatal piglets caused hepatic injury. In a model of chronic exposure to Al in PNAC the study suggested that at high dose intravenous infusion of Al in neonatal piglets Al accumulated in bile, serum, liver tissues and urine. Further investigations with low Al content in PN showed that the reduction in Al level in PN solutions reduced the severity and incidence of PNAC (Alemmari et al., 2012). The effects were evaluated by measuring the combination of clinical outcomes related with PNAC comprising serum direct bilirubin levels, serum total bile acids levels and morphological changes in the liver tissues.

Such evidence suggests a correlation between aluminum levels and PNAC in neonates but the exact mechanism of action of Al in the PNAC development is unknown. Therefore, a step further into this is by what mechanism Aluminum contributes in the development of PNAC is required.

2.5 The role of transporters in PNAC:

Hepatocyte transporters have a key role to play in hepatic xenobiotic exposure and xenobiotic clearance. As well, their co-ordinated action is essential for bile formation and for the biliary secretion of various ions and xenobiotics (Pauli-Magnus and Meier, 2006). Cholestasis results from the disruption of biliary secretion of bile acids. The intracellular levels of bile acids need to be regulated tightly. This can be brought about by the regulation of the transcription factors and of genes expressing important proteins involved in synthesis and transport of bile acids (Mil et al., 2005). Transporter function can be regulated by both exogenous and endogenous substances at any level of gene expression, i.e. transcriptional, posttranscriptional, translational or posttranslational. For example, downregulation of the NTCP gene following an inflammatory stimulus occurs at the transcriptional level, whereas mRNA levels of MRP2 and BSEP are unchanged while protein expression levels were reduced suggesting posttranscriptional regulation by the inflammatory stimulus (Elferink et al., 2004). Xenobiotics have also been shown to regulate transporter function at the translational and posttranslational levels (Williams et al., 2000). Many xenobiotics are known to inhibit transporter function through reversible inhibition, either through competitive or noncompetitive mechanisms.

Hepatocyte transporters:

Specific transporters mediate the enterohepatic circulation of bile acids. These transporters are expressed in both the enterocytic and hepatocellular polarized epithelial barrier and function in a concerted manner to recycle bile acids between the liver and intestine (Meier and Stieger, 2002). The major hepatocyte transporters are presented in the Figure 2.4. The major two processes involved in enterohepatic recirculation of bile acids are secretion from the liver and absorption from the intestine (Alrefai and Gill, 2007). At the basolateral surface of the hepatocellular epithelium, Na+ taurocholate co-transporting peptide (NTCP) and organic anion transport polypeptides (OATPs) ensure bile acid transport from the blood into the hepatocyte. Excretion of bile salts into the bile requires the function of the bile salt excretory pump (BSEP) and multi-drug resistant proteins (MRPs) expressed in the apical portion of the hepatocellular epithelium (Alrefai and Gill, 2007). Bile acids via the bile ducts are presented to the intestinal lumen where they facilitate the absorption of lipids and cholesterol. Finally, the majority of bile acid is reabsorbed through apical sodium dependent bile acid transporter (ASBT) and sodium barrier (Alrefai and Gill, 2007).

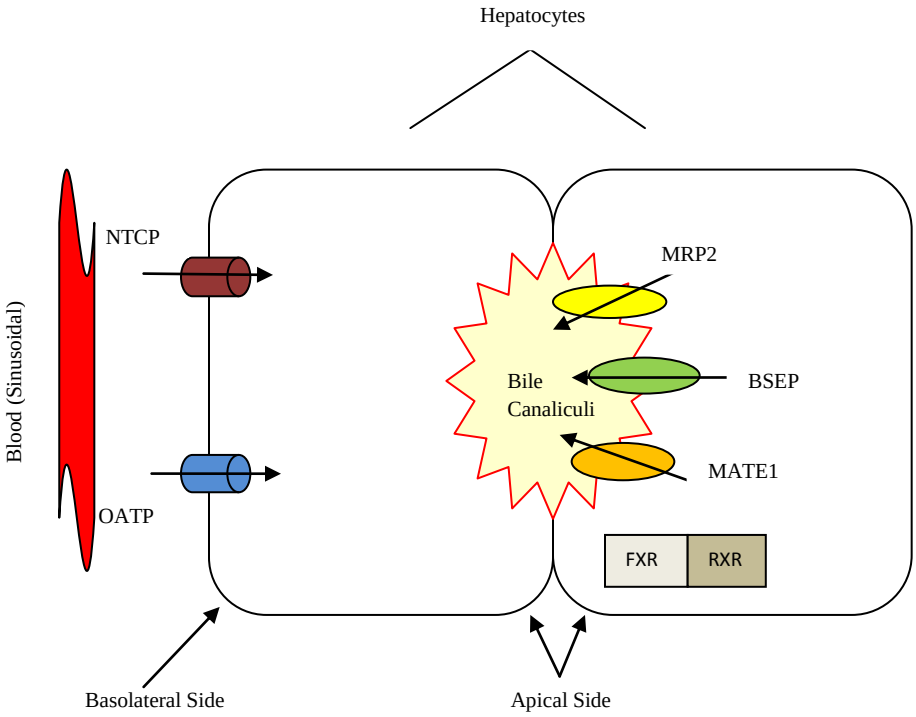

Figure 2.4: Major hepatocyte transporters. Uptake transporters are present on the basolateral membrane whereas the efflux transporters are present on apical membrane with arrowheads pointing to the direction of xenobiotic transport.

2.5.1 Canalicular hepatic transporters of Bile acids:

The most critical component of enterohepatic circulation is the canalicular bile acid transport as it is the rate-limiting step in bile formation as well as hepatic excretion (Trauner and Boyer, 2003). The major transporters involved include BSEP and MRP2. BSEP is responsible for the efflux of monovalent bile acids whereas MRP2 exports divalent bile acids (Trauner and Boyer, 2003).

BSEP (Bile salt excretory pump):

BSEP is exclusively expressed in the liver (Gerloff et al., 1998). It belongs to the ATP Binding Cassette (ABC) superfamily and is a member of the multidrug resistance protein family (Meier and Stieger, 2002). It has 12 transmembrane spanning domains and Walker A and B motifs as intracellular nucleotide-binding domains, which help the binding and hydrolysis of ATP (Arerese et al., 2004). BSEP expression occurs during the postnatal period and upon hepatocellular de-differentiation (eg. cell culture of primary hepatocytes) BSEP expression becomes down regulated (Rippin et al., 2000; Tomer et al., 2003). Defects in the functioning of BSEP lead to decrease bile flow and, hence, cholestasis (Oude et al., 2006; Suchy et al., 2006). Mutations in the BSEP gene results in progressive familial intrahepatic cholestasis (PFIC) type 2 and are associated with permanent cholestasis since birth (Jansen and Sturum, 2003).

MRP2 (Multi-drug resistant protein 2):

There are 12 members in human the ABCC subfamily which include nine MRPs, cystic fibrosis transmembrane conductance regulator (CFTR) and two sulfonyl-urea receptors, SUR1 and SUR2. In terms of structure MRP2 contains two parts: transmembrane domain (TMD) and nucleotide-binding domain (NBD). The ATP binds to the NBDs which cause its hydrolysis and this is crucial for the transport of substances across the plasma membrane (Haimeur et al., 2004). Organic anionic compounds, glutathione, glucuronic acid or sulphate conjugates of drugs and drugs metabolites are substrates of MRP transporters (Funk, 2008). MRP2 (ABCC2), which is present on the canalicular membrane, has significance in xenobiotic elimination. Originally it was designated as the canalicular multispecific organic anion transporter (cMOAT).

The expression of MRP2 can be modulated in different physiopathological conditions (Payen et al., 2002). MRP2 is down-regulated in cholestasis (Zollner et al., 2001). Various experimental studies have shown that in cholestasis the reduced expression of MRP2 leads to impaired excretion of bile salts and organic anions like bilirubin (Trauner et al.,1998; Trauner et

al.,1999). Further, mutations in MRP2 can lead to defective transporter expression. For example, Dubin–Johnson syndrome causes increase levels of conjugated bilirubin in the serum and is often associated with the hepatocytes inability to secrete conjugated bilirubin into the bile resulting in conjugated hyperbilirubinemia. A defect in the MRP2 gene causes this autosomal recessive disease (Paulusma et al., 1999).

