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THE ROLE OF SPHINGOSINE KINASE 2 IN ALCOHOLIC LIVER DISEASE

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of  
Philosophy at Virginia Commonwealth University

by

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## List of Abbreviations

ABC	ATP-Binding Cassette
ACC-1	acetyl-CoA carboxylase-1
ADH	alcohol dehydrogenase
AKT	protein kinase B
ALD	alcoholic liver disease
ALDH	acetaldehyde dehydrogenase
ALP	alkaline phosphatase
ALT	alanine aminotransferase
ApoE	apolipoprotein E
AST	aspartate aminotransferase
ATF4	activating transcription factor 4
ATF6	activating transcription factor 6
AUD	alcohol use disorder
BSA	bovine serum albumin
C1P	ceramide 1-phosphate
CA	cholic acid
CBA	conjugated bile acid
CD	Crohn's disease
CDCA	chenodeoxycholic acid
CHOP	C/EBP homologous protein
<i>CYP27A1</i>	sterol 27-hydroxylase
<i>CYP7A1</i>	cholesterol 7 $\alpha$ -hydroxylase

<i>CYP7B1</i>	oxysterol 7 $\alpha$ -hydroxylase
<i>CYP8B1</i>	sterol 12 $\alpha$ -hydroxylase
DAMPs	danger-associated molecular patterns
DCA	deoxycholic acid
DSS	dextran sulfate sodium
EIF2	eukaryotic translation-initiation factor 2
ER	endoplasmic reticulum
ERAD	endoplasmic reticulum associated degradation
ERK	extracellular regulated protein kinases
FGF15/19	fibroblast growth factor 15/19
FGFR4	fibroblast growth factor receptor 4
FITC	fluorescein isothiocyanate
FTY720	fingolimod
FXR $\alpha$	farnesoid X receptor $\alpha$
G6pc	glucose 6-phosphatase
GPCR	G-protein coupled receptor
H&E	hematoxylin and eosin
HDAC	histone deacetylase
HDL	high density lipoprotein
<i>Hmgcs2</i>	3-hydroxy-3-methylglutaryl-CoA synthase 2
HNF-4 $\alpha$	hepatocyte nuclear factor 4 $\alpha$
HPH	hypoxia-mediated pulmonary hypertension
HSCs	hepatic stellate cells

IBAT	ileal sodium dependent bile acid transporter
IBD	inflammatory bowel disease
IHC	immunohistochemistry
IL-1 $\beta$	interleukin-1 $\beta$
IL-6	interleukin 6
IRE1 $\alpha$	inositol-requiring enzyme 1 $\alpha$
JNK1/2	c-Jun N-terminal kinase
LCA	lithocholic acid
LDL	low-density lipoprotein
LPS	lipopolysaccharide
LRH-1	liver-related homolog-1
MAPK	mitogen-activated protein kinase
MCP-1	monocyte chemoattractant protein-1
MMP	matrix metalloproteinase
NAD/H	nicotinamide adenine dinucleotide
NAFLD	non-alcoholic fatty liver disease
NASH	non-alcoholic steatohepatitis
NF $\kappa$ b	nuclear factor kappa-light-chain-enhancer of activated B cells
NIAAA	National Institute on Alcohol Abuse and Alcoholism
NKT	natural killer T cell
NPC	Niemann-Pick type C
Pck1	phosphoenolpyruvate carboxykinase 1
PERK	protein kinase R-like endoplasmic reticulum kinase

PKR	protein kinase R
<i>PNPLA3</i>	patatin-like phospholipase domain-containing protein 3
PPAR $\gamma$	peroxisome proliferator-activator receptor gamma
ROS	reactive oxygen species
RT-PCR	real-time polymerase chain reaction
S1P	sphingosine 1-phosphate
S1PR	sphingosine 1-phosphate receptor
SGPL1	sphingosine 1-phosphate lyase 1
SHP	small heterodimer partner
SLC10A1	sodium taurocholate cotransporting polypeptide
SphK2	sphingosine kinase 2
SphK2 <sup>-/-</sup>	sphingosine kinase 2 deficient
Spns2	spinster 2
SSP	S1P phosphatase
STAT3	signal transducer and activator of transcription 3
TLR4	Toll-like receptor 4
TNF- $\alpha$	tumor necrosis factor- $\alpha$
UC	ulcerative colitis
UPR	unfolded protein response
VLDL	very-low-density lipoprotein
WHO	world health organization
WT	wild type
XBP1	X-box binding protein

## Abstract

### THE ROLE OF SPHINGOSINE KINASE 2 IN ALCOHOLIC LIVER DISEASE

Eric K. Kwong, B.S.

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University

Virginia Commonwealth University, 2019

Major Director: Huiping Zhou

Professor, Department of Microbiology and Immunology

Alcoholic liver disease (ALD) is one of the most common liver diseases worldwide characterized by the accumulation of lipids within the liver, inflammation and the possibility of progressing to cirrhosis and liver failure. More importantly, there are currently no effective treatments for ALD and liver transplantation remains the only therapeutic option for end-stage liver disease. Previous studies have shown that ALD is a result of a combination of endoplasmic reticulum (ER) stress, lipid metabolism dysregulation and inflammation. It has been previously reported that alcohol disrupts gut microbiota homeostasis and causes increased endotoxins that contribute to the pathology of ALD. However, the detailed mechanism(s) underlying ALD and disease progression is poorly understood. We have discovered that sphingosine kinase 2 (SphK2) deficient (SphK2<sup>-/-</sup>) mice on an alcohol diet exhibit increased steatosis and inflammation compared to wild type mice. Sphingosine 1-phosphate receptor 2 (S1PR2) and SphK2 have been previously shown to play a key role in nutrient metabolism and signaling. However, their roles in alcohol-induced liver injury have not been characterized.

The overall objective of this study is to determine the molecular mechanism(s) by which disruption of S1PR2-mediated SphK2 signaling contributes to ALD. The effects of alcohol on mouse primary hepatocytes and cultured RAW264.7 macrophages were examined. The acute on chronic alcohol mouse model from NIAAA that recapitulates the drinking pattern of human ALD patients was used to study the effects of SphK2 deficiency in ALD. In addition, 60-day chronic alcohol mouse model was used to determine whether a more severe form of ALD was present in SphK2<sup>-/-</sup> mice. The results indicated that SphK2<sup>-/-</sup> mice on an alcohol diet exhibited an increased amount of hepatic steatosis compared to wild type mice. Genes regulating lipid metabolism were also dysregulated in SphK2<sup>-/-</sup> mice. SphK2<sup>-/-</sup> mice also had increased inflammation and liver injury as shown by an upregulation of inflammatory markers and increased levels of liver enzymes. Moreover, SphK2 protein expression levels were downregulated in the human livers of alcoholic cirrhotic and hepatocellular carcinoma (HCC) patients. These findings contribute to a greater understanding of the pathophysiology of ALD and could provide information on the development of novel therapeutics against ALD.

## Chapter 1: Background

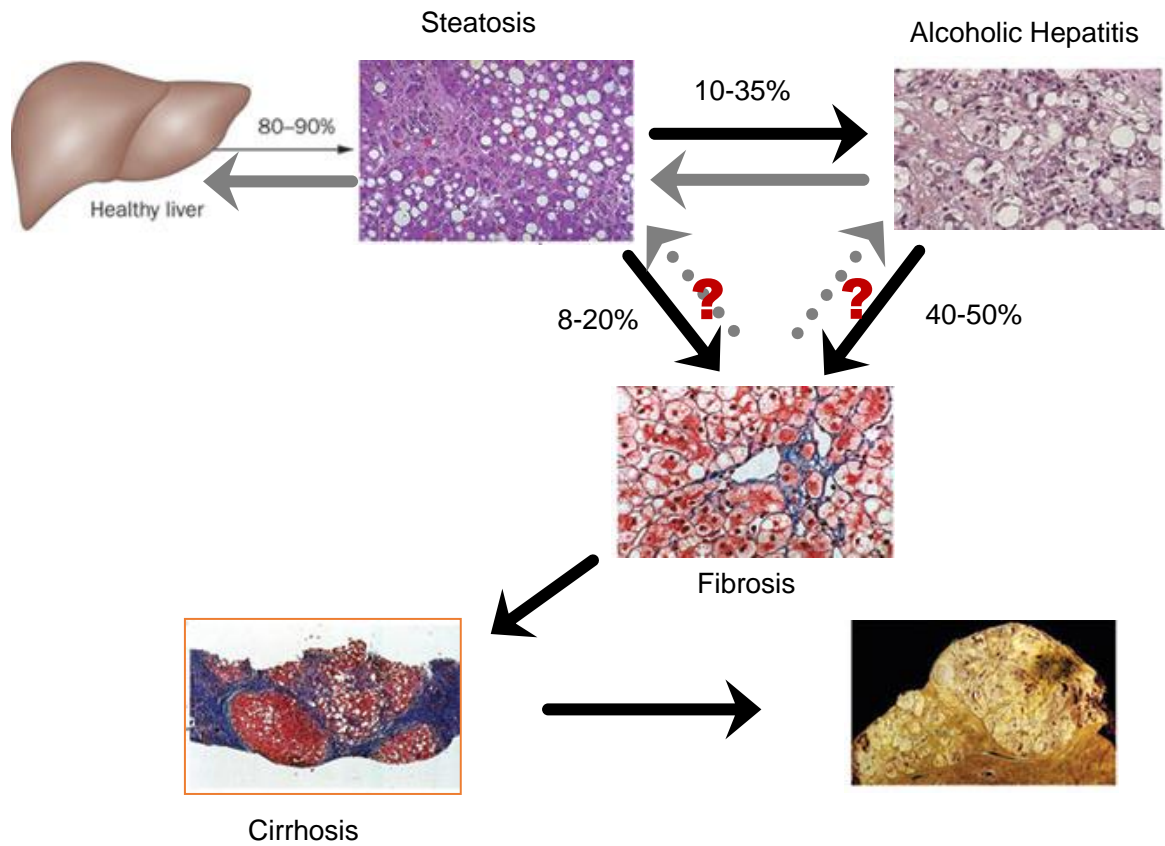
### I. Alcoholic Liver Disease

#### A. Epidemiology and Disease Spectrum

Alcoholic liver disease (ALD) is one of the most common chronic liver diseases worldwide along with non-alcoholic fatty liver disease (NAFLD) and hepatitis C [1, 2]. ALD exists as a clinical spectrum of disease ranging from steatosis, alcoholic hepatitis (AH), fibrosis, cirrhosis and hepatocellular carcinoma (Figure 1) [3, 4]. The World Health Organization (WHO) estimates that 3.3 million deaths are attributed to alcohol use and that alcohol use disorder (AUD) is one of the leading causes of preventable diseases [5]. Moreover, nearly 50% of liver cirrhosis is due to alcohol abuse. The National Institute of Alcoholism and Alcohol Abuse (NIAAA) defines one standard drink in the United States as 14g of alcohol which is equivalent to 12 ounces of beer (5%), 5 ounces of wine (10%) and 1.5 ounces of hard liquor (40%). AUD is defined as greater than 3 drinks per day in males and greater than 2 drinks per day in females or binge drinking (greater than 5 drinks for males and 4 drinks for females consumed over 2 hours) [6].

The disease burden and economic cost of AUD are substantial and ever increasing. In 2010, alcohol abuse cost the United States \$249 million and 75% of the cost is related to binge drinking. It is estimated that 88,000 annual deaths in the United States are alcohol-related and that it is the third leading preventable deaths second to tobacco and poor dietary habits [5]. Although alcohol abuse does not always translate to clinically significant ALD as only 10-20% of chronic alcohol users develop advanced stages of liver disease, cirrhosis remains as the 12th leading cause of deaths in the United States [5]. Treatment options remain limited and





**Figure 1. The spectrum of ALD.** ALD consists of the early stage steatosis followed by more advanced stages including AH and fibrosis. Advanced stages of ALD could progress to compromised liver function leading to cirrhosis and hepatocellular carcinoma.

prolonged abstinence remains the only effective therapy to attenuate alcohol-induced liver injury [7].

## **B. Environmental and Genetic Factors**

It has long been observed that the majority of chronic heavy alcohol users do not develop advanced stages of ALD such as AH, and cirrhosis [8]. Perhaps other factors such as genetics, environmental, behavior, and manner of drinking may play an important role in the development of ALD. 90% of these patients develop fatty liver, also known as hepatic steatosis [9]. However, steatosis is only the early stage of ALD and generally presents with no clinical symptoms. Interestingly, only 10-20% progress to advanced stages of ALD which suggests that there may be an underlying genetic component [10].

Although there is a clear positive relationship between the amount of alcohol consumed and the increased relative risk of developing ALD, there is extensive variability between individuals when taken into account for the amount of alcohol consumed. For example, the female gender is associated with an increased risk for developing ALD with lower amounts of alcohol, presumably due to an increased body fat percentage and lower activity levels of the enzyme alcohol dehydrogenase [11, 12]. It is also interesting to note that the behavioral pattern and method of alcohol consumption could factor into the development of ALD. It is believed that binge drinking generally increases the risk of developing AH and cirrhosis compared to partitioning the same amount of alcohol consumed over the course of the week [13-15]. Additionally, whether the consumption of alcohol with a meal decreases the risk of ALD compared to drinking alone is uncertain. The impact of drinking pattern on the development of ALD is left up for much debate as currently there are no studies that seem to suggest or prove this notion.

Obesity is a well-known important risk factor in the development of ALD. Studies have shown that patients who are obese for ten years and consume heavy amounts of alcohol are two-fold more likely to develop cirrhosis [16-18]. Coexisting liver disease also influences the likelihood of developing ALD. Those who have chronic liver diseases such as hepatitis B or C have accelerated rates of developing ALD leading to liver fibrosis, cirrhosis and hepatocellular carcinoma (HCC) [19, 20]. Cigarette smoking and hepatotoxic drugs have also shown to increase the risk of cirrhosis in alcoholic individuals [21, 22].

Genetic and molecular studies underscored the importance of genetic variability in ALD. Twin studies show that monozygotic twins have a higher concordance rate for developing cirrhosis than dizygotic twins [23]. Moreover, a specific subset of genes may influence behavior or propensity to consume alcohol [24]. Although controversial, it has been suggested that polymorphisms in alcohol dehydrogenase and acetaldehyde dehydrogenase enzymes may influence the susceptibility for AUD. A textbook example is an East Asian population for coding allelic variations on the alcohol dehydrogenase enzyme. This ethnic group contains a polymorphism that encodes a variant alcohol dehydrogenase enzyme that allows the conversion of alcohol to the toxic metabolite acetaldehyde at a much higher efficiency [25-27]. These individuals will exhibit signs of flushing along with nausea, headache, and vomiting when small quantities of alcohol are consumed giving rise to the colloquial term "Asian glow" [28-31]. In addition, about 50% of these individuals will code for another variant acetaldehyde dehydrogenase enzyme which worsens alcohol metabolism by inefficiently converting acetaldehyde to acetate [26]. These individuals have been hypothesized to be much less likely to develop alcoholism or AUD. Candidate genes have been linked to AUD and polymorphisms in these genes responsible for oxidative stress, inflammation or sensitivity to endotoxins have been implicated in the development of ALD [32]. Patatin-like phospholipase domain-containing

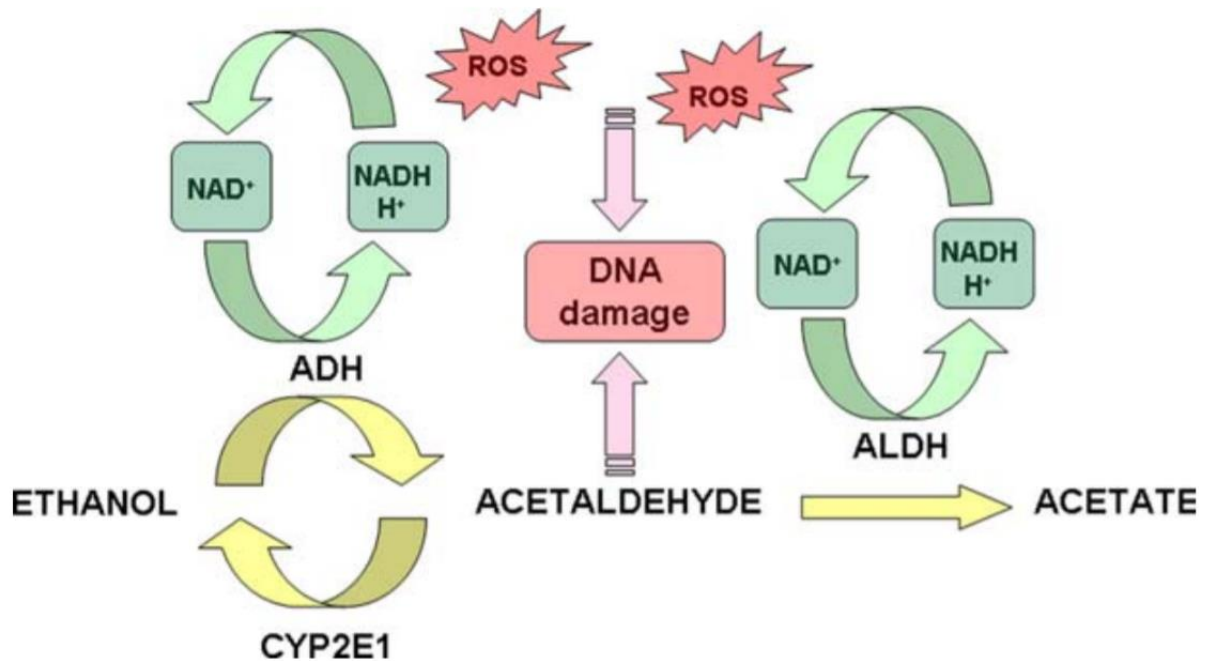
protein 3 (*PNPLA3*) gene has been shown to be associated with ALD severity in Caucasians [33, 34].

### **C. Pathophysiology of Alcohol-induced Liver Injury**

The molecular mechanisms of ALD are complex and not fully understood. Numerous studies have been performed to elucidate the underlying mechanisms and are believed to be caused by a combination of oxidative stress, endoplasmic reticulum (ER) stress, and inflammation [35-37]. Moreover, pathologic contributions from the gut (gut-liver axis) promote transient bacteremia and endotoxemia also sensitize hepatocytes to alcohol-induced injury [38, 39].

Oxidative stress is the result of a disturbance in homeostasis favoring the generation of free radicals that damage cellular components over the biological system's ability to neutralize these reactive oxygen species (ROS) [40]. The first step of alcohol metabolism occurs by the conversion of alcohol to acetaldehyde by alcohol dehydrogenase. Subsequent oxidation of acetaldehyde to acetate occurs by acetaldehyde dehydrogenase [41-43]. Each of these oxidation steps produces ROS and damage cellular components (Figure 2) [44]. Alcohol may also be metabolized by the cytochrome P450 system by the enzyme CYP2E1 further generating ROS [45-47].

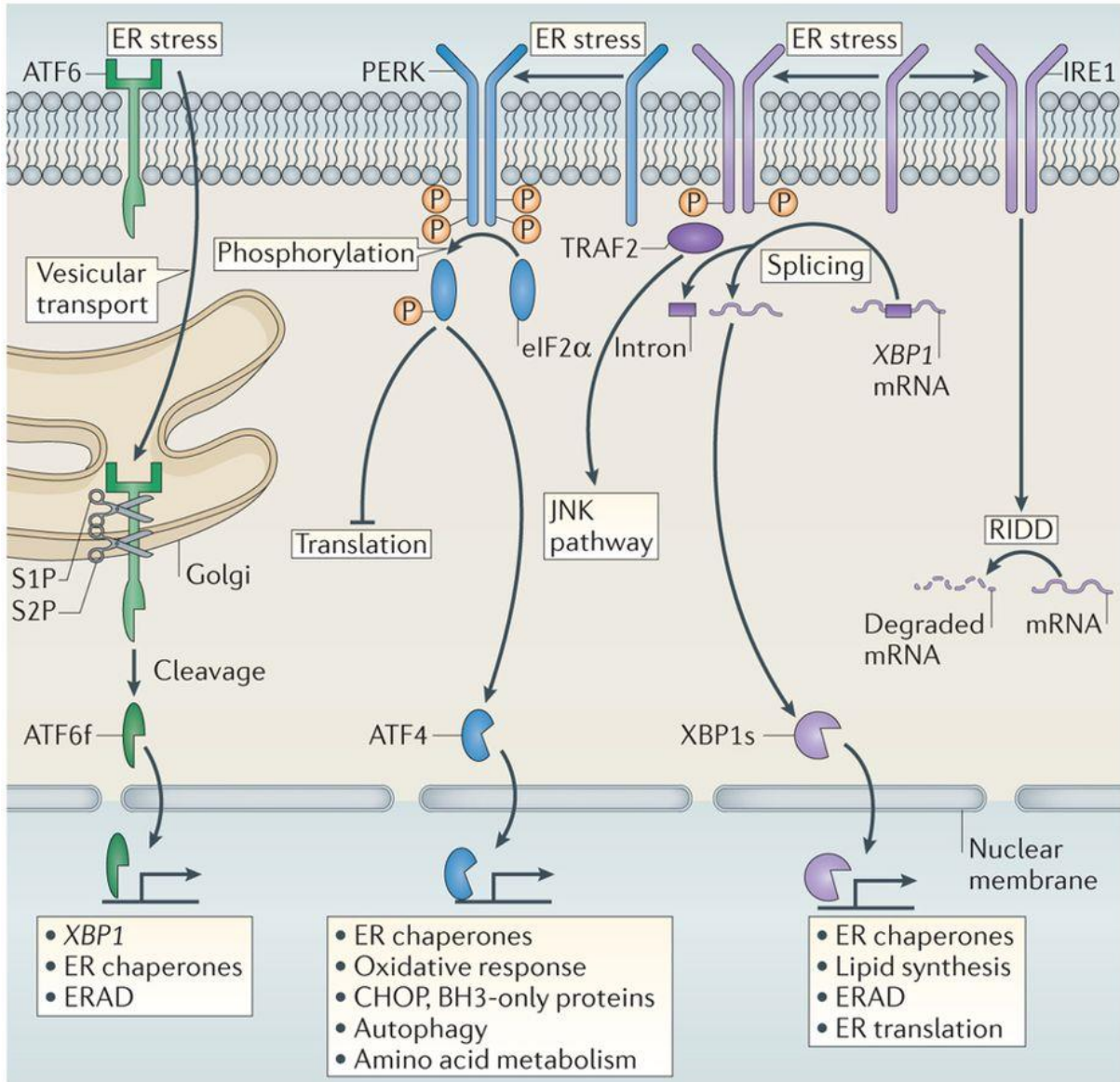
Alcohol may induce the misfolding of cellular proteins and cause ER stress [48]. Alcohol promotes the formation of protein adducts which disrupts the formation and structure of proteins [49]. This imposes stress upon the cellular system to alleviate the accumulation of misfolded proteins in the ER. Under ER stress, protein quality control is maintained through various signaling pathways collectively known as the unfolded protein response (UPR) [50]. One



**Figure 2. Alcohol metabolism and induction of oxidative stress.** Main molecular mechanisms which alcohol causes oxidative stress. ADH, alcohol dehydrogenase; ALDH, aldehyde dehydrogenase; NAD, nicotinamide adenine dinucleotide; NADH, the reduced form of NAD; ROS, reactive oxygen species. Adapted from [44].

mechanism is the phosphorylation and activation of the protein kinase R (PKR)-like endoplasmic reticulum kinase (PERK) and its subsequent enzymatic phosphorylation of the alpha subunit of the eukaryotic translation initiation factor 2 (EIF2) which halts protein synthesis [51]. The termination of protein translation prohibits the synthesis of nascent protein to relieve the burden of misfolded proteins in the ER. Under prolonged stress, the cell may deem the accumulation of misfolded proteins as a significant threat and may activate activating transcription factor 4 (ATF4) which upregulates C/EBP homologous protein (CHOP) to signal apoptosis [52]. Alternatively, activating transcription factor 6 (ATF6) is activated by ER stress and induces the synthesis of chaperone genes to aid in the folding of misfolded proteins. Inositol-requiring enzyme 1  $\alpha$  (IRE1 $\alpha$ ) can be activated by ER stress and contains two functional enzymatic domains. It has the capability to auto-phosphorylate and oligomerize. Upon activation, it carries out an endonuclease RNA splicing activity to cleave an intron from the X-box binding protein 1 (XBP1) mRNA, allowing it to code for a functional transcription factor [53-55]. XBP1 promotes the endoplasmic reticulum-associated degradation (ERAD) pathway where misfolded proteins are designated for ubiquitination and degradation by the proteasome. Figure 3 summarizes the UPR pathways when challenged with ER stress.

Recent studies have shown the importance of liver-resident macrophages (Kupffer cells) and infiltrating macrophages have in mediating ALD. After exposure to alcohol, Kupffer cells are sensitized to LPS and *via* Toll-like receptor 4 (TLR4), increase the production of inflammatory cytokines and chemokines [56-62]. Moreover, it has been shown that microRNA155 is positively correlated to Kupffer cell sensitivity to LPS. MicroRNA155 upregulation in Kupffer cells stabilizes TNF mRNA which in turn, heightens the inflammatory response [63]. Other microRNAs have been implicated in ALD, but their roles are less clear [64, 65]. Interestingly, the polarization of infiltrating macrophages (M1 vs. M2 subtypes) also play a role in alcohol-induced injury and ALD. Alcohol shifts the macrophage population towards M1 which increases the production of



**Figure 3. Summary of ER stress and the unfolded protein response.** ER stress can activate ATF6 through proteolytic cleavage by site 1 protease (S1P) and site 2 protease (S2P) to upregulate ER chaperones. Alternatively, PERK can phosphorylate eIF2 $\alpha$  to halt protein synthesis. ER stress can also phosphorylate IRE1 to cleave XBP-1 to its active form to upregulate lipid synthesis and ERAD. Adapted from [66].