MATE1 (Human multidrug and toxin extrusion 1):

OCT transporters mediated the hepatocellular entry of organic cations but it was not clear how they exited the hepatocyte. Recently, MATE transporters were found to be responsible for the removal of organic cations from hepatocytes. MATE transporters belong to the Solute Carrier (SLC) superfamily of transporters. MATE is also known to be the transporter responsible for executing the last step of excretion for organic cations that are toxic (Hiasa et al., 2006). Mammalian MATE1 was the first to be identified from the multidrug and toxin extrusion (MATE) protein family (Hiasa et al., 2006). It is predominantly expressed in bile canaliculi and causes organic cation excretion by H^+/OC electro neutral exchange (Inui et al., 2000; Koepsell, 1998; Koepsell, 2004).

2.5.2 Sinusoidal hepatic transporters of bile acids:

Finally, to complete the enterohepatic circulation, bile acids are cleared from the portal blood in the liver. And this hepatic uptake is supported by dedicated transporters in the sinusoidal membrane; mainly the NTCP.

NTCP (Na^+ taurocholate co-transporting pepetide):

NTCP is expressed at the sinusoidal membrane and is the principal uptake transporter for bile acids (Hagenbuch et al, 1999). NTCP mediates sodium dependent uptake activity (Kullak-Ublick et al., 2004; Meier and Steiger, 2002; Trauner and Boyer, 2003). NTCP is also known to demonstrate decreased expression upon de-differentiation of hepatocytes (Rippin et al., 2001; Liang et al., 1993). NTCP belongs to the SLC10A gene family and is also denoted as SLC10A1 (Hagenbuch and Dawson, 2004). NTCP mediates the transport of conjugated as well as unconjugated bile acids. Also, it transports sulphated bile acids and steroid sulphates (Kullak-Ublick et al.,1997). High levels of bile acids have shown to suppress the gene expression of NTCP (Anwar, 2004).

2.5.3 Liver-enriched transcription factor:

FXR (Farnesoid X receptor):

FXR is the master regulator of bile acid transport and metabolism. It is the chief sensor of intracellular bile acid levels and main executor of bile acid-induced transcriptional programs (Kalaany and Mangelsdorf, 2006). Bile acids are ligands of FXR and upon binding activates FXR to effect the transcriptional regulation of a number of genes. CDCA is the most efficient activator of FXR followed by DCA and CA (Eloranta and Kullak-Ublick, 2007). FXR gets activated following bile acid binding, which heterodimerizes with the retinoid X receptor (RXR). The heterodimer binds to its consensus sequence on the promoter regions of genes such as BSEP (Redinger., 2003; Arrese et al., 2004). This has been indicative of the fact that on induction of BSEP by FXR leads to accumulation of hydrophobic bile acids in the hepatocytes (Arrese et al., 2004).

2.5.4 Drug-induced cholestasis through inhibition of transporters:

Malfunctioning of the transporters responsible for the production and maintenance of bile flow may result in cholestatic liver disease. Drug induced cholestasis may be the result of inhibition of hepatobiliary transporters mediated by the drug or its metabolites. Drug mediated disturbances in transporters can lead to intracellular accumulation of bile acid constituents, which may result in cholestatic liver cell damage (Pauli-Magnus and Meier, 2006). The inhibition of BSEP on the canalicular membrane by various drugs is thought to result in cholestasis (Pauli-Magnus and Meier, 2006). Rifampicin, cyclosporine A, bosentan, erythromycin, troglitazone and glibenclamide have shown to inhibit BSEP (Steiger et al., 2000; Fattinger et al., 2001; Funk et al., 2001). Several studies suggest that cyclosporine reduces MRP2 expression leading to reduced bile flow (Padda et al., 2011). Further, the cholestatic effect is enhanced with the co-administration of sirolimus (Bramow et al., 2001). Cholestatic drugs like cephtriaxone and diclofenac, which are MRP2 drug substrates, are known to induce cholestasis (Padda et al., 2011). Estrone-3-sulfate is NTCP drug substrate that can also produce cholestasis (Padda et al., 2011). Hepatic accumulation of sulindac causes canalicular bile salt transport inhibition and thus contributing to cholestasis (Giroux et al., 1982; McIndoe et al., 1981).

2.6 Inflammation Induced Cholestasis:

The potent inducers of inflammation-induced cholestasis are the proinflammatory cytokines, which are produced in response to different non-infectious and infectious stimuli. Clinically, intra/extra-hepatic infections, drug-induced hepatic injury, alcohol-induced hepatic injury, total parenteral nutrition and postoperative surgeries may lead to inflammation-induced cholestasis. Pathophysiologically, the systemic release of proinflammatory cytokines is the common denominator underlying all these aetiologies resulting in cholestasis.

2.6.1 Mediators of inflammation induced cholestasis.

The early reactions of innate immunity and the later responses of acquired immunity mediate defense against infectious or non-infectious stimuli (Trauner et al., 1999). Innate immunity, also known as natural immunity; provides first line of defence against microbes. The principal components of innate immunity encompass 1) physical barriers, such as epithelia and chemical barriers, comprising of anti-microbial chemicals produced by the epithelial membranes, 2) immune cells (macrophages, neutrophils, dendritic cells and natural killer cells) and 3) proteins called cytokines (Abbas et al., 2005). Another form of immunity develops as a response to infection and is known as acquired immunity. The acquired immunity is able to recognize and react to a large number of microbes and in addition is able to distinguish between various closely related microbes or molecules (Flajnik et al., 2004).

2.6.2 The effect of inflammation on hepatic biliary transporters:

The pro-inflammatory cytokines are TNFα, IL-1 and IL-6 released from epithelial cells and immune cells such as tissue macrophages may be responsible for inflammation induced cholestasis (Gabay and Kushner, 1999; Baumann and Gauldie, 1994). The expression, activity and function of various transporters at cellular membranes can be altered by these proinflammatory cytokines (Ling et al., 2012). Various studies in vitro and in vivo have displayed that transporter expression changes during inflammatory conditions. For example, in cancers of pancreas, stomach, breast and thyroid, the expression of SMCT1 is down regulated (Park et al., 2008; Porra et al., 2005; Thangaraja et al., 2009). MCT1 expression is down regulated in intestinal inflammation, which may lead to inflammatory bowel disease (Thibault et al., 2007). In mice, renal tubular glucose transporters SGLT2, SGLT3 and GLUT2 were down regulated by inflammatory stimuli, which were subsequently accompanied by decrease in plasma glucose levels (Ueno et al., 2004). Various transporters in mammary glands are also shown to be

altered following LPS induced inflammation. At different stages of lactation, glucose, fatty acids and L-carnitine transporter expression changes (Ling et al., 2012). Such evidence suggests similar effects of inflammation on hepatic biliary transporters.

In patients with inflammatory processes or extrahepatic infections inflammation induced cholestasis is a common complication. Within hepatocytes signalling pathways are induced either directly or via proinflammatory cytokines which are activated by endotoxins (Kosters and Karpen, 2010). In response to the inflammatory signalling, the expression and the function of hepatocyte transporters is suppressed. Inflammation can further lead to repressed activity of nuclear transcriptional regulators that are essential for gene expression of various hepatocyte transporters (Kosters and Karpen, 2010).

In mammals, LPS activates TLR4 signal transducing receptors. Kupffer cells respond to the circulating LPS by producing increase levels of cytokines (Kosters and Karpen, 2010). These in turn activate the hepatocyte membrane transporters and this further leads to altered transporter function (Mulder et al., 2009; Trauner et al., 1999). Studies in rodents have shown that LPS and the various cytokines decrease the expression of Ntcp, the basolateral transporter and of Bsep, the canalicular transporter (Geier et al., 2005; Ghose et al., 2004; Green et al., 1996). In hepatocytes, transporter activities of sodium-taurocholate co-transporting polypeptide (NTCP) and organic anion-transporting polypeptides (OATP) have been decreased by TNF α and IL-6 (Vee et al., 2009).

2.7 Models to assess mechanisms leading to neonatal cholestasis:

Both in vitro and in vivo model systems have been employed to understand the risk factors and pathophysiological mechanisms involved in cholestasis. Various studies have used several animal models (rodents, rabbits, neonatal piglets) while trying elucidating molecular mechanisms involved in TPN-induced liver injury (Burrin et al., 2000; Burrin et al., 2003; Wykes et al., 1993; Loff et al., 1998). A widely used model, the TPN fed piglet, provides the most relevant system to understand the underlying mechanisms of human neonatal cholestasis, given the spontaneous nature of the development of cholestasis, unlike rodent models typically require genetic manipulation to express the cholestatic phenotype (Wang et al., 2006). Nonetheless, the administration of parenteral nutrition leads to cholestasis has been confirmed with the help of mouse models. These models are an efficient way of measuring transaminase levels, bile flow and also the expression of hepatic biliary transporters critical in maintaining the

bile acid homeostasis (Carter and Shulman, 2007). Mouse models are criticised for their short duration of TPN and failure to demonstrate reproducible cholestasis. Rabbit models tend to mimic the clinical presentation of hepatobilitary alterations noted in children and such models receive greater attention due to their ability for long-term TPN administrations (Loff et al., 1998).

The use of animal models for the study of hepatotoxicity is limited, though, by the animal welfare and ethical issues. Therefore, *in vitro* liver preparations are popularly being used as they offer a diverse array of approaches to investigate the toxicity (Gronberg et al., 2002). Three major *in vitro/ex vivo* liver models are available for the study of hepatotoxicity. These include: 1) liver cell culture model, 2) isolated and perfused organ models and 3) liver slices. Each model has its own advantages. Cell culture models offer greater efficiency to assess cellular metabolism and cytotoxicity. Whereas, isolated and perfused organs are best used to assess physiological as well morphological parameters. Efficiency of liver slices is based on tissue morphology and cellular assays.

2.8 Hypothesis:

The etiology of PNAC is due to multiple factors (Merritt, 1986). An important factor being studied is the aluminum contamination of PN solutions. Aluminum related adverse effects on the liver are well documented (Stein, 1978). Cholestatic changes are caused by the accumulation of the aluminum in the liver (Dermican, 1998; Klein, 1998). Despite intensive investigation into aluminum toxicity the mechanism of action still remains unclear and ill defined. As cholestasis involves reduced bile flow, alteration in hepatic biliary transporters as an underlying mechanism of aluminum induced intrahepatic cholestasis is possible. The purpose of the study would be to see if aluminum like many other xenobiotics alters the expression of hepatic biliary transporters and thus contribute to cholestasis. As well, inflammation is a component of cholestasis so the effects of aluminum may be exacerbated by the underlying inflammation. Therefore, the other purpose of the study would be to see if LPS has an additive effect on aluminum induced changes in mRNA expression of hepatic biliary transporters (BSEP, NTCP, MRP2, MATE1) and FXR.

1) Aluminum alters the mRNA expression of hepatic biliary transporters (BSEP, NTCP, MRP2, MATE1) and FXR.
2) LPS (inflammatory stimulus) has additive effect on aluminum induced changes in mRNA expression of hepatic biliary transporters (BSEP, NTCP, MRP2, MATE1) and FXR.

2.9 Objectives:

1) To conduct cytotoxicity assays to determine whether Aluminum (Al) and Al with LPS (an inflammatory stimulus) cause direct toxicity of HEPG2 cells and to identify the non-toxic Al and LPS concentration for further studies.
2) To evaluate the influence of Aluminum (Al) and Al with LPS on mRNA expression of hepatic biliary transporters (BSEP, NTCP, MRP2, MATE1) and FXR in HEPG2 cells using real-time RT-PCR analysis.

3. Materials & Methods

3.1 Materials

EMEM cell culture medium was purchased from American Type Culture Collection (ATCC) (Rockville, Maryland, USA). T-75 flasks, sterile 15 mL and 50 mL polypropylene centrifuge tubes, 6 well plates and 96 well plates, and eppendorf tubes were purchased from ThermoFisher Scientific (Ottawa, Ontario, Canada). Ribonucleic acid (RNA) isolation kit was purchased from Qiagen Inc (Toronto, Ontario, Canada). SyberGreen RT-PCR kits were acquired from Applied Biosystems (Foster City, California, USA).

3.2 Gene expression methods employed in assessing the effects of Aluminum and LPS exposure on transporter expression.

3.2.1 *Cell Culture*

HEPG2, a human liver carcinoma cell line that has phenotypic characteristics of hepatocytes *in vivo, was* purchased from ATCC at passage number 74. HEPG2 cells were cultured as specified by ATCC in Eagle's Minimum Essential Medium (EMEM) with 10% fetal bovine serum (FBS). The cells were incubated at 37˚C under an atmosphere of 95% air and 5% CO_2 in a humidified incubator. Cell culture media was changed every 2-3 days. Cells growing in T-75 flasks were passaged using 0.25-% trypsin, 0.03-% of EDTA solution. The passaged cells were resuspended in T-75 flasks containing cell culture medium. These cells were allowed to grow up to 70-80% confluence after which the cells were subcultured at a ratio of 1:4 using 0.25% of trypsin.

3.2.2 *Primer Design*

Gene sequences for each respective transporter were obtained from the National Center for Biotechnology Information Genebank (NCBI;http://www.ncbi.nih.gov) with primer sequences designed using Primer3 software (http://www.broad.mit.edu/cgi-bin/primer/primer3), a web-based primer design program. All primers were designed to be between 18-20 base pairs as appropriate for the Applied Biosystems real-time PCR platform. The primer sequences and the amplicon sizes are given in Table 3.1

Table 3.1. Primer details for mRNA expression analysis of transporters using QRT-PCR.

Gene	Accession number	Forward Primer	Reverse Primer	T_m (^{0}C)	Base pair size
β-Actin	NM_001101.3	ttgctatccaggctgtgc	atgtcacgcaagatttcc	86.1	235
FXR	BC144184	cagcagcctgaagagtgg	gctcatccccttttgatcc	58.0	185
BSEP	NM_003742	cgcttgtctacggtcagagc	atcctggtagctccctctgc	61.0	222
MATE1	NM_018242	tgtcactggtgtctcagtgg	gtaagcctggacacatctgg	58.2	216
MRP2	NM_000392	acgacctccgagagaagc	ccagcctctgtcacttcg	58.0	190
NTCP	NM_003049	cctcagcattgtgatgacc	ggtgcaaggaatgaacc	57.0	170

3.2.3 *RNA Isolation*

Total RNA was extracted using RNeasy Midi isolation kits as per the manufacturer's directions. Frozen cell pellets were thawed for 10-15 minutes at room temperature. Cell lysate was homogenized in 350 µL of lysis buffer RLT containing β-ME using a Polytron cell homogenizer. One volume of ethanol (70% EtOH) was added to the homogenized suspension to precipitate the nucleic acids. The sample was then applied to the Midi column and series of buffers were used for washing away the cellular contaminants (including genomic DNA) as per the manufacturer's instructions. The purified RNA sample was eluted from the column using RNase-free water (30 µL).

Total RNA concentration and purity was determined by measuring the absorbance of a diluted sample of RNA (RNA:RNase free water) at 260 nm with a Nanoview UV spectrophotometer (GE Healthcare Life Sciences, Quebec, Canada) according to equation 1:

$$[RNA] = \frac{40\mu g}{mL} * A260 * Dilution\ Factor \qquad \text{(Equation 1)}$$

RNA purity was assessed by measuring the absorbance ratio of a diluted sample of RNA (RNA:10 mM TrisCl (pH 7.5)) at 260 nm and 280 nm. Pure RNA has an absorbance ratio of 1.9 – 2.1. Total RNA was stored at -80°C until analysis.

3.2.4 *Two-step Quantitative Real-Time Reverse Transcription-Polymerase Chain Reaction (QRT-PCR)*

Reserve transcription combined with the polymerase chain reaction (RT-PCR) offers the best sensitivity, reproducibility and dynamic range of any standard technique and hence proved to be the most powerful method to quantify gene expression. The relative expressions of hepatic biliary transporters were determined using QRT-PCR on an Applied Biosystems Real-Time PCR system with the use of High-Capacity cDNA Reverse Transcription kits and Power SYBR Green PCR Master Mix.

Two-step QRT-PCR starts with the reverse transcription of total RNA to cDNA using a reverse transcriptase. High-Capacity cDNA Reverse Transcription kit uses the random primer scheme for initiating cDNA synthesis. The kit contents were allowed to thaw on ice and then the total volume of components needed to prepare the RT mix was calculated as per Table 3.2. Finally, RNA was added to reverse transcription reactions and the samples were loaded on the thermal cycler to perform reverse transcription.

Table 3.2. Preparation of 2X RT Master Mix (per 20 µL reaction):

Components	**Volume (µL) /Reaction Kit**
10X RT Buffer	2.0
25X dNTP Mix (100mM)	0.8
10X RT Random Primers	2.0
MultiScribe™ Reverse Transcriptase	1.0
RNase Inhibitor	1.0
Nuclease Free Water	3.2
Total per Reaction	10.0

Following the first-strand synthesis reaction, cDNA was taken into a separate tube for qPCR reaction and as per Table 3.3 the reactions were performed.

Table 3.3 QRT-PCR master mix reaction components.