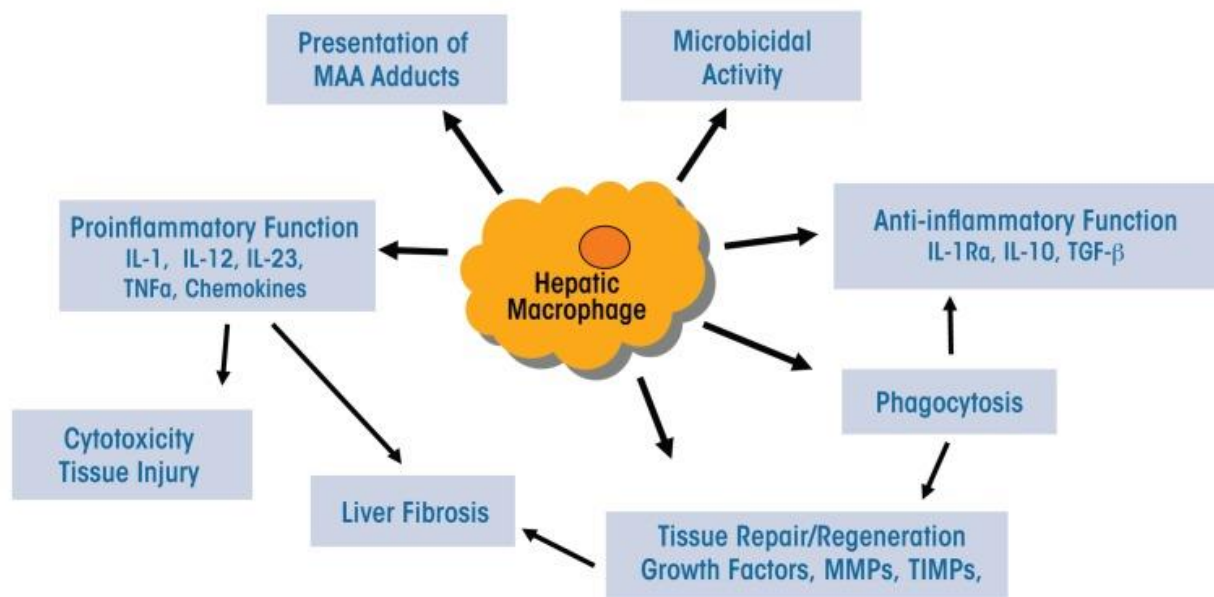
inflammatory cytokines and reactive oxygen species [67-69]. The role of hepatic macrophages have on ALD is illustrated in Figure 4 and Figure 5 [70]. Fibrosis in the liver from hepatocyte injury is associated with the immune response and macrophages. In addition to macrophages, it is thought that alcohol-induced fibrogenesis is mediated in part by activated hepatic stellate cells (HSCs). HSCs migrate, proliferate and deposit collagen at the site of injury [71, 72]. Although the mechanism of liver fibrogenesis is poorly understood, evidence suggests that IL-22 is a hepatoprotective cytokine produced by inflammatory cells that attenuate HSC activation and matrix production [73-75].

#### **D. Experimental Models for ALD**

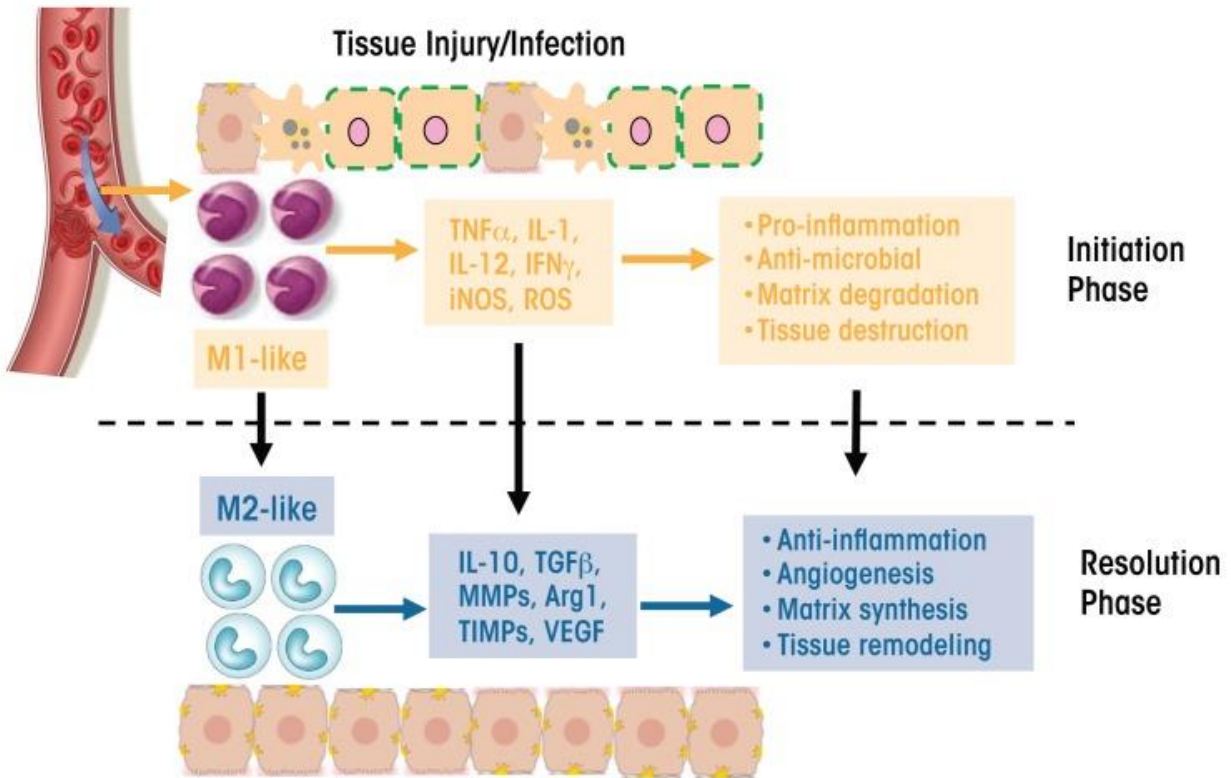
In the past several decades, researchers have attempted to generate viable experimental models for ALD. Many of these in vivo experimental mouse models are sufficient in producing early stages of ALD including hepatic steatosis and to some degree steatohepatitis [76]. However, there is no mouse model that can recapitulate the full human disease spectrum of ALD. Part of the challenge is the complexity by which ALD is developed and is multifactorial. As mentioned earlier, environmental and genetic factors play a significant role in the development of ALD. For this reason, researchers often select mouse strains of the C57BL background as they are genetically predisposed to drinking alcohol [77-79]. This eliminates experimental obstacles such as requiring an acclimation period for mice to drink alcohol.

Majority of ALD mouse models utilize an alcohol diet (Lieber-DeCarli) which contains all the caloric and water needs of mice (Figure 6). This offers the benefit of being able to adjust the alcohol content by volume while controlling for the number of calories taken by mice. It is common to feed mice 5% alcohol by volume for 4 to 16 weeks to produce varying degrees of





**Figure 4. Macrophage functions in ALD.** Macrophages fulfill a variety of functions in the context of ALD, including both proinflammatory and anti-inflammatory functions, depending on the state of the disease. These activities include the production of proinflammatory cytokines (e.g., interleukin [IL]-1, -12, and -23; tumor necrosis factor alpha [TNF- $\alpha$ ]) and chemokines, as well as of anti-inflammatory cytokines (e.g., IL-10, IL-1 receptor a [IL-1Ra], and transforming growth factor beta [TGF- $\beta$ ]). Other relevant activities include presentation of malondialdehyde-acetaldehyde (MAA) adducts and microbicidal and phagocytotic activity, as well as tissue repair and regeneration through the production of growth factors, matrix metalloproteinases (MMPs), and tissue inhibitors of metalloproteinases (TIMPs). Adapted from [70].



**Figure 5. Schematic representation of macrophage plasticity and its involvement in tissue injury.** Macrophages recruited to the site of an injury or infection during the initiation phase of the inflammatory reaction have an M1 phenotype. They produce proinflammatory and stress mediators and cytokines, such as tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), interleukin (IL)-1 and -12, interferon  $\gamma$  (IFN $\gamma$ ), an enzyme generating nitric oxide (iNOS), and reactive oxygen species (ROS). These macrophages have pro-inflammatory and antimicrobial effects and lead to matrix degradation and tissue destruction. During the resolution phase of the injury, these M1 macrophages are converted into an M2 phenotype with a different cytokine and chemokine repertoire, including IL-10, transforming growth factor  $\beta$  (TGF- $\beta$ ), matrix metalloproteinases (MMPs), arginase 1 (Arg1), tissue inhibitors of metalloproteinases (TIMPs), and vascular epithelial growth factor (VEGF). These M2 macrophages have anti-inflammatory effects and promote blood-vessel formation (angiogenesis), matrix synthesis, and tissue remodeling. Adapted from [70].



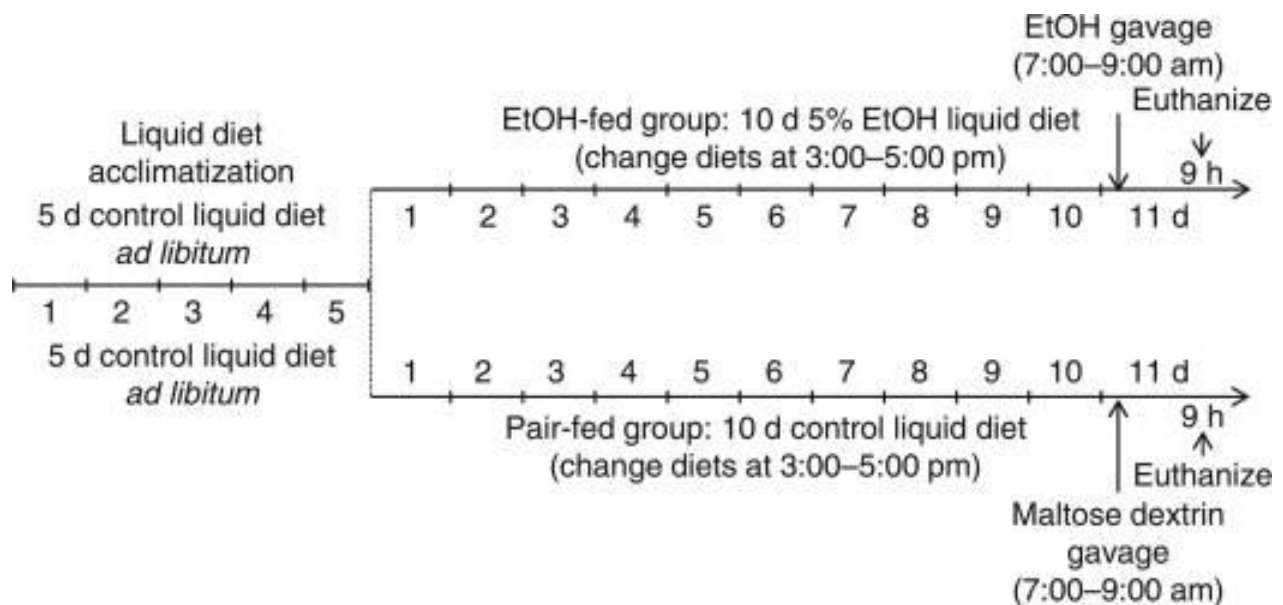
**Figure 6. Cage and feeding tube apparatus.** Photo shows a commercially available feeding tube containing Lieber-DeCarli alcohol diet that hangs over the lip of the cage.

steatosis and liver injury. Longer feeding windows produce a greater extent of steatosis but may increase mortality [80-85]. A popular mouse model developed by Bin Gao at the NIH/NIAAA called the chronic and binge alcohol feeding (the NIAAA model) utilizes the Lieber-DeCarli diet along with oral delivery of a bolus of alcohol (Figure 7). This model comprises feeding mice 5% Lieber-DeCarli alcohol diet for 10 days followed by oral gavage of 31.5% alcohol (5g/kg) on day 11 [86]. The advantage of the NIAAA model is a short feeding window while producing steatosis and liver injury. There is also a marked increase in blood alcohol content along with a rise in liver enzymes ALT and AST. This model is attractive for its relative ease to perform. However, no models that utilize the Lieber-DeCarli diet can produce liver fibrosis. To date, the only mouse model that can produce some degree of liver fibrosis is through intragastric fusion (Tsukamoto-French model). The significant drawback is that it is difficult to perform, requires extensive medical care and is associated with a high mortality rate [87-90].

## **II. Bile Acids in Lipid Metabolism**

### **A. Introduction**

Bile acids are synthesized in the liver from cholesterol and help promote the digestion and absorption of dietary fats and fat-soluble vitamins (A, D, E, and K) in the intestines. The synthesis of bile acids is one main mechanism of excreting excess cholesterol from the body [91]. Although crucial to proper nutrient absorption, bile acids may have toxic effects on the liver and other various organ systems. It is becoming increasingly evident that bile acids are important signaling molecules that exert various biological effects by activating different signaling pathways. However, the concept of bile acids acting as signaling molecules is recent. It was not until the past two decades that studies have reported that bile acids act as natural ligands for the farnesoid X receptor  $\alpha$  (FXR $\alpha$ ) [92-94]. Following this discovery, bile acids have



**Figure 7. Overview of the NIAAA model procedure.** Mice are initially fed the control Lieber-DeCarli diet *ad libitum* for 5 d to acclimatize them to a liquid diet and tube feeding. Afterward, alcohol (EtOH)-fed groups are allowed free access to the alcohol Lieber-DeCarli diet containing 5% (vol/vol) alcohol for 10 d, and control groups are pair-fed with the isocaloric control diet. At day 11, alcohol-fed and pair-fed mice are gavaged in the early morning with a single dose of alcohol (5 g kg<sup>-1</sup> body weight) or isocaloric maltose dextrin, respectively, and euthanized 9 h later. This model can be extended to longer periods of chronic feeding (up to 8 weeks) plus single or multiple binges. All of the animal experiments were approved by the NIAAA Animal Care and Use Committee. Adapted from [86].

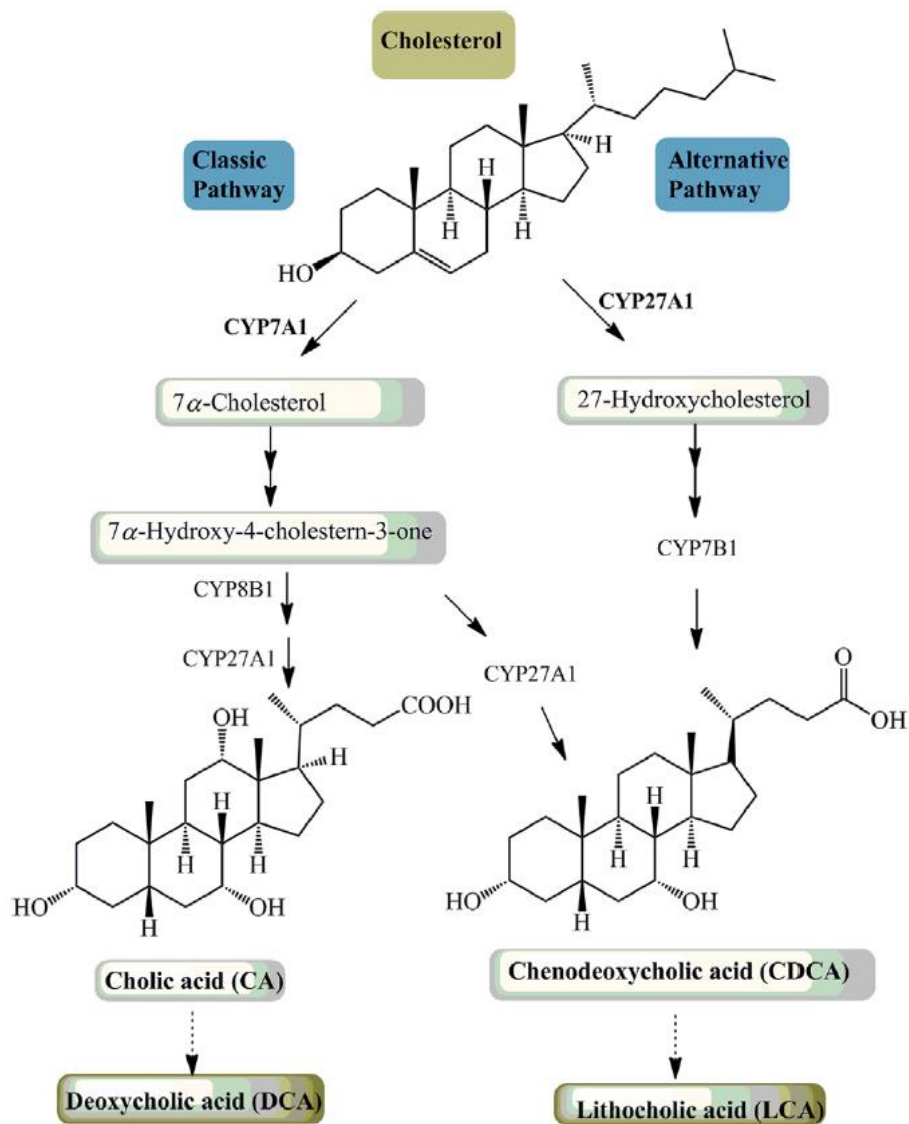
been shown to activate other nuclear receptors (pregnane X receptor, vitamin D receptor), G protein-coupled receptors (GPCRs) (G-protein-coupled bile acid receptor 5 (TGR5), muscarinic receptor 2 (M2), sphingosine-1-phosphate receptor 2 (S1PR2)) and cellular signaling pathways (c-Jun N-terminal kinase (JNK1/2), protein kinase B (AKT), and extracellular regulated protein kinases (ERK1/2)) [95, 96]. Bile acids have also been implicated in the inflammatory response and various liver diseases, as well as the promotion of cancers such as colorectal cancer and cholangiocarcinoma [97, 98]. The emerging role of bile acids as hormones and nutrient signaling molecules helped contribute to our understanding of glucose and lipid metabolism.

## **B. Enterohepatic Circulation and Bile Acid Synthesis**

Bile acids are synthesized from cholesterol in the hepatocytes and are actively transported into the bile duct system using ATP-binding cassette (ABC) transporter after conjugation with glycine or taurine. Hepatocytes secrete bile acids *via* bile salt export proteins (BSEP, ABCB11) along with phosphatidylcholine by ABCB4 and cholesterol by ABCG5/ABCG8 [99, 100]. As detergent molecules, bile acids keep cholesterol in solution within the gallbladder by forming micelles with cholesterol and phospholipids. The ratio of conjugated bile acids, cholesterol and phospholipids is highly regulated and excess cholesterol has been linked to an increased risk for cholesterol gallstone formation [101]. Bile is stored in the gallbladder and excreted into the duodenum in response to eating to activate pancreatic lipases and solubilize lipids to promote dietary fat absorption. Approximately 95% of bile acids are reabsorbed through the ileum by ileal sodium-dependent bile acid transporter (IBAT, SLC10A2) [102, 103]. Bile acids reabsorbed from the intestines travel through the portal blood and return to the liver *via* the sodium taurocholate cotransporting polypeptide (NTCP, SLC10A1) [104]. A small portion of primary bile acids is converted into secondary bile acids by anaerobic gut bacteria, which can be either passively absorbed from the large intestine or secreted in the feces. During

enterohepatic circulation, bile acids lost through fecal excretion must be replenished by de novo bile acid synthesis.

Bile acids are direct end-products of cholesterol catabolism. In humans, two primary bile acids, CA (3 $\alpha$ , 7 $\alpha$ , 12 $\alpha$ -trihydroxycholanoic acid or cholic acid) and CDCA (3 $\alpha$ , 7 $\alpha$ -dihydroxycholanoic acid or chenodeoxycholic acid), are formed in the liver through two synthetic pathways, the neutral pathway and the acidic pathway (Figure 8). The neutral pathway, also called the classic pathway, is the major pathway of generating bile acids for humans under physiological conditions and produces both CA and CDCA. The initiation of bile acid synthesis involves the enzyme cholesterol 7 $\alpha$ -hydroxylase (CYP7A1) to catalyze the 7 $\alpha$ -hydroxylation of cholesterol. In this rate-limiting step, *CYP7A1* gene expression is tightly regulated at the transcriptional level and by a negative feedback mechanism involving bile acids, glucagon, tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and fibroblast growth factor 15/19 (FGF15/19). In ileocytes, bile acids stimulate the production of FGF15/19 which can bind to the fibroblast growth factor receptor 4 (FGFR4)/ $\beta$ -Klotho complex on the cell membrane of hepatocytes and regulate bile acids and carbohydrate metabolism *via* activating several signaling cascades including JNK1/2 and ERK1/2 [105-107]. Activation of the JNK1/2 pathway has been shown to repress *Cyp7a1* gene expression in hepatocytes [108]. FGFR4 and  $\beta$ -Klotho null mice have been shown to contain increased *Cyp7a1* mRNA levels and bile acid levels. These results demonstrate the critical role FGF15/19, an FXR target gene, plays in the regulation of *Cyp7a1* and bile acid synthesis. In addition, FXR $\alpha$  can induce the expression of an atypical orphan nuclear receptor, small heterodimer partner (SHP). SHP has no DNA-binding domain and functions as a common transcriptional repressor of nuclear receptors. SHP can form a heterodimer with several transcription factors, including hepatocyte nuclear factor 4 $\alpha$  (*Hnf-4 $\alpha$* ) and liver-related homolog-1 (*Lrh-1*), to inhibit their transactivation activities, which results in inhibiting *Cyp7a1* and sterol 12 $\alpha$ -hydroxylase (*Cyp8b1*) transcription [109, 110].



**Figure 8. Bile acid synthesis and metabolism.** Two major pathways are involved in bile acid synthesis. The neutral (or classic) pathway is controlled by CYP7A1 in the ER. The acidic (or alternative) pathway is initiated by sterol CYP27A1 in mitochondria. CYP8B1 is required to synthesize CA. Oxysterol 7 $\alpha$ -hydroxylase (CYP7B1) is involved in the formation of CDCA in the acidic pathway. The neutral pathway is also able to form CDCA by CYP27A1. Primary bile acids are metabolized by gut bacteria to form the secondary bile acids, DCA and LCA. Adapted from [111]



The acidic pathway is initiated by sterol 27-hydroxylase (CYP27A1) in the mitochondrial inner membrane and has been shown to be more active in cirrhosis and various liver diseases [112, 113]. Since cholesterol concentration is very low in the inner mitochondrial membrane, the rate-limiting step in the acidic pathway may be the transport of cholesterol into the mitochondrion. The acidic pathway generates mostly CDCA. In addition to the liver, CYP27A1 is ubiquitously expressed in most tissues including the macrophages. CYP27A1 can catalyze cholesterol to form oxysterols by introducing a hydroxyl group to the carbon at either the 27 or 25 position in cholesterol [114-116]. The products, 27-hydroxycholesterol and 25-hydroxycholesterol, are known to be regulatory oxysterols that are important in maintaining cholesterol and fat levels in the liver [117]. The primary bile acids CA and CDCA are converted into deoxycholic acid (DCA) and lithocholic acid (LCA) respectively by a small population of intestinal anaerobic bacteria.

### **C. Bile Acids and Lipid Metabolism**

The role of bile acids regulating lipid metabolism in humans has been well established. Cholesterol gallstone patients treated with CDCA experience a decrease in hepatic very-low-density lipoprotein (VLDL) production and plasma triglyceride levels. Patients with hypercholesterolemia received bile acid binding resins, which increased serum levels of VLDL-triglycerides and high-density lipoprotein (HDL)-cholesterol while reducing low-density lipoprotein (LDL)-cholesterol levels [118-120]. It has been proposed that bile acids regulate hepatic lipid homeostasis through the coordinate regulation of *FXR*, *SHP*, *LXR*, and *SREBP1c* genes. In this model, FXR-induced activation of SHP interacts with LXR to suppress the transcriptional activity of SREBP-1c, a transcription factor known to induce genes involved in fatty acid, triglyceride and VLDL biosynthesis [121-123]. Moreover, FXR induces the expression of genes that regulate lipoprotein and triglyceride metabolism, including ApoA-V, ApoC-II,

ApoC-III, ApoE, PPAR $\alpha$  and syndecan-1 [124-126]. FXR<sup>-/-</sup> mice have been shown to accumulate hepatic lipids, cholesterol and triglycerides, whereas wild type mice exhibit decreased plasma cholesterol and triglycerides upon treatment with bile acids or FXR agonists [127]. The FXR inducible gene, *FGF19*, has been shown to repress lipogenesis and increase metabolism. FGF19 transgenic mice exhibited resistance to diet-induced obesity and insulin resistance [128-131]. Interestingly, overexpressing CYP7A1 in mice prevents fat-induced obesity and insulin resistance [132].

### **III. Role of Sphingosine Kinase 2 in Hepatic Lipid Metabolism and Liver Disease**

#### **A. Introduction**

Sphingosine forms the backbone of most sphingolipids and was initially recognized as a component of the plasma membrane lipid bilayer. The advent of large scale data analyses including genomics and proteomics paved way for the identification of many regulatory receptors and enzymes involved in sphingolipid metabolism [133]. Since then, sphingosine and its derivative sphingolipids have emerged as important signaling molecules in the regulation of different biological processes including migration, differentiation, cell survival and lipid metabolism. The phosphorylated derivative of sphingosine, sphingosine 1-phosphate (S1P), has attracted the attention of investigators for its potency as an activator of cell signaling and regulator of cell survival, growth, and immune cell trafficking which are important in inflammatory diseases and various cancers [134, 135]. In addition, studies with genetically modified mouse models have provided physiological insight into the functions of sphingolipids and have yielded many important advances in the understanding of the role of sphingolipid-mediated signaling pathways in various human diseases, including inflammation, cancer, pulmonary arterial hypertension, diabetes, NAFLD, and metabolic diseases [136-138].

S1P represents a key signaling sphingolipid molecule. It is exclusively formed by the phosphorylation of sphingosine *via* the action of sphingosine kinases (SphKs). Two major isoforms of SphK (SphK1 and SphK2) have been isolated and characterized. SphK1 and SphK2 have diverse and compensatory biological activities. S1P is not only an intracellular messenger, but also a natural ligand for five specific cell surface G-protein-coupled receptors (GPCRs). During the last two decades, numerous agonists and antagonists of S1P receptors (S1PRs) and chemical inhibitors of SphKs have been developed. One notable discovery is FTY720 (fingolimod), a modulator of the S1PRs except S1PR2, has been approved for the treatment of multiple sclerosis [139]. However, the role of SphKs and S1P in lipid metabolism remains largely unknown.

## **B. Sphingosine Lipid Metabolism**

Sphingolipids are lipids that contain the sphingoid backbone and can be N-acylated to form ceramide which occupies a central position in the biosynthetic pathway of sphingolipid metabolism [140]. The first step in *de novo* synthesis of sphingolipids is the condensation of serine and palmitoyl CoA by the enzyme serine palmitoyltransferase to form 3-ketosphinganine. This reaction takes place in the ER and represents the rate-limiting step of this biosynthetic pathway. Subsequent reactions involve the N-acylation of 3-ketosphinganine and reduction to sphinganine. Sphinganine is further fatty acylated by ceramide synthesis to generate dihydroceramide followed by desaturation by dihydroceramide desaturase to form ceramide, which is the precursor of the majority of complex sphingolipids [141]. Ceramide is a membrane-bound molecule with very low aqueous solubility. It requires transport from ER to the Golgi complex by ceramide transfer protein or vesicular transport, where it serves as a substrate for the production of sphingolipids such as glycosphospholipids, sphingomyelin and sphingosine. In

addition, ceramide can be converted to an important cell signaling molecule, ceramide 1-phosphate, by ceramide kinase. Furthermore, sphingosine can be converted to S1P by SphKs [142, 143].