Components	Volume(µL) /Reaction	Final Concentration
2X Quantitect SYBR Green	12.5	1X
Left Primer	2	1µM
Right Primer	2	1µM
Template cDNA	2	≤500 ng
RNase-free water	6.25	-
Total per reaction	25	-

Optimization and validation of the primers for its most favorable annealing temperature and highest primer efficiency was performed. A single specific melting peak for the optimal annealing temperature was produced, which was not seen in a negative control (sample with no RNA template). QRT-PCR products were further resolved by horizontal 2% (w/v) agarose gelelectrophoresis for a single band at the specified amplicon size correlating to the single, specific product melt peak, to assure a single PCR product as identified by a single band on the gel. From the slope of a 3-point standard curve the primer efficiency was calculated using serial dilutions of control RNA and efficiencies of 1.8 to 2.2 were considered as optimal. Using the comparative C_T or $2^{-\Delta\Delta CT}$ method, primers showing closer efficiency to β-actin's efficiency which is an internal standard, were used for QRT-PCR reaction. The reactions were quantified following determination of the threshold cycle (C_T; the amplification cycle when PCR products are first detected above baseline fluorescence) and fluorescence was measured from the intercalation of SYBR green dye into the double stranded product after the primer elongation phase. A nontemplate negative control was incorporated into all analysis runs.

PCR reactions consisted of an initial activation step (1 cycle at 95ºC for 15 min) followed by a three step thermal cycling (40 cycles; denaturing at 94ºC for 15s, annealing at 60ºC for 30s, and extension at 60º for 30s). Finally, a melt curve analysis from 65ºC to 95ºC at 0.5ºC/s was performed.

3.3 Experimental procedures performed in assessing the effects of aluminum and LPS exposure on transporter expression

3.3.1 Aluminum & LPS Cytotoxicity Study.(Pilot Study1)

The combined cytotoxicity of aluminum and LPS was assessed using the MTT cytotoxicity assay. The purpose of using MTT cytotoxicity assay was to determine a range of concentrations for combination of $AlCl_3$ with LPS over a time period of 12h and 24h that gave cell viabilities >85%. The cytotoxicity assessments were done in the presence of 50 µM CDCA (chenodeoxycholic acid) which is used to up regulate the expression of BSEP in HEPG2 cell line (Jinghua et al, 2002). Previous optimization experiments in the laboratory showed 1µg/mL of Lipopolysacchride (LPS) induces maximal cytokine (TNF-α) expression without the loss of cell viability. Therefore LPS at 1 µg/mL was combined with different concentrations of Aluminum (0, 10, 25, 50, 75 & 100 µg/mL). Aluminum and LPS stock solutions were prepared at concentration of 400 µg/mL and 2 µg/mL, respectively. Therefore, 53.66 mg of $AlCl_3.6H_2O$ was weighed out and dissolved in 15 mls of cell media to give a final concentration of 400 µg/mL elemental aluminum. As LPS is known to react with the glass, the glassware was first coated with dichloromethylsilane (silanized) for handling the LPS stock and working solutions.

HepG2 cells were plated in triplicate in 96-well plates at a cell density of 300,000 cells/mL. In each well of 96-well plates, 100 µL of cell suspension was pipetted and the plates incubated at 37°C, 5% CO_2 for 24 hours. The cell culture media was replaced with fresh media after 24 h and subsequently, the cells were exposed to different concentrations of Aluminum and LPS for 12h and 24 hr. Cells in media with CDCA was taken to be the negative control. At a given incubation time, 15 µL of the 5 mg/mL MTT solution was added per well and the plate was further incubated for an additional 3 hours. After that 150 µL of acidified isopropanol was added per well and the plate was shaked on an orbital shaker at 50 rpm at room temperature for 1 hour. Inhibition of cell growth is calculated according to Equation 2 (using the MS-Excel):

$$Relative\ viability\ (\%) = \frac{Absorbance(Treatment)}{Absorbance(Control)} * 100 \qquad \text{(Equation 2)}$$

Where OD refers to the Optical Density as determined using a plate reader at 570 nm.

3.3.2 Optimization of time exposure (Pilot Study 2).

Using the MTT cytotoxicity assay, range of concentrations for combination of $AlCl_3$ with LPS over a time period of 12h and 24h that gave cell viabilities >85% were determined in pilot study 1 and then these were used to optimize the time exposure. All the assessments were done in the presence of 50 μM CDCA (chenodeoxycholic acid) which is used to up regulate the expression of BSEP in HepG2 cell line (Jinghua et al, 2002). Therefore, LPS at 1 μg/mL was combined with concentrations of aluminum (0, 10 & 50 μg/mL). Aluminum and LPS stock solutions were prepared at concentration of 200 μg/mL and 2 μg/mL, respectively. The mRNA analysis was carried out with the standard gene β-actin and MRP2 to determine whether the concentrations of $AlCl_3$ were appropriate.

HEPG2 cells were plated in triplicate in 96-well plates at a cell density of 300,000 cells/mL. In each well of 96-well plates, 100 μL of cell suspension was pipetted and the plates incubated at 37°C, 5% CO_2 for 24 hours. The cell culture media was replaced with fresh media after 24 h and subsequently, the cells were exposed to different concentrations of aluminum and LPS for 12h and 24 hr. Cells in media with CDCA was taken to be the negative control.

This was then followed by RNA isolation using the RNeasy Midi isolation kits. This was then followed by 2 step quantitative real-time reverse transcription polymerase chain reaction (QRT-PCR).

3.3.3 Main Study

Using the MTT cytotoxicity assay, range of concentrations for combination of $AlCl_3$ with LPS over a time period of 12h and 24h that gave cell viabilities >85% were determined in pilot study 1 and then time exposure optimization was done using pilot 2. The pilot studies suggested the following conditions: LPS at 1 μg/mL combined with Aluminum (0, 10 & 50 μg/mL) over an exposure period of 24 h. Aluminum and LPS stock solutions were prepared at concentrations of 200 μg/mL and 2 μg/mL, respectively. All the assessments were done in the presence of 50 μM CDCA (chenodeoxycholic acid) which is used to up regulate the expression of BSEP in HepG2 cell line (Jinghua et al, 2002). The mRNA analysis was carried out with the standard gene β-actin and other genes including BSEP, MATE1, MRP2 and NTCP and FXR.

HepG2 cells were plated in triplicate in 96-well plates at cell density of 300,000 cells/mL. In each well of 96-well plates, 100 μL of cell suspension was pipetted and the plates incubated at 37°C, 5% CO_2 for 24 hours. The cell culture media was replaced with fresh media after 24 h and

subsequently, the cells were exposed to different concentrations of Aluminum and LPS for 24 hr. Cells in media with CDCA was taken to be the negative control.

This was then followed by RNA isolation using the RNeasy Midi isolation kits. This was then followed by 2 step quantitative real-time reverse transcription polymerase chain reaction (QRT-PCR) as explained in the above section.

4. Results

4.1 Pilot Study 1: To determine the cytotoxicity and concentrations of aluminum and LPS that gave cell viability greater than 85%.

To determine the concentrations of aluminum and LPS that gave cell viabilities greater than 85%, HepG2 cells were exposed to various concentrations of aluminum and a constant concentration of LPS (1 µg/mL). Using the MTT assay the combined cytotoxicity of Aluminum and LPS was assessed and the range of concentrations for the combination over a time period of 12h and 24h were calculated using the following equation:

$$Relative\ viablity\ (\%) = \frac{Absorbance(Treatment)}{Absorbance(Control)} * 100$$

Where OD refers to the Optical Density as determined using a plate reader at 570 nm.

Table 4.1 & table 4.2 show that over 12h as well as 24h time period, cell viabilities were >85% with Al at 50 µg/mL and lower concentrations when combined with LPS at 1 µg/mL. Al concentrations exceeding 50 µg/mL gave unacceptable toxicities.

Table 4.1 Cell viabilities for various concentrations of aluminum and LPS over a period of 12 hrs. (Each observation indicates the measure of optical density (OD) at 570 nm)

	LPS 1µg/mL					
	Al 100 µg/mL	Al 75 µg/mL	Al 50 µg/mL	Al 25 µg/mL	Al 10 µg/mL	Al 0 µg/mL
	0.385	0.419	0.594	0.539	0.633	0.59
	0.506	0.527	0.716	0.65	0.622	0.649
	0.429	0.452	0.602	0.508	0.573	0.656
Average	0.440	0.466	0.637	0.566	0.609	0.632
Toxicity	27	23	0	6	0	0
Viability	73	77	106	94	101	105

Table 4.2: Cell viabilities for various concentrations of aluminum and LPS over a period of 24 hrs. (Each observation indicates the measure of optical density (OD) at 570 nm)

	LPS 1µg/mL					
	Al 100 µg/mL	Al 75 µg/mL	Al 50 µg/mL	Al 25 µg/mL	Al 10 µg/mL	Al 0 µg/mL
	0.312	0.336	0.471	0.399		0.481
	0.293	0.367	0.6	0.52	0.435	0.403
	0.269	0.376	0.498	0.509	0.521	0.471
Average	0.291	0.360	0.523	0.476	0.478	0.452
Toxicity	43	30	0	7	7	12
Viability	57	70	102	93	93	88

4.2 Pilot Study 2: mRNA analysis with β-actin and MRP2 using various concentrations of aluminum combined with LPS to optimize the time exposure.

Using the MTT cytotoxicity assay, range of concentrations for combination of $AlCl_3$ with LPS over a time period of 12h and 24h that gave cell viabilities >85% were determined in pilot study 1 and then these were used to optimize the time exposure. HepG2 cells were exposed to different concentrations of Aluminum and LPS (LPS at 1 µg/mL was combined with concentrations of Aluminum (0, 10 & 50 µg/mL) for 12h and 24 hr.

The mRNA analysis was carried out with the standard gene β-actin and MRP2 to determine whether the concentrations of $AlCl_3$ were appropriate. The mRNA expression studies revealed that the MRP2 expression levels were found to be similar at 12 hr time point and 24 hr time point. Therefore, for the further study the 24 hr time period was chosen.

4.3 Aluminum and LPS at different combination of concentrations differentially alters the mRNA expression hepatic biliary transporters.

To determine whether aluminum alone or in combination with LPS alters the biliary transporters involved in production and maintenance of bile flow, HepG2 cells were exposed to aluminum and LPS for 24hrs and mRNA expression levels were evaluated. Figure 4.1 illustrates the relative mRNA expression levels of the transcription factor. The mRNA expression of FXR was slightly increased (not statistically significant) in a dose-dependent manner with treatment of aluminum alone. When combined with LPS, a slight downregulation of FXR expression was observed, while LPS alone caused no effect. In HepG2, NTCP mRNA expression showed differential changes with different treatments, but this change was not statistical significant (Figure 4.2). There was increase in expression level compared to control when aluminum alone

was used. But downregulation in expression was seen when combined with LPS. Al at 10 μg/mL with LPS at 1μg/mL gave more downregulation compared to the Al at 50 μg/mL with LPS at 1μg/mL. LPS at 1 μg/mL showed slight decrease in expression.

MRP2 expression in HepG2 cells was shown to be unaffected. No significant change was observed in the relative levels of MRP2 mRNA in HepG2 cells after treatment with either aluminum and/or LPS (Figure 4.3). At higher concentrations aluminum alone and with LPS caused an increase in MATE1 mRNA expression in HepG2 as illustrated in Figure 4.4. LPS also increased MATE1 expression in HEPG2 cells.

A concentration-dependent increase in BSEP mRNA expression was observed in HepG2 exposed to aluminum alone and in combination with LPS stimulation (Figure 4.5). However, BSEP mRNA was not significantly affected by LPS alone.

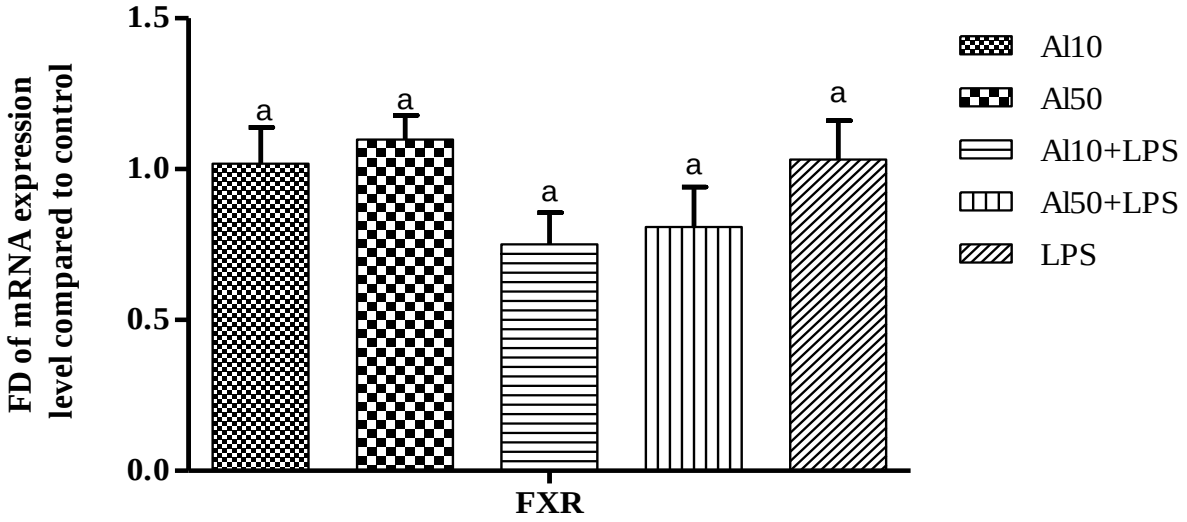

Figure 4.1: Mean ± SEM mRNA expression of Farnesoid X receptor (FXR). Expression was normalised to β-actin and fold difference (FD) determined by using $2^{-\Delta\Delta CT}$ method. FD in expression of various transporters in HepG2 cells incubated with Al 10 µg/mL, Al 50 µg/mL, LPS 1 µg/mL, Al 10 µg/mL with LPS 1 µg/mL and Al 50 µg/mL with LPS 1 µg/mL for 24 h. One-way ANOVA with Tukey's post hoc test was used for the comparisons between the different set of treatments and bars with the same letters indicate no significant difference, $P < 0.05$.

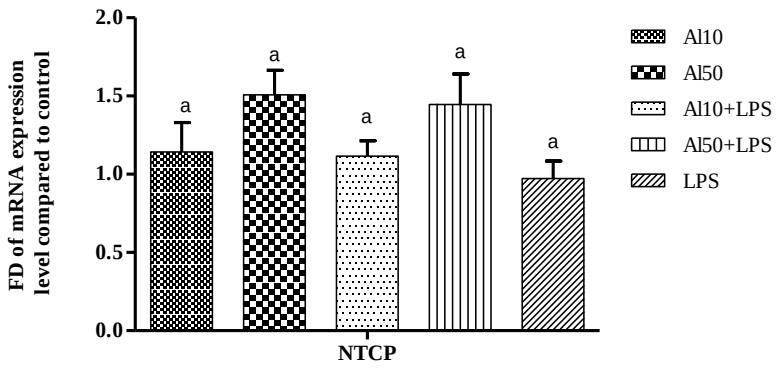

Figure 4.2: Mean ± SEM mRNA expression Na+ taurocholate co-transporting peptide (NTCP). Expression was normalised to β-actin and fold difference (FD) determined by using $2^{-\Delta\Delta CT}$ method. FD in expression of various transporters in HepG2 cells incubated with Al 10 µg/mL, Al 50 µg/mL, LPS 1 µg/mL, Al 10 µg/mL with LPS 1 µg/mL and Al 50 µg/mL with LPS 1 µg/mL for 24 h One-way ANOVA with Tukey's post hoc test was used for the comparisons between the different set of treatments and bars with the same letters indicate no significant difference, $P < 0.05$.

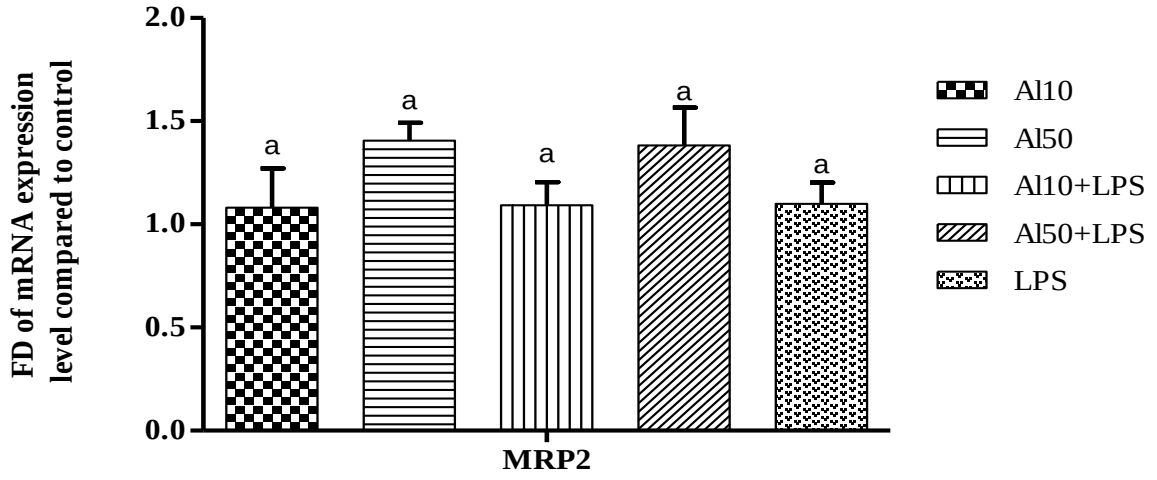

Figure 4.3: Mean ± SEM mRNA expression of Multi-drug resistant protein (MRP2). Expression was normalised to β-actin and fold difference (FD) determined by using $2^{-\Delta\Delta CT}$ method. FD in expression of various transporters in HepG2 cells incubated with Al 10 µg/mL, Al 50 µg/mL, LPS 1 µg/mL, One-way ANOVA with Tukey's post hoc test was used for the comparisons between the different set of treatments and bars with the same letters indicate no significant difference, $P < 0.05$.