### **C. Sphingosine Kinases**

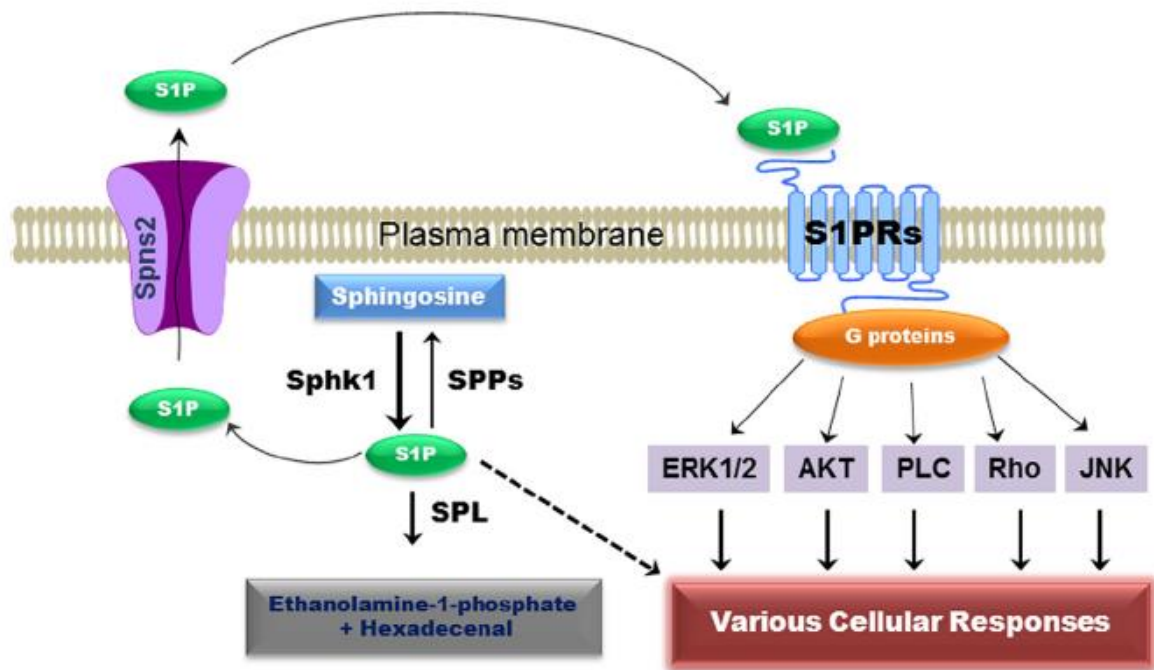
SphKs belong to the class of lipid kinases that contain five conserved domains and are evolutionarily conserved. Two mammalian isoforms have been identified, SphK1 and SphK2 [144]. Human SphKs are encoded by two distinct genes, SPHK1 and SPHK2, which are located on chromosome 17 and 19, respectively [145]. Human SphK1 and SphK2 share 80% similarity in amino acid sequence and 50% similarity in nucleotide sequence identity. SphK2 has 200 additional amino acids at the N-terminal region, containing a nuclear targeting sequence. The predicted molecular weights for SphK1 and SphK2 are 42 kDa and 68 kDa, respectively [146]. SphK1 is highly expressed in cells in the lung and spleen, whereas SphK2 is more abundant in liver, kidney, brain, and heart [147]. Moreover, the intracellular localization of SphK1 and SphK2 is closely linked to their physiological functions. SphK1 predominately resides in the cytoplasm under normal physiological conditions and upon various stimuli such as activation of mitogen-activated protein kinase (MAPK) by cytokines and growth factors, SphK1 is activated and translocated from the cytosol to the plasma membrane to carry out its catalytic conversion of sphingosine to S1P. Activation of SphK1 is associated with the promotion of cell proliferation, survival, migration, differentiation, angiogenesis, and inflammation [148-150]. In contrast, SphK2 is mainly localized in the nucleus [151]. Nuclear S1P produced by SphK2 has been shown to inhibit histone deacetylases (HDAC1/2) activity, leading to increased histone acetylation and increased gene transcriptional activity [152]. The subcellular localization of SphK2 and its function are less well-studied compared to those of SphK1. In addition, SphK2 is also found in

mitochondria, where it binds with high affinity and specificity to prohibitin 2, a highly conserved protein that regulates mitochondrial assembly and function [153]. Activation of SphK2 has also been shown to increase mitochondrial membrane permeability and promote cytochrome c release [154, 155].

#### **D. Sphingosine 1-Phosphate Signaling**

S1P is a simple sphingolipid but potent activator of cellular signaling pathways. S1P plays a role in various cellular processes including cell proliferation, differentiation, angiogenesis, inflammation, and cancer [156]. Like most signaling molecules, intracellular S1P level is tightly regulated by its synthesis and degradation. S1P is exclusively synthesized *via* phosphorylation of the 1-hydroxyl group on sphingosine either by SphK1 or SphK2 in response to diverse stimuli, including inflammatory cytokines, growth factors, and activation of GPCRs. S1P can be converted back to sphingosine by S1P specific phosphatase in the cytosol or degraded by S1P lyase to alcoholamine phosphate and hexadecanal [157]. Unlike sphingosine, which is sufficiently hydrophobic to diffuse across membranes, S1P is a more hydrophilic molecule, which requires specific transporters to be exported to the extracellular space. Several membrane-associated transporters have been identified as active S1P transporters, including ATP-binding cassette (ABC) transporters, ABCA1, and ABCC1, and spinster homologue 2 (SPNS2) [158-161]. The exported S1P can activate the S1P-specific GPCRs on the cell membrane to induce various physiological responses [162].

Identification of the S1P-specific GPCRs represents an important milestone in understanding S1P-mediated biological functions (Figure 9). Since the discovery of the first



**Figure 9. Sphingosine-1-phosphate-mediated signaling pathways.** Intracellular S1P is synthesized from sphingosine by SphK1. S1P can be converted back to sphingosine by S1P phosphatases (SPPs) or degraded to ethanolamine-1-phosphate and hexadecenal by SPL. Intracellular S1P can directly activate various cellular signaling pathways or be exported out of cells by specific transporters in the cell membrane. Extracellular S1P exerts its biological functions through activation of five G-protein coupled receptors, which are coupled to different G proteins and activate different cellular responses. Adapted from [111].

S1PR, S1PR1 (formerly named as EDG1) in 1998 [163], a total of five GPCRs have been identified as S1P-specific receptors (S1PR1-5) [152, 164-166]. S1PRs are differentially expressed in different tissues and the expression levels vary under different physiological and pathological conditions [167]. S1PR1 is ubiquitously expressed, and deletion of S1PR1 is embryonically lethal [168]. S1PR1 has been shown to play a key role in immune cell trafficking and angiogenesis [169, 170]. S1PR2 is important for the development of the auditory and vestibular system [171]. Unlike S1PR1, deletion of S1PR2 is not lethal. However, mice, deficient in S1PR2 have been shown to develop spontaneous seizures [172]. S1PR3 is highly expressed in the brain, heart, lung, spleen, kidney, liver, intestine and skeletal muscle [173]. Moreover, it plays a role in the vascular endothelial function and lung barrier integrity [174]. S1PR4 is expressed in leukocytes and regulates T cell cytokine production [175]. S1PR5 is highly expressed in oligodendrocytes. However, the function of S1PR5 remains unknown [176]. The various biological functions of S1P in different cells and tissues are largely due to the different expression patterns of S1PR subtypes and the various G proteins they couple with [177]. The crystal structure of S1PR1 made a significant advancement in the understanding of S1P-mediated signaling [178]. The S1P receptor subtype-specific agonists and antagonists have become novel therapeutic candidates for various diseases [179, 180].

### **E. SphKs and S1P in Lipid Metabolism**

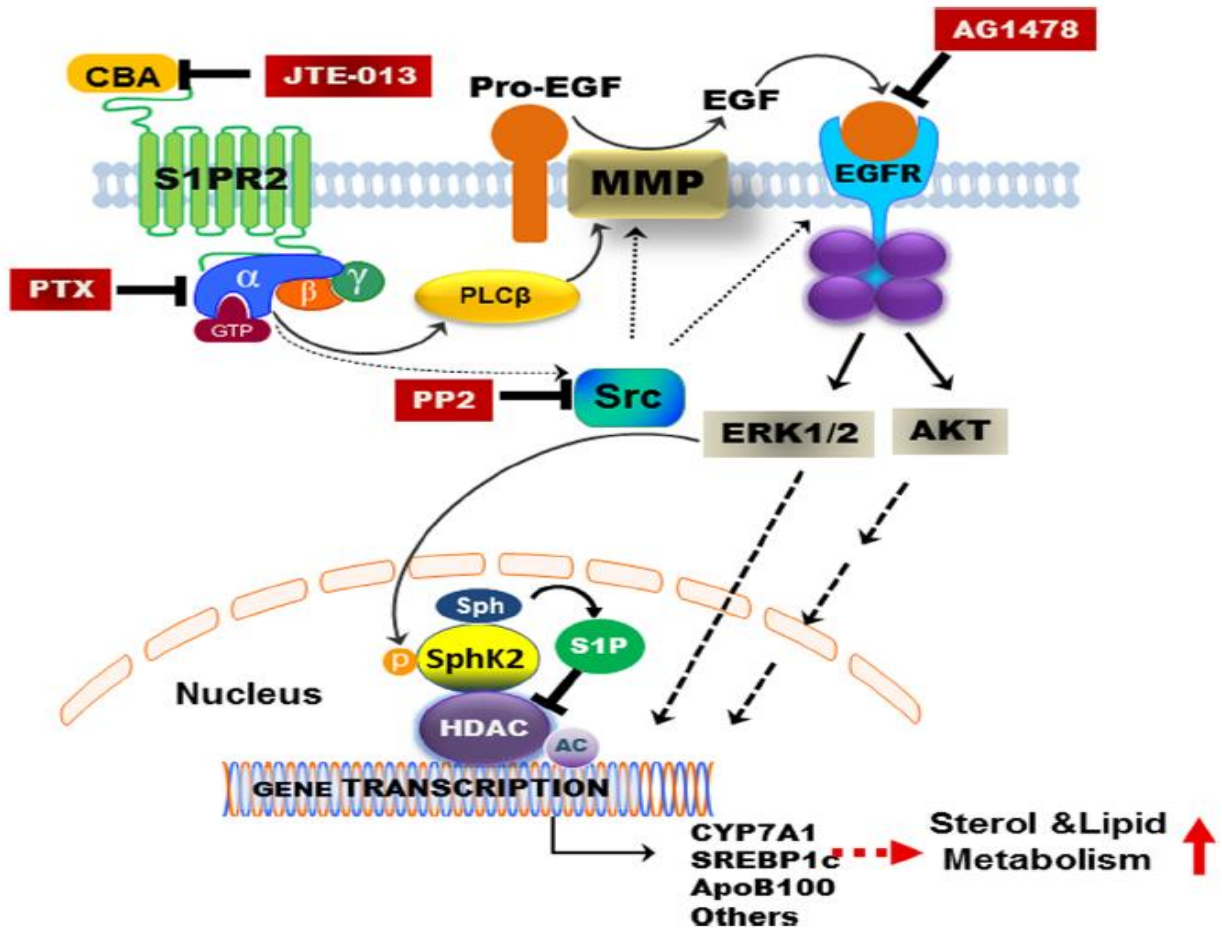
Dyslipidemia associated with metabolic diseases is complex and involves dysregulation of metabolic pathways of various lipid species [181]. In addition to phospholipid metabolites, sphingolipid metabolites are also involved in the regulation of metabolic lipid homeostasis [141]. The function of SphKs in regulating cell proliferation, differentiation and migration as well as the inflammatory response has been studied extensively, but only until recently have studies

demonstrated an eminent role for SphKs in regulating lipid metabolism [182-185]. S1P is present in the circulation and bile and mainly associated with HDL and albumin [156]. HDL-mediated release of S1P has been shown to have protective effects against atherosclerosis [186, 187]. S1P-mediated activation of the inflammatory response represents a key event in atherosclerotic disease progression. Both pharmacologic inhibition and genetic silencing of S1PR2 attenuated atherosclerotic lesion formation in apolipoprotein E knockout mice (ApoE<sup>-/-</sup>) [188]. Interestingly, several studies have shown that FTY720, a synthetic S1P analogue, reduces atherosclerosis in rodent models [189-193]. A recent study has shown that the SphK1/S1P axis plays a critical role in hypoxia-mediated pulmonary hypertension (HPH). Pharmacological inhibition of SphK1 and S1PR2 or genetic deletion of SphK1 prevented the development of HPH in rodent HPH models [194]. FTY720 is also found to reduce cholesterol and sphingolipid accumulation in Niemann-Pick type C (NPC) mutant fibroblasts by upregulating the expression of NPC1 and NPC2 and reducing cholesterol accumulation [195].