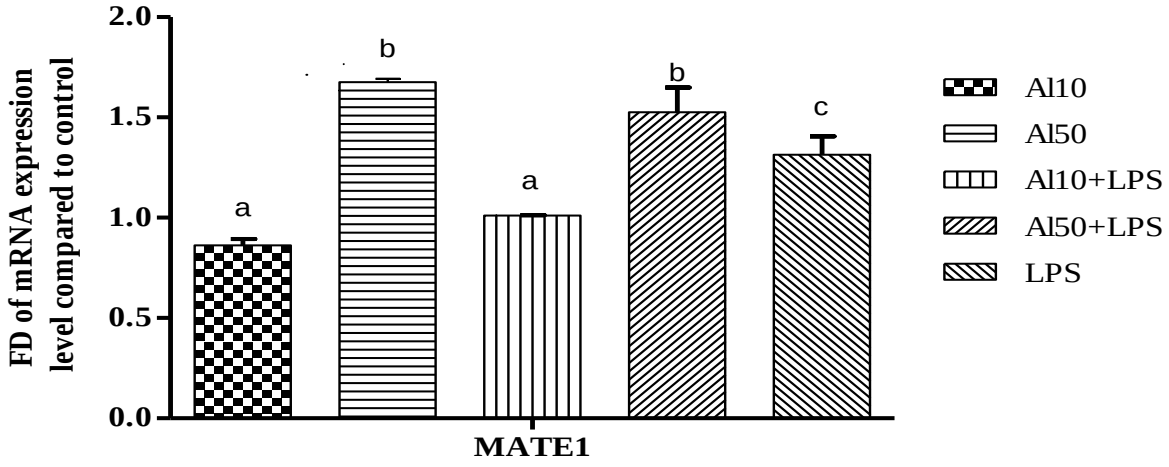

Figure 4.4: Mean ± SEM mRNA expression of Human multidrug and toxin extrusion 1(MATE1). Expression was normalised to β-actin and fold difference (FD) determined by using $2^{-\Delta\Delta CT}$ method. FD in expression of various transporters in HepG2 cells incubated with Al 10 µg/mL, Al 50 µg/mL, LPS µg/mL, Al 10 µg/mL with LPS 1 µg/mL and Al 50 µg/mL with LPS 1 µg/mL for 24 h. One-way ANOVA with Tukey's post hoc test was used for the comparisons between the different set of treatments and bars with the same letters indicate no significant difference, $P < 0.05$.

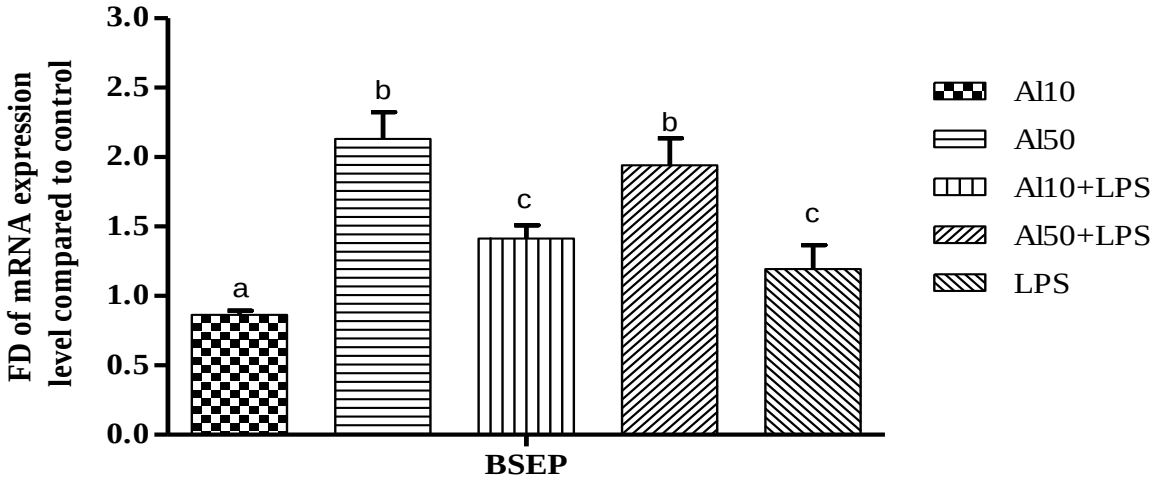

Figure 4.5: Mean ± SEM mRNA expression of Bile salt excretory pump (BSEP). Expression was normalised to β-actin and fold difference (FD) determined by using $2^{-\Delta\Delta CT}$ method. FD in expression of various transporters in HepG2 cells incubated with Al 10 µg/mL, Al 50 µg/mL, LPS 1 µg/mL, Al 10 µg/mL with LPS 1 µg/mL and Al 50 µg/mL with LPS 1 µg/mL for 24 h. One-way ANOVA with Tukey's post hoc test was used for the comparisons between the different set of treatments and bars with the same letters indicate no significant difference, $P < 0.05$.

5. Discussion

Aluminum contamination of parenteral nutrition solutions is thought to be an important risk factor contributing in parenteral nutrition induced intrahepatic cholestasis. Aluminum as a risk factor may influence a number of hepatocellular functions to lead to cholestasis but one important function is the potential for aluminum to cause dysfunction of those transporters responsible in the maintenance of bile flow. As well, inflammation is a component of cholestasis so the effects of aluminum may be exacerbated by underlying inflammation. In my study, the aim was to determine the effect of aluminum and an inflammatory stimulus, LPS, alone or in combination on the mRNA expression of hepatic biliary transporters (FXR, NTCP, MRP2, MATE1 and BSEP) as a contributing factor to parenteral nutrition induced intrahepatic cholestasis using the human liver carcinoma cell line, HepG2. My data suggest that for the canalicular hepatic transporters MATE1 and BSEP, aluminum at higher concentration alone as well as with LPS caused increased mRNA expression levels. In addition to this, BSEP mRNA expression was preserved and that of MATE1 was increased on LPS exposure. Given the particular importance of BSEP in the maintenance of bile flow and reported effects of drug-induced inhibition of BSEP to cause hepatic cholestasis, the influence of aluminum on BSEP (and MATE1) protein expression and activity warrant investigation. Inhibition of BSEP function (and possibly MATE1) by aluminum contamination of total parenteral nutrition formulations may explain, in part, the intrahepatic cholestasis associated with parenteral nutrition.

Hepatocyte transporters are of paramount importance in hepatic drug exposure and drug clearance. Their co-ordinated action is essential for bile formation and for the biliary secretion of various ions and xenobiotics (Pauli-Magnus and Meier, 2006). Following passage across the gastrointestinal wall, the first organ to be exposed to absorbed nutrients, drugs, metals and other xenobiotics is the liver. Efficient scavenging mechanisms extract these absorbed elements from the portal blood flow for hepatocellular metabolism and/or secretion into the bile (Diaz, 2000). Therefore, the liver is considered to be major detoxifying organ, and extraction from the blood is mediated by the parenchymal cells of the liver (hepatocytes) arranged with defined polarity that maintains a microvilli rich, specialized apical canalicular membrane and basolateral sinusoidal membrane domains, each studded with specific transporter proteins (Evans, 1980). NTCP and OATP's are the major sinusoidal transporters in the hepatocytes that execute the first process involved in hepatic elimination i.e. uptake of various endogenous and xenobiotics into the

hepatocyte from the circulating blood. Internalized compounds may undergo assimilation and then secreted into the bile canaliculus by the canalicular export pumps BSEP, MRP2 and MATE. Bile acids then via bile ducts are delivered to the intestinal lumen where they emulsify cholesterol and lipids facilitating their absorption.