Obesity is closely associated with diabetes, NAFLD and non-alcoholic steatohepatitis (NASH), and cardiovascular diseases [196]. Numerous studies have reported that dysregulation of sphingolipid metabolism is linked to diabetes [136]. However, most studies in this area are focused on ceramide [197-199], and only a few studies have examined the involvement of SphKs/S1P signaling in obesity-related diseases. It has been reported that the expression of SphK1, but not SphK2, is elevated in mouse 3T3-L1 adipocytes during adipogenesis and in ob/ob mouse adipose tissue [200]. Recently, several studies reported that SphK/S1P-signaling plays an important role in hepatic lipid metabolism and insulin resistance [201]. However, there is a discrepancy regarding the role of SphK1 in the regulation of hepatic lipid metabolism in recent studies. NAFLD is characterized by the aberrant accumulation of lipids in hepatocytes. Hepatic lipid homeostasis is controlled by the balance of hepatic fatty acid synthesis, dietary fat intakes, adipocyte lipolysis, fatty acid oxidation, and secretion of hepatic lipids [202]. Kowalski,



et al. reported that high-fat-high-glucose diet feeding resulted in the accumulation of hepatic lipids and the reduction of total hepatic SphK activity, which was correlated to the down-regulation of SphK1, but not SphK2 [185]. However, hepatic overexpression of SphK1 only reduced hepatic triglycerides in low-fat diet-fed mice, but not in high fat diet-fed mice. SphK1/S1P-mediated signaling has been suggested to promote hepatic steatosis and inflammation. Expression of hepatic SphK1 is elevated in both high-fat-high-glucose-fed mice and human NASH patients [203]. SphK1<sup>-/-</sup> mice were protected from high-fat-high-glucose diet-induced hepatic inflammation and lipid accumulation [203]. Interestingly, another recent study done by Chen, et al. reported that deletion of SphK1 ameliorated hepatic steatosis in high-fat-diet-fed mice by the down-regulation of peroxisome proliferator-activated receptor gamma (PPAR $\gamma$ ) expression in the liver [182]. Furthermore, SphK1/S1P-mediated pro-steatotic effect is dependent on S1PR2/S1PR3, not S1PR1 [182]. To solve these discrepancies, more in-depth mechanistic approaches will be needed, including liver/hepatocyte-specific deletion and overexpression of SphK1. In contrast to SphK1, the physiological and pathological function of SphK2 is less well-characterized. The role of SphK2 in regulating immune cell function and the inflammatory response is controversial in different disease settings [204, 205]. Specific chemical inhibitors of SphK2 have been developed as potential tumor suppressors [206-210]. Our recent study demonstrated that SphK2 is a key regulator of hepatic lipid metabolism (Figure 10) [183]. Both S1PR2<sup>-/-</sup> and SphK2<sup>-/-</sup> mice fed on a high-fat-diet rapidly develop overt fatty livers compared to wild type mice. Interestingly, key lipid metabolism genes such as *Srebp-1c*, *Fas*, *Ldlr*, *Fxr*, and *Ppar $\gamma$*  are significantly downregulated in both S1PR2<sup>-/-</sup> and SphK2<sup>-/-</sup> mice [183]. Our recent RNA-seq data further indicated that overexpression of S1PR2 significantly upregulated the expression of SphK2 and key genes in hepatic lipid metabolism. Similarly, another study reported by Lee et al. indicated that activation of SphK2 by ER stress improves hepatic steatosis and insulin resistance [184]. High-fat diet-induced ER stress results in the upregulation of SphK2 through the activation of ATF4. Consistent with our findings, this study



**Figure 10. Conjugated bile acids regulate sterol and lipid metabolism *via* activating S1PR2.** In hepatocytes, CBA activates the S1PR2 on the cell membrane, which induces the activation of ERK1/2 through activation of G<sub>i</sub> protein. Activation of G<sub>i</sub> will further induce activation of SRC, which can activate the matrix metalloproteinase (MMP) and EGFR. EGFR-induced ERK1/2 activation can directly activate gene transcription in the nucleus or further induce the activation of nuclear SPHK2 and the increase of nuclear S1P levels. Nuclear S1P is a strong inhibitor of nuclear HDAC1/2. Inhibition of HDAC will increase the acetylation and transcription of a lot of genes involved in nutrient and lipid metabolism such as *CYP7A1*, *SREBP1c* and *APOB-100*. Adapted from [111].

demonstrated that SphK2 is an important regulator of hepatic fatty acid metabolism. In addition, SphK2-mediated activation of AKT signaling pathways protects mice against high-fat diet-induced glucose intolerance and insulin resistance [184].

#### **IV. Role of Sphingosine 1-Phosphate Signaling in the Gut**

##### **A. Role of S1P in Gastrointestinal Diseases**

Several studies have demonstrated a critical role for S1P in endothelial barrier function and inflammation [211, 212]. Activation of S1P-mediated signaling pathway has been linked to colitis, inflammatory bowel disease and colorectal cancer [213]. The chronic state of inflammation in the gut increases the relative risk of developing cancer, and there has been an impetus for finding suitable pharmacologic targets to attenuate the inflammatory response in the gut. However, S1P activation is not all deleterious and S1P has been shown to have a protective role in various tissues including the heart, brain, lung, and kidney [214-217]. S1P enhances endothelial function in the lung and attenuates acute lung injury in animal models [218]. In addition, S1P has been shown to protect the heart from ischemia-reperfusion injury [219]. Despite the wealth of literature on S1P's role in promoting endothelial barrier integrity, only recently have studies turned to elucidate the role of S1P in intestinal epithelial barrier function.

Inflammatory bowel disease (IBD) is a disease caused by a dysregulation of host immune function in the gastrointestinal tract and affects up to 0.5% of the population in western countries. IBD is subdivided into two disease entities, ulcerative colitis (UC) and Crohn's disease (CD) [220, 221]. UC is characterized by continuous inflammation of the mucosa with crypt abscesses while CD is more characteristic of skip lesions in the GI tract with cobblestoning

[222, 223]. Symptoms of IBD include diarrhea, bloody stool, and abdominal pain and medical management of IBD involves immunosuppression [224]. The early drugs used to treat IBD utilizes glucocorticoids, sulfasalazine/5-aminosalicylic acid and methotrexate to attenuate the inflammatory response [225-227]. However, these drugs act nonspecifically, and unwanted systemic side effects were apparent. With advances in immunotherapy, the next generation of drugs were specific monoclonal antibodies directed at TNF- $\alpha$  such as infliximab and adalimumab [228]. However, anti-TNF- $\alpha$  proved to be effective in only a subset of patients and the efficacy diminished with time. With the increased knowledge in the pathophysiology behind IBD and its cause is due to lymphocyte trafficking and immune cell dysfunction, the quest for drug targets that directly inhibit these pathways has received substantial attention.

In this regard, S1P and S1PR1 have the potential to be an effective drug target due to its role in lymphocyte egress and T cell differentiation [229]. Moreover, S1PR2, S1PR3, and S1PR5 have been suggested to play a role in macrophage and natural killer cell trafficking [180]. Clinical studies have shown that interleukin 6 (IL-6), TNF- $\alpha$ , nuclear factor kappa-light-chain-enhancer of activated B cells (NF $\kappa$ B), and signal transducer and activator of transcription 3 (STAT3) expression in IBD patients are elevated [230].

Accordingly, S1P has been shown to mediate TNF- $\alpha$  activation and subsequently the NF $\kappa$ B pathway. Genetic studies supported this notion when SphK1 deficient mice were partially protected against dextran sulfate sodium (DSS)-induced colitis [231]. Furthermore, data demonstrating the importance of S1P in IBD is a pediatric case study on IBD analyzing the gene expression levels of proteins involved in S1P metabolism. The critical findings of this study showed an upregulation of S1P synthetic genes (SphK1, SphK2), signaling (S1PR1, S1PR2, S1PR4) and degradation (SGPL1) in colon biopsies of IBD patients with moderate to severe

symptoms compared to control or patients in remission. Ceramide and ceramide 1-phosphate (C1P) levels were significantly elevated in IBD patients compared to control [232].

Chronic intestinal inflammation has been linked to colorectal cancer and reports demonstrated that S1P mediates pro-inflammatory cytokines such as TNF- $\alpha$  [233, 234]. Interestingly, colon biopsies from colorectal cancer patients show an elevation of SphK1 levels [235]. It is believed that the NF $\kappa$ B and STAT3 are activated which enhances the survival of intestinal epithelial cells. In a feedback loop, NF $\kappa$ B and STAT3 induce pro-inflammatory cytokines IL-6 and TNF- $\alpha$ , effectively reinforcing inflammation-induced tumorigenesis [236]. S1P and SphK1 have been implicated in colorectal cancer through its association with TNF- $\alpha$ . TNF- $\alpha$  promotes the translocation of SphK1 to the plasma membrane to produce S1P. Moreover, it has been suggested that SphK1 and intracellular SphK1 can stimulate the E3 ligase activity of TRAF2, contributing to the activation of NF $\kappa$ B pathway leading to inflammation and anti-apoptotic signals [237].

## **B. Molecular Mechanisms of S1P signaling in Gut/Liver Axis**

There are numerous studies demonstrating the causal relationship of diseases affecting the gut also impacts the liver. Since blood from the GI tract drains to the liver *via* the hepatic portal system, bacterial products, cytokines and various biological signal molecules in the gut could very well produce a disease state in the liver [238, 239]. With the ever-increasing body of knowledge on S1P in gut and liver pathology, we will highlight potential mechanisms of how S1P signaling could produce pathologies in both the gut and liver.

Recent evidence supports the notion that there is a strong interaction between the gut microbiota and the liver. Receiving about 70% of the blood from the intestines, the liver

encounters the majority of bacterial-derived products and antigens from the gut [240]. Concurrently, inflammation is a critical component of liver disease progression with the activation of intrahepatic macrophages, Kupffer cells and the release of pro-inflammatory cytokines [241]. The role of the Gut/Liver axis is critical in understanding the pathogenesis of ALD. Alcohol disrupts the intestinal barrier *via* damaging intestinal integrity, tight junctions and changing the gut microbiome. Bacterial endotoxins such as lipopolysaccharide (LPS) drains to the portal circulation and sensitizes liver macrophages to release cytokines, chemokines and reactive oxygen species [242, 243]. Interestingly, S1P has been shown to promote intestinal epithelial cell proliferation through the activation of S1PR2 [244]. Evidence also suggests the activation of Akt signaling pathway *via* S1P protects intestinal stem cells from apoptosis [245].

Bile acids have been shown to mediate S1P signaling, and novel experiments have demonstrated a unique role for primary and secondary bile acids in regulating liver disease *via* gut microbiota. Mouse models for liver cancer metastasis show that secondary bile acids produced by commensal gut bacteria from primary bile acids promote metastatic liver cancer *via* suppression of natural killer T (NKT) cells. Treating mice with an antibiotic cocktail to deplete the commensal gut microbiota upregulated NKT cells and promoted a liver-specific antitumor effect [246]. As secondary bile acids are generated from gut bacteria, these results demonstrate an important role for the gut microbiota in regulating gut and liver disease. Moreover, it would be interesting for future studies to determine the underlying mechanisms and receptors that mediate the effects of bile acids in other gut and liver-related disorders.

## **V. Research Objectives**

Our previous studies identified sphingosine 1-phosphate receptor 2 (S1PR2) and sphingosine kinase 2 (SphK2) knockout (S1PR2<sup>-/-</sup> and SphK2<sup>-/-</sup>) mice develop overt fatty liver

with two-week high-fat diet feeding, which is associated with significant down-regulation of hepatic lipid metabolism genes [183]. In addition, it has been reported that the activation of SphK2 in response to ER stress ameliorates hepatic steatosis [184]. The development of ALD appears to be due to a combination of lipid metabolism dysregulation, ER stress, and inflammation. ER stress, induced by alcohol and its metabolites, promote the formation of protein adducts and accumulation of unfolded or misfolded proteins, which further activate the unfolded protein response (UPR) [48, 50]. The initial activation of the UPR increases the degradation of misfolded proteins *via* the proteasome to restore ER homeostasis. Moreover, inflammation has been suggested to play a critical role in the progression of ALD from steatohepatitis to cirrhosis and cancer [247]. The recruitment of immune cells to the liver and pro-inflammatory cytokines have been proposed to contribute to hepatocyte injury and fibrosis [248]. Our preliminary data suggest that disruption of SphK2 sensitizes hepatocytes to alcohol-induced lipid accumulation and injury, indicating that SphK2 plays a crucial role in the regulation of hepatic lipid metabolism and inflammatory response in ALD. The **overall goal** of this proposed project is to define the role of SphK2 in alcohol-induced liver injury and further identify the mechanisms by which disruption of the S1PR2/SphK2-mediated signaling pathways lead to the progression of ALD. Accomplishing the proposed aims will provide insight into the molecular mechanisms of ALD and could identify potential therapeutic targets for the treatment of ALD.

## Chapter 2: Materials and Methods

### I. Reagents

Oil Red O, LPS and common laboratory chemicals were purchased from Sigma Aldrich (St. Louis, MO). Lieber-Decarli Diet was purchased from Bioserv (Flemington, NJ). SphK2 antibody was purchased from proteintech (Chicago, IL). BD Matrigel Basement Membrane Matrix was purchased from BD Biosciences (Bedford, MA). Dulbecco's PBS (DPBS), 100 x Penicillin/ Streptomycin, 100 x GlutaMAX, 1M HEPES, 50x B-27 Supplement and 100x N-2 Supplement were obtained from Life Technologies, Gibco (Waltham, MA). Heat Inactivated Fetal Bovine Serum (FBS) was from Atlanta Biologicals (Flowery Branch, GA). Recombinant murine epithelial growth factor (EGF), recombinant murine Noggin and recombinant human R-Spondin-1 were purchased from Pepro Tech (Rocky Hill, NJ).

### II. Animal Studies

C57BL/6J wild-type (WT) mice and SphK2 knockout mice (both male and female, 8-10 weeks old) were purchased from The Jackson Laboratory (Bar Harbor, ME) and housed under a 12-hour light/12-hour dark cycle with free access to water and normal chow. The acute on chronic model of alcohol feeding (NIAAA model) has been previously described [86]. The 60-day chronic alcohol feeding was performed by feeding mice *ad lib* Lieber-DeCarli control diet or 5% alcohol by volume Lieber-DeCarli diet following the manufacturer's protocol for 60 days. At the end of the experiment, mice were sacrificed for subsequent biochemical analysis. All animal study protocols were approved by the Institutional Animal Care and Use Committee of Virginia Commonwealth University (Richmond, VA).



### **III. Histological and immunohistochemical staining**

Tissues (liver, small intestine) were processed for Hematoxylin and eosin (H&E) and Masson's Trichrome staining at the clinical pathology laboratory at the Medical College of Virginia Hospital (Richmond, VA). Frozen tissue sections cut to 10µm in thickness and preserved in 3.7% formaldehyde for 20 minutes were used for Oil Red O (ORO) staining and fluorescent immunohistochemistry (IHC). Oil Red O staining was performed on frozen tissue sections with an initial 2x H<sub>2</sub>O wash followed by a wash with 60% isopropanol for 30 sec. Slides are stained with a working solution of 0.3% ORO dissolved in 60% isopropanol for 15 min. Slides are followed by 2x H<sub>2</sub>O wash, 60% isopropanol wash for 30 sec and stained with hematoxylin for 1 min. Fluorescent IHC was performed by blocking frozen liver sections in 2% bovine serum albumin (BSA) with 0.1% Triton-X for 30 min followed by overnight incubation of indicated antibodies at 4°C. F4/80 and CD11b Alexa Fluor® 488 Goat anti-mouse and Alexa Fluor® 594 Goat anti-rabbit secondary antibodies (Thermo Fischer, Waltham, MA) were used. Staining images were all taken using a Zeiss Axio Scope A1 microscope (Carl Zeiss, Germany) and analyzed using ZEN software (Carl Zeiss, Germany).

### **IV. Isolation of intestinal crypts and intestinal organoid culture**

The small intestine was cut open longitudinally and washed with ice-cold PBS HEP-17-1007-R2 to remove intestinal contents. After removal of intestinal villi with a sterile cell scraper, remains were cut into small pieces, washed with 10% FBS-Dulbecco's PBS (DPBS) several times, transferred into 2 mM EDTA-DPBS and incubated at 4C on a shaker for 30 min. After sedimentation, EDTA was removed, followed by vigorously pipetting up and down using 10% FBS-DPBS to dissociate intestinal crypts. After passing through a 100 µm cell strainer, the cell suspension containing the intestinal crypts was centrifuged and then washed with Basic

Medium (Advanced DMEM/F12 medium with the supplement of 1x B27, 1x N2, 500 mM N-Acetyl-L-cysteine, 10 mM HEPES, 1x GlutaMAX and 1x Penicillin/Streptomycin). The isolated crypts were either resuspended with BD Matrigel Basement Membrane Matrix for intestinal organoid culture or lysed with TRIzol Reagent for RNA isolation. The crypts were cultured in Intestinal Organoid Medium (Basic Medium supplemented with 30 ng/mL recombinant murine EGF, 100 ng/mL recombinant murine Noggin and 500 ng/mL recombinant human R-Spondin-1). Three dimensional-cultured intestinal organoids were photographed every 2 days for 10 days using a 10x or 20x objective lens of an Olympus 1X71 microscope (Olympus Corp., PA). Cultured organoids were harvested for RNA isolation or immunofluorescence staining from the frozen section.

## **V. Isolation of primary mouse hepatocytes**

Hepatocytes were isolated by a two-step collagenase perfusion system. Trypan blue exclusion was used to determine cell viability (>90%) before plating monolayers on collagen-coated plates (60-mm or 6-wells). Cells were cultured in serum-free Williams" E medium containing dexamethasone (0.1  $\mu$ M), penicillin (100 units/mL), and thyroxine (1  $\mu$ M). Media was changed 4 hours after plating. Kupffer cells were isolated using a two-step collagenase perfusion followed by centrifugation in a density-gradient made by Percoll.

## **VI. Biochemical analyses of serum**

Serum alkaline phosphatase (ALP), aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities were determined at the clinical pathology laboratory at Hunter Holmes McGuire VA Medical Center (Richmond, VA).

## **VII. RNA Isolation and RT-PCR**

Total RNA was extracted using Trizol reagent (ThermoFisher) following the manufacturer's protocol. cDNA synthesis and Quantitative RT-PCR analysis of relative mRNA expression levels of target genes were normalized to *Hprt1* as an internal control. Primer sequences will be provided upon request.

## **VIII. Immunoblotting**

Cells were lysed in cold RIPA buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 1X Protease Inhibitor) and centrifuged for 15 min at 4°C. Tissue samples were homogenized in cold RIPA buffer using a tissue grinder. Protein concentrations were determined using the Bio-Rad Protein Assay reagent and Bradford protein assay. Samples were resolved by SDS-PAGE and transferred to nitrocellulose membranes. After blocking with 5% skim milk in TBS-T (150 mM NaCl, 50 mM Tris-HCl, pH 8.0, and 0.05% Tween 20), the membranes were probed with the indicated primary antibodies overnight at 4°C followed by incubation with a horseradish peroxidase-conjugated secondary antibody. The antibody-antigen complexes were detected using the ECL system.

## **IX. FITC-Dextran Permeability Assay**

FITC-Dextran solution (100mg/ml) was prepared in PBS. 60mg/100g of FITC-Dextran was administered to mice by oral gavage, and blood samples were taken after 3 hours. The serum concentration of FITC-dextran was measured using Victor Multilabel Plate Counter

(PerkinElmer, Waltham, MA) with an excitation wavelength of 490 nm and an emission wavelength of 530 nm.

## **X. Human Liver Samples**

Frozen patient liver tissues were obtained through the Liver Tissue Cell Distribution System (Minneapolis, MN) funded by NIH Contract# HSN276201200017C.

## **XI. Statistical Analysis**

Results are presented as the mean  $\pm$  SE and are from at least three independent experiments. One-way analysis of variance and post-test was performed to analyze the differences between multiple groups by GraphPad Prism (version 5; GraphPad Software Inc., San Diego, CA). P-values of 0.05 were considered statistically significant.

## Chapter 3: SphK2 Deficiency Promotes Hepatic Steatosis and Dysregulation of Hepatic Lipid Metabolism

### I. Rationale

With the ever-increasing epidemic of obesity worldwide, significant efforts have been made to determine the underlying mechanisms of lipid metabolism and to discover potential drug targets. Many investigators have turned to the liver as it is the central organ responsible for the metabolism of nutrients. With the advent of computational biology, RNA sequencing and next-generation sequencing, these novel techniques allowed researchers to analyze large data sets to identify potential organisms and genes that may play a key role in nutrient partitioning [249]. Traditionally, our understanding of bile acids and sphingolipids was quite limited. Bile acids were detergent molecules that were synthesized by the liver to emulsify fats and aid in its absorption. Sphingolipids were merely structural molecules for cellular components. However, in the past several decades, bile acids and sphingolipids were proved to be potent signaling molecules that play a role in various cellular functions and disease processes [111, 250].

Our laboratory has a longstanding interest in S1P-mediated signaling in various gastrointestinal disorders. As such, our previous studies identified sphingosine 1-phosphate S1PR2 and SphK2 knockout ( $S1PR2^{-/-}$  and  $SphK2^{-/-}$ ) mice develop overt fatty liver with two-week high-fat diet feeding, which is associated with significant down-regulation of hepatic lipid metabolism genes [183]. We were able to demonstrate that S1PR2 activates SphK2 to generate nuclear S1P and inhibit HDAC1/2 allowing the transcription of key genes in hepatic lipid metabolism [183]. Moreover, it has been reported that the activation of SphK2 in response to ER stress ameliorates hepatic steatosis [184].

In this study, we attempt to define the role of alcohol in SphK2<sup>-/-</sup> mice and elucidate potential mechanisms by which alcohol damages hepatocytes. Here we show potential genes and pathways that S1PR2 regulates through RNA sequencing along with the role it may have in alcohol-induced liver injury. In addition, we adopted the NIAAA chronic and binge mouse model for our in vivo studies. We also show the phenotypic liver differences between wild type and SphK2<sup>-/-</sup> mice fed an alcohol diet and the gene expression changes that are associated with SphK2 deficiency.

## II. Results

### **S1PR2 overexpression in mouse primary hepatocytes regulate key pathways in bile acid and lipid metabolism**

S1PR2 is a GPCR that is activated by conjugated bile acids and mediates the downstream activation of sphingosine kinase 2 (SphK2) [183, 251]. Previously in our laboratory, we have shown that S1PR2-mediated activation of SphK2 upregulates several important hepatic lipid metabolism genes *via* S1P-mediated inhibition of HDAC1/2 [183]. To gain a more unbiased and inclusive understanding of the biological pathways and genes S1PR2 regulate, we overexpressed S1PR2 in mouse primary hepatocytes, and total RNA was isolated. RNA sequencing was subsequently performed to determine relevant genes that were enriched in various biological pathways.

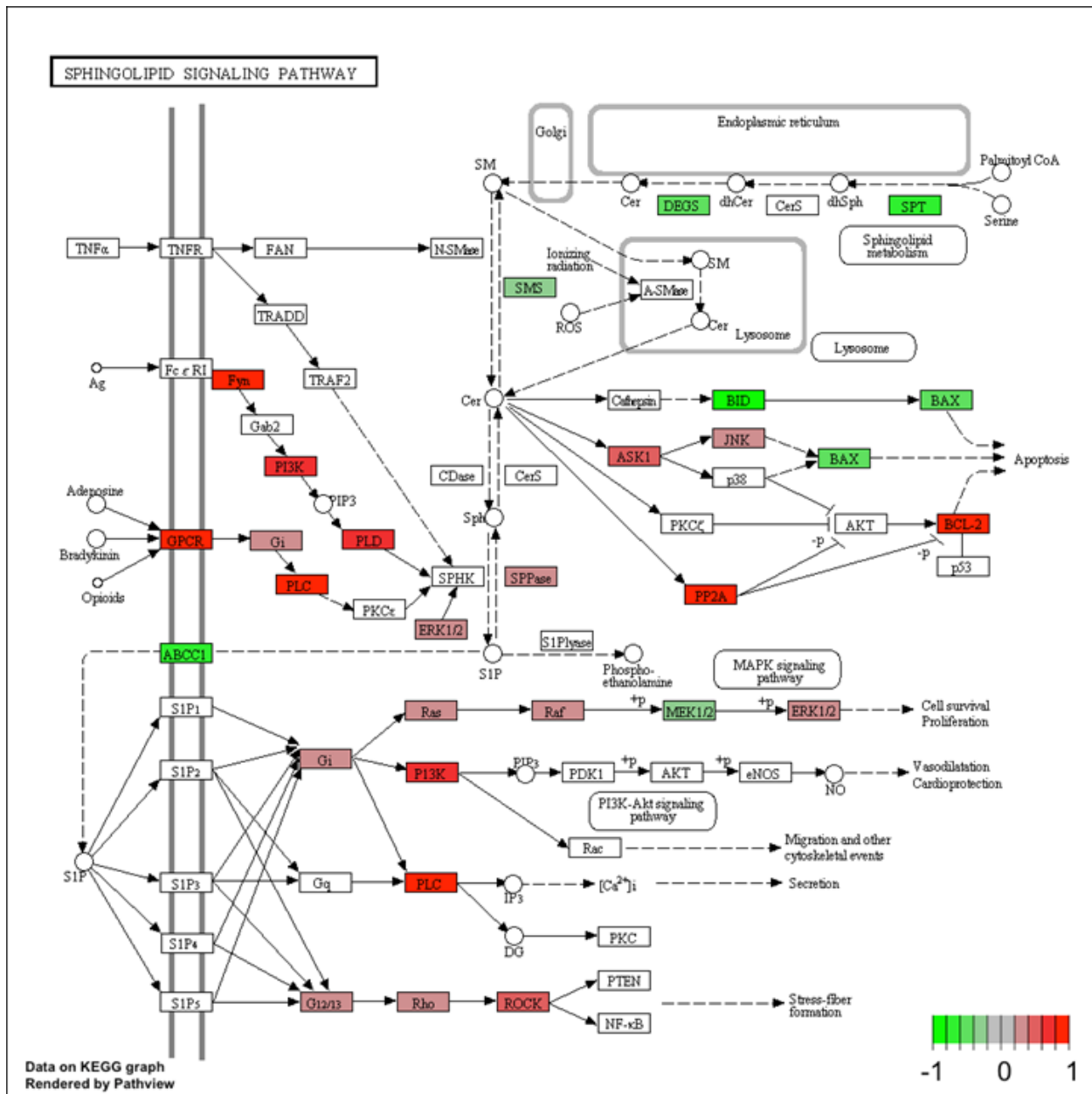
Kyoto Encyclopedia of Genes and Genomes (KEGG), a collection of genomes and biological pathways, was used to map pathways that were regulated by S1PR2 overexpression in mouse primary hepatocytes. Figure 11 shows various KEGG pathways that were identified to have been significantly regulated by S1PR2 overexpression including sphingolipid signaling, PPAR signaling, MAPK signaling, AMPK signaling, bile acid secretion and NAFLD. Genes highlighted in green and red indicate downregulation and upregulation, respectively. Interestingly, these pathways play a key role in biological processes that are responsible for cellular growth, nutrient partitioning, and energy metabolism. More importantly, there was a significant induction of long-chain fatty acid oxidation genes. This is consistent with the enhanced lipid accumulation in the livers of S1PR2<sup>-/-</sup> and SphK2<sup>-/-</sup> mice.