An essential role of sinusoidal and canalicular transporters is evident from the pathology associated with the disruption of their function (Alrefai and Gill, 2007). The pathophysiology of cholestasis is implicated by the malfunctioning of hepatic transporters. For example, MRP2 expression is altered in various rat models of cholestasis (Trauner et al., 1997). Additionally, the expression of Ntcp and Oatp1 is downregulated in cholestasis and this suggests a co-ordinated alteration of transporter expression in cholestatic rats (Gartung et al., 1996; Dumont et al., 1997). Various exogenous and endogenous compounds can cause alterations in transporter expression and hence influence their functions. Metalloid salts like sodium arsenite can induce MRP2 expression in primary human and rat hepatocytes in contrast to cadmium chloride which was inactive (Vernhet et al., 2001).

In general, a literature review failed to provide a significant body of supportive evidence for the potential of aluminum to contribute to intrahepatic cholestasis, particularly through mechanisms implicating the inhibition of hepatocellular transporters involved in bile flow. Consequently, for initial evaluations of my thesis work focused primarily on providing *in vitro* support for the ability of aluminum to inhibit transporters in the hepatocyte. Since inflammation is a component of cholestasis, these evaluations included the use of an inflammatory stimulus. Positive outcomes would then support the use of appropriate *in vivo* models to investigate the hypothesis. In the literature, LPS from *E. coli* is extensively used for inflammatory challenges inducing cytokine release in mammalian cells. Also, the HepG2 cell line has been used in previous investigations as a cell culture model to examine the effects of various xenobiotics as well as cytokine exposure on epithelial cell function and gene expression. Therefore, optimization experiments were conducted using HepG2 cells: 1) to identify the non-toxic aluminum and LPS concentrations, and 2) to determine the optimum time exposure. Optimized concentrations of aluminum and LPS were employed to determine whether aluminum might lead to differential changes in mRNA expression of hepatic biliary transporters, NTCP, MRP2, MATE1 and BSEP, and the nuclear receptor, FXR, in HepG2.

I evaluated the mRNA expression of FXR, as this nuclear receptor is a major transcriptional regulator of the transporters involved in the maintenance of bile flow. Upon activation by bile acid binding FXR plays a significant role in the regulation of bile acid homeostasis. When bile acid levels are high FXR protects the liver from the harmful effects of bile acids by inhibiting synthesis and stimulating excretion of bile acids. The inverted repeat-1 motif is the preferred DNA binding sequence for FXR within its target promoters and FXR binds to it as a heterodimer with another nuclear receptor, RXR (Laffitte et al., 2000). BSEP expression is stimulated by FXR in response to bile acids via interaction of FXR-RXR heterodimer with IR-1 element of BSEP gene (Ananthanarayan et al., 2001; Schuetz et al., 2001; Plass et al., 2002). Therefore, high levels of bile acids stimulate their own hepatocanalicular clearance. Similarly, MRP2 expression is also activated by bile acids and FXR through an atypical ER-8 (everted repeat-8) (Kast et al., 2002). In cholestatic rodent models, NTCP expression is suppressed at mRNA as well as protein levels due to activation of FXR by increased intracellular bile acid levels (Fickert et al., 2001). In my study, the mRNA expression of FXR was slightly increased (not statistically significant) in a dose-dependent manner with treatment of aluminum alone. When combined with LPS, a slight downregulation of FXR expression was observed, while LPS alone caused no effect (Figure 4.1). These data suggest aluminum has little influence on the transcriptional expression of levels of FXR and is unlikely to mediate any effect through alterations in FXR expression.

In HepG2, NTCP mRNA expression showed differential changes with different treatments, but this change was not statistical significant. Uptake of bile salts and organic anions at the hepatocellular sinusoidal membrane is markedly impaired in cholestasis in the rat, in part due to downregulation of *Ntcp* expression (Geier et al., 2003). A similar decrease in the *Ntcp* expression has been reported in percutaneous liver biopsy samples of cholestatic liver disease patients (Hagenbuch and Dawson, 2004). Downregulation of NTCP is a protective adaptation as it lowers the hepatocellular load of potentially cytotoxic bile salts. NTCP, through the action of FXR, has been shown to be downregulated by bile acids. The mechanism explaining the observation involves the induction of the repressor heterodimer partner (SHP). This further interferes with the activity of the RAR/RXR heterodimer that controls *Ntcp* gene expression. The inflammatory cytokine interleukin, (IL)-1β, also downregulates the binding of RAR:RXR complex resulting in downregulation of Ntcp expression (Li et al., 2002). In ethinylestradiol-induced cholestasis, rat

liver *Ntcp* expression is associated with decreased binding of HNF1 (hepatocyte nuclear factor 1) and C/EBP (CCAAT/enhancer binding protein). Similarly, decreased binding of transactivators such as C/EBP, RAR/RXR heterodimer and HNF-1 results in decreased Ntcp transcription in endotoxin-induced cholestasis and toxic liver injury (Denson et al., 2001). The failure to observe changes in NTCP expression may suggest that aluminum does not affect important transactivators or repressors of transcription. However, LPS treatment of HepG2 caused a slight decrease in the mRNA expression of NTCP, which is consistent with the reported literature.

In the present study, MRP2 expression in HepG2 cells was shown to be unaffected. MRP2 is one of the most important canalicular transport proteins which mediate the transport of a variety of amphipathic compounds including organic anions, glutathione and glucuronic acid conjugates (Dietrich et al, 2001). No significant change was observed in the relative levels of MRP2 mRNA in HepG2 cells after treatment with either aluminum and/or LPS. LPS-induced inflammatory responses *in vivo* and *in vitro* have been extensively studied. Downregulation of Mrp2 following LPS administration has been reported in many *in vivo* studies in the rats (Vos et al., 1998). However, in human liver slices MRP2 at mRNA level is unaffected by the LPS treatment but downregulated at the protein level (Elferink et al., 2004). This suggested that the impairment of MRP2 expression in humans by LPS is regulated by posttranscriptional process (Elferink et al., 2004). In the rat, MRP2 downregulation is due to suppression of RXR/RAR by IL-1β, but in humans this regulatory pathway is apparently not present for MRP2 (Hartmann et al., 2002). The posttranscriptional mechanisms involved in the downregulation of MRP2 in humans have been further confirmed by the evaluation of liver biopsies of patients with inflammation-induced icteric cholestasis (Zollner et al., 2001).

I expected aluminum alone to downregulate MRP2 expression. A previous study in our laboratory demonstrated that infusion of parenteral nutrition solutions containing aluminum to neonatal piglets caused the downregulation of the canalicular transporter, MRP2 (Alemmari, unpublished data). The aluminum content of the parenteral nutrition infusion was proportional to the severity of canalicular microvilli damage (Alemmari et al., 2012). Bile acid transporter proteins like MRP2 are expressed in these microvilli rich domains so microvilli damage was suspected to cause the loss of bile acid transporters and reduced bile flow. Furthermore, in a rat model, an intraperitoneal administration of aluminum resulted in the downregulation of hepatic Mrp2 expression (Gonzalez et al., 2004). The failure of aluminum to alter the mRNA expression

of MRP2 in HepG2 cells may be due to the possibility that aluminum's effect on MRP2 expression in humans may involve posttranscriptional process as observed with LPS treatment. Additionally, HepG2 cells do not maintain a strong constitutive expression of MRP2, and any influence of aluminum on MRP2 expression may be obscured by these low constitutive MRP2 expression levels. Evaluations (at the mRNA and protein level) in a cell line that maintains MRP2 expression may identify an effect of aluminum on this transporter.

In my study, a concentration-dependent increase in BSEP mRNA expression was observed in HepG2 exposed to aluminum alone and in combination with LPS stimulation. However, BSEP mRNA was not significantly affected by LPS alone, which is consistent with observations in *in vitro* experiments reported in the literature (Elferink et al., 2004). BSEP mRNA is induced with elevation in bile acid levels as observed with dietary challenges (Wolters et al., 2002) or under cholestatic conditions (Zollner et al., 2003). This induction is due to the direct activation of the rodent and human BSEP genes by bile acid binding to and activation of the nuclear receptor, FXR (Ananthanarayanan et al., 2001). The BSEP induction is not a universal property for all the bile acids and is related to the FXR ligand specificity (Parks et al., 1999). As HepG2 cells lack constitutive BSEP expression, CDCA was used to up-regulate the expression of BSEP in these cells. In my study, the increase in BSEP mRNA levels by aluminum treatment is not likely to involve transcriptional upregulation by activation of FXR due to the presence of the strong binding ligand of FXR, CDCA, in the cell culture system.

BSEP does undergo considerable posttranscriptional regulation. Endotoxin-induced cholestasis in rodents is caused by the downregulation of Bsep (Vos et al., 1998), but more recent studies show that BSEP expression is relatively preserved in endotoxin and other cholestatic models of hepatic injury (Lee et al., 2000). In humans hepatic BSEP mRNA expression was unaltered but inflammation induced the downregulation of BSEP at the protein level in human liver (Elferink et al., 2004). Interestingly, Tazuke et al examined the expression of bile canalicular transporters with total parenteral nutrition administration and showed that MDR1 mRNA expression increased whereas mRNA expression of MRP2 and BSEP failed to show any significant change (Tazuke and Tietelbaum, 2008). In my study the aluminum mediated upregulation of BSEP mRNA may suggest a compensatory mechanism, possibly in response to posttranscriptional effects of aluminum, and a co-ordinated regulation of liver detoxifying transport proteins in response to aluminum (Figure 4.5).

Very limited information exists about MATE1 expression during inflammatory state as well as with xenobiotic exposure. At higher concentrations aluminum alone and with LPS caused an increase in MATE1 mRNA expression in HepG2. LPS also increased MATE1 expression in HEPG2 cells. These observations may suggest aluminum (and inflammation) may induce compensatory mechanisms in the hepatocyte to maintain cellular homeostasis. The exact mechanism underlying these observations is uncertain and warrants further investigation.

There have been studies on aluminum toxicity in HepG2 cells that have shown that iron homeostasis, membrane lipids, Ca^{+2} mediated processes and Mg^{+2} catalysed reactions appears to be the target of Al toxicity and contribute to various Al induced abnormalities (Zatta et al., 2002). Al disrupts oxidative phosphorylation, triggers oxidative stress and as a result an anaerobic respiratory regime is adopted by HepG2 cells to generate ATP (Mailloux and Appanna, 2007). Also, the non essential amino acid, L-carnitine is involved in the transport of fatty acids into the mitochondria (Muniyappa, 2010) and its homeostasis is key regulator in lipid metabolism (Vaz and Wanders, 2002). A decrease in L-carnitine levels in Al exposed HepG2 cells suggest Al to be a contributing factor to dyslipidemia. The underlying molecular mechanism involves the reduced activity and expression of enzymes involved in L-carnitine synthesis, namely butyrobetaine-aldehyde-dehydrogenase (BADH) and butyrobetaine deoxygenase (BBDOX) (Mailloux et al., 2006). Al toxicity also promotes mitochondrial dysfunction, i.e. the inability to perform the TCA cycle and thereby limiting L-carnitine requirements. Mitochondrial dysfunction further leads to lipid accumulation and slows down fatty acid metabolism. This can lead to various liver abnormalities and this could be another mechanism how aluminum may cause cholestasis.

Study Limitations

In the present study, a number of major limitations preclude any affirmative judgements with the observed outcomes. One significant limitation was the choice of the *in vitro* cell line used in the study. HepG2 cells lack constitutive BSEP expression and its expression requires induction by addition of CDCA. Furthermore, HepG2 cells tend to exhibit weak expression of influx transporters (Le Vee et al., 2006). Consequently, the effects of aluminum exposure on mRNA expression may be obscured by the already low transporter expression levels and the maximally induced BSEP expression in this cell line. Recently, primary hepatocytes have been demonstrated to be a useful and adequate tool for research related to activity and regulation of

hepatic drug transporters. They exhibit constituent activity of both sinusoidal as well as canalicular transporters (Jigorel et al., 2005; Payen et al., 2000) and also retain the regulatory pathways of transporter expression (Payen et al., 2000). However, their scarce and unpredictable availability limits their use. Other alternative immortalized hepatic cell lines have been used for toxicological studies (Knasmuller et al., 2004; Xue et al., 2004). In this context, HepaRG cell line has been shown as a promising substitute for the HepG2 cell line. Another consideration is the complexity of the in vitro system. Various cell types in the liver co-ordinate the defence against xenobiotics or inflammatory processes. Therefore, it becomes important that these studies be conducted in a system which has all different cell types present. An example of such a system is precision cut liver slices. This *in vitro* model has been validated in many laboratories working on inflammation-induced reactions in the liver (Olinga et al., 1998; Olinga et al., 2001). It has been reported that NTCP levels in human liver slices are maintained much better as compared to those in cultured hepatocytes (Kwekkeboom et al., 1989). Obviously, the system selected for *in vitro* evaluations can have a significant impact on study outcomes.

Another important limitation of the study was its exclusive focus on the evaluation of mRNA expression. As discussed above, previous studies with MRP2 and BSEP show that mRNA levels are preserved following exposure to inflammation or other cholestasis inducing agents, whereas the protein levels are altered indicating that posttranscriptional processes are involved in the regulation of these transporters (Elferink et al., 2004). Therefore, further investigation is needed at the protein level (and activity level) to determine whether aluminum may influence hepatocellular transporter expression and function. Determination of mRNA levels alone is not adequate enough to understand the effect of aluminum and LPS on transporter expression. Although mRNA levels are not directly proportional to the expression level of the proteins they code for, mRNA expression is commonly used as a proxy for estimating functional differences occurring at the protein level.

Also, the use of CDCA in my experiments could be another limiting factor. CDCA was used in the study to induce the expression of BSEP in HepG2 cells. However, we know that FXR is a key regulator of bile acid homeostasis and bile acids are the most effective activators of FXR (Kullal-Ublick et al., 2004). Bile salt transporter genes that are directly or indirectly regulated by FXR include BSEP, MRP2 and NTCP (Ananthanarayanan et al., 2001). Therefore, it is quite

possible that addition of CDCA to the cell media might have ameliorated the effect of aluminum on transporter expression, thereby, confounding experimental outcomes.

6. Conclusions and Future Work

The purpose for this study was to conduct simple initial studies into the possible influence of aluminum on the expression of hepatic biliary transporters as a contributing factor to intrahepatic cholestasis. The incidence of TPN-related hepatobiliary complications is common in both adults and infants on TPN and the etiology of PNAC is multifactorial. Previous work in *in vivo* models suggested that one of the potential contributing factors is the aluminum contamination of TPN solutions. The mechanism by which aluminum contributes to the PNAC development, though, was unknown. To provide some initial critical information regarding the role of aluminum as a potential risk factor contributing to cholestasis and the possible underlying mechanism, cytotoxicity studies were conducted to determine whether aluminum and aluminum with LPS (an inflammatory stimulus) cause direct toxicity of HepG2 cells and also to evaluate the influence of aluminum and LPS on the mRNA expression of hepatic biliary transporters (BSEP, MRP2, MATE1, NTCP) and nuclear transcription factor (FXR) in HEepG2 cells using real-time RT-PCR analysis. Since inflammation is a component of cholestasis, these investigations also involved the use of an inflammatory stimulus to determine whether the effects of aluminum were exacerbated by underlying inflammation. My study concludes that aluminum and LPS at different combination of concentrations differentially alters the mRNA expression of hepatic biliary transporters. For a couple of key transporters (BSEP and MATE1) the differences between the various treatment groups were statistically significant. Therefore, these transporters warrant further study.

These observations coupled with the known limitations of the study also suggest that further study of the influence of aluminum on hepatocellular expression as an underlying mechanism of cholestasis is warranted using a model system that better reflects the liver *in vivo*. The effect of aluminum and aluminum with LPS on hepatic biliary transporters commands further evaluations at the level of protein expression and transporter activity, as posttranscriptional processes may be involved. Studies at the protein levels may also help identify whether the observed changes in mRNA expression levels in the present study are reflected through changes in protein expression. Furthermore, with NTCP, MRP2 and FXR the difference between the different treatment groups did not reach statistical significance. This could be attributed to the weak expression of biliary transporters in the HepG2 cell line. Hence, it warrants that mRNA expression levels for these transporters need to be studied using a more

efficient cell line. In this context, it may be important to consider the newly characterized hepatoma HepaRG cell line as it has been shown to express specific liver functions including biliary transporters at relatively high levels in contrast to HepG2 cells. Also, the use of in vivo models to evaluate the mRNA expression is necessary. In our laboratory, Alemmari et al have shown that parenteral aluminum induces liver injury in a newborn piglet model and that the reduced aluminum contamination reduces parenteral nutrition associated liver injury (Alemmari et al., 2011; Alemmari et al., 2012). Therefore, the existing tissues from his study where microstructural changes in the canalicular membrane were observed needs further evaluation for the mRNA expression of the hepatic biliary transporters. Once the effect of aluminum on these transporters is established at mRNA as well as protein levels, in *in vivo* as well as *in vitro*, then we would have experimental support assisting us to understand the mechanism by which aluminum may be a potential risk factor contributing to cholestasis. Additional information of aluminum contamination of TPN solutions needs thorough examination so that TPN can be given safely and efficiently to the adults and infants.

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