One challenge with high throughput experiments and interpreting large data sets is managing redundancy. To circumvent this potential issue, we used REVIGO which compiles redundant gene ontology (GO) terms under the same biological processes using a simple algorithm that groups based on semantic similarity. A scatter plot for the RNA sequencing data that was used to create the KEGG pathways in Figure 10 was generated through multidimensional scaling, and similar GO terms are grouped closer together in the plot based on similar semantics (Figure 12). The color of the biological processes cluster corresponds to the p-value input. The biological processes that are most enriched and clustered together are stress response and cellular metabolism. This simplified summary of biological processes that S1PR2 governs is in congruence with the KEGG pathways obtained. In addition to mapping pathways, we also ranked the top 6 most significant genes based on the p-value of 3 key pathways in lipid metabolism (MAPK signaling, AMPK signaling and sterol synthesis) (Figure 13). Interestingly, key genes in glucose homeostasis and gluconeogenesis, glucose 6-phosphatase (*G6pc*) and phosphoenolpyruvate carboxykinase 1 (*Pck1*), are upregulated. For sterol synthesis, 3-hydroxy-3-methylglutaryl-CoA synthase 2 (*Hmgcs2*) is upregulated 3-fold while *Cyp26a1* and *Apob* are reduced by about 2-fold.

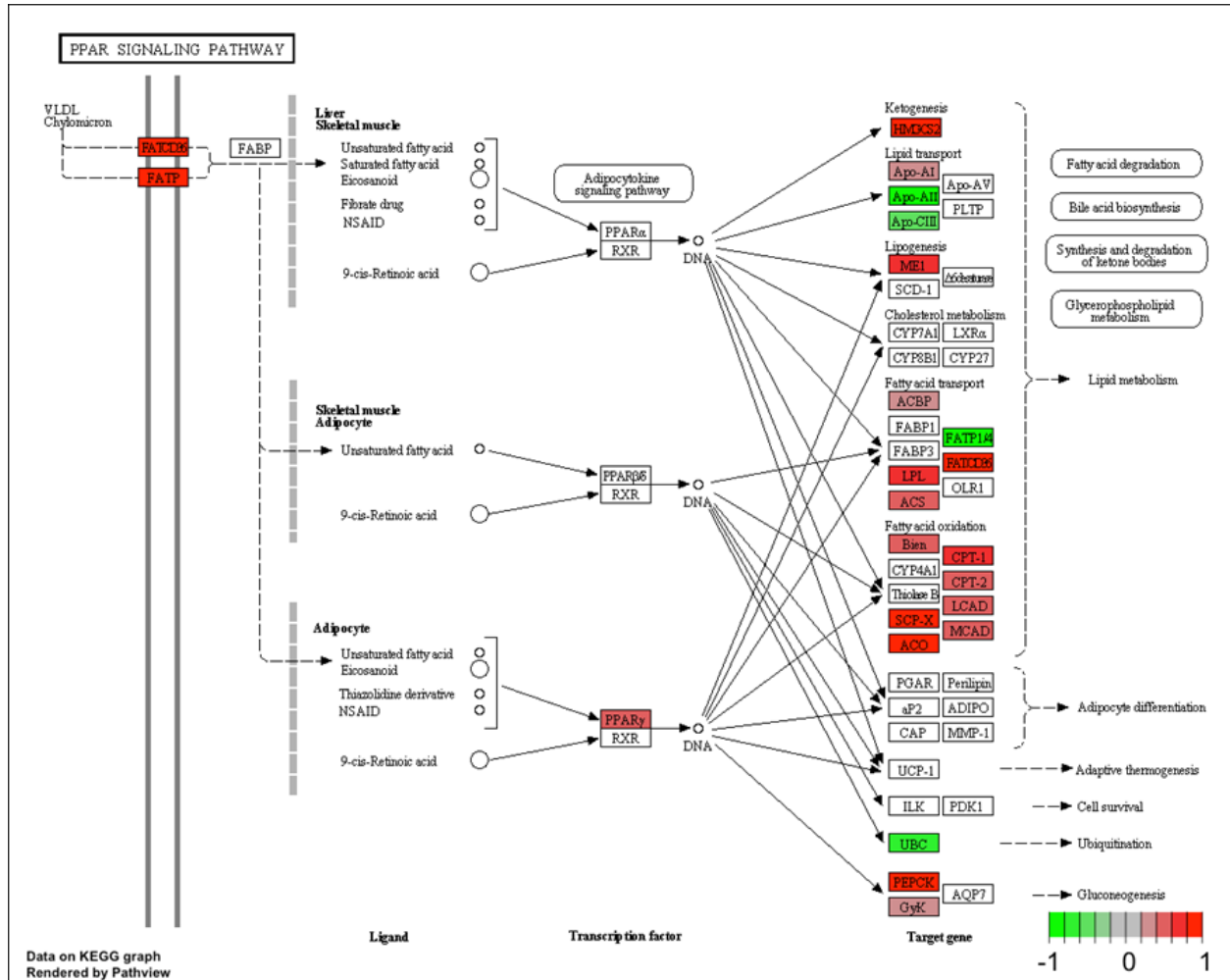
### **Characterization of the SphK2<sup>-/-</sup> mouse liver on an alcohol diet using the chronic and binge mouse model (the NIAAA model)**

We wanted to determine the effects of alcohol on liver injury in SphK2 deficient mice. Based on the literature, variations on the Lieber-DeCarli alcohol liquid diet composed of 1-5% alcohol by volume have been published. The advantage of the Lieber-DeCarli alcohol diet is that the caloric and water requirements are combined with alcohol versus other drinking studies that replace water with a fraction of alcohol. This allows close monitoring of not only the amount of alcohol consumed but also the caloric intake which may also be a confounding variable in

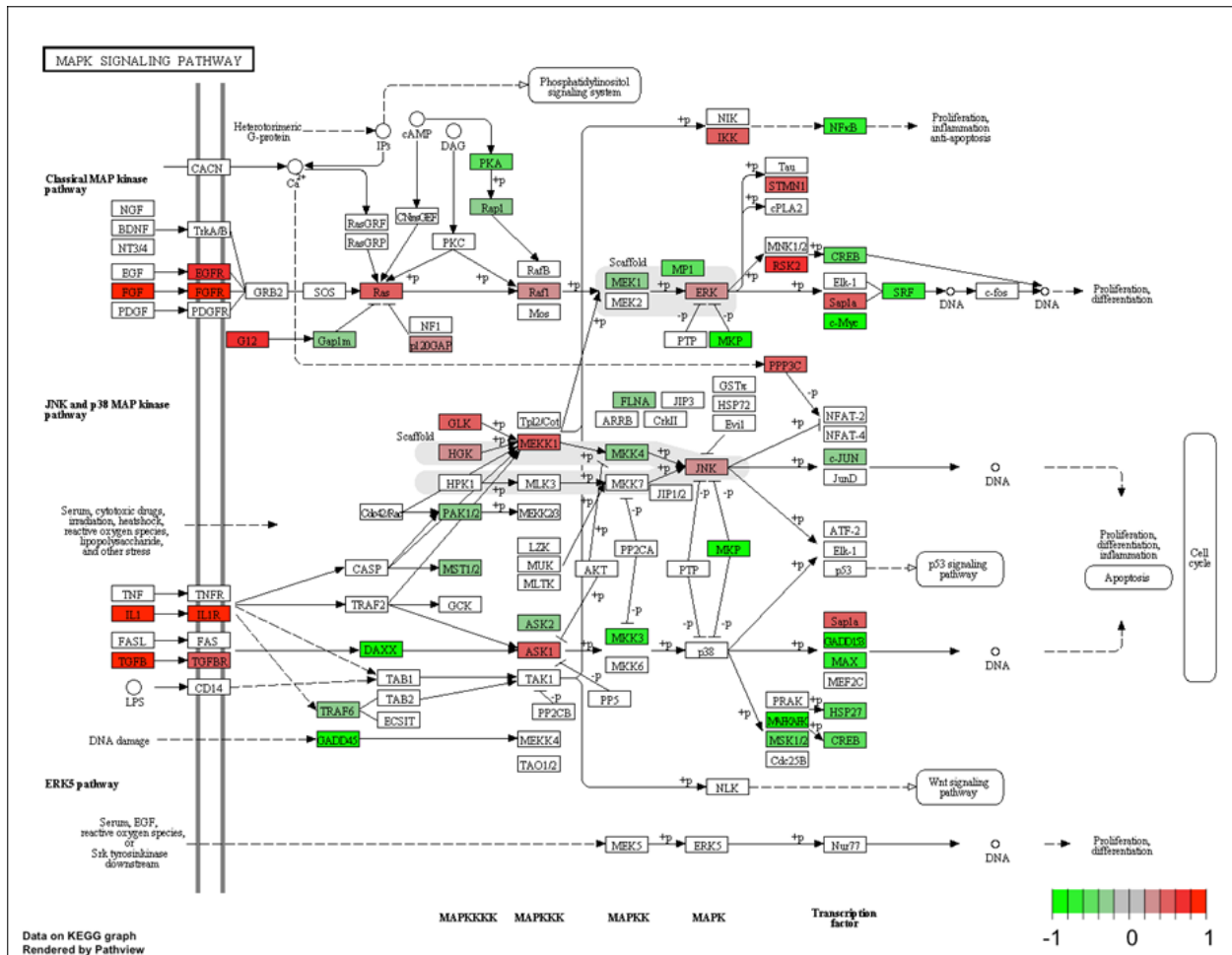




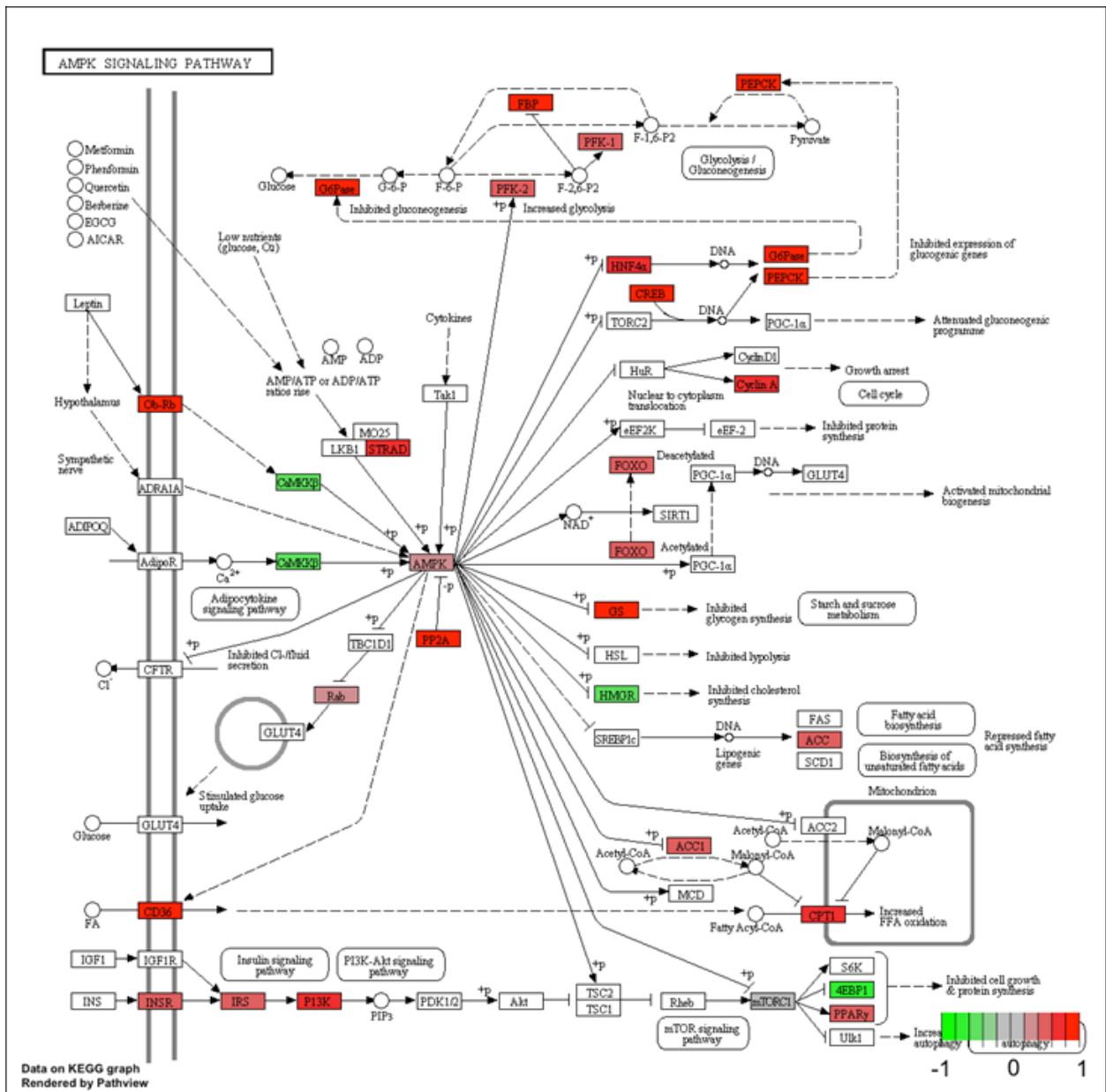
**Figure 11. KEGG Pathways that are regulated by S1PR2.** S1PR2 overexpression regulated genes in the sphingolipid signaling pathway. Genes highlighted in green and red indicate downregulation and upregulation, respectively.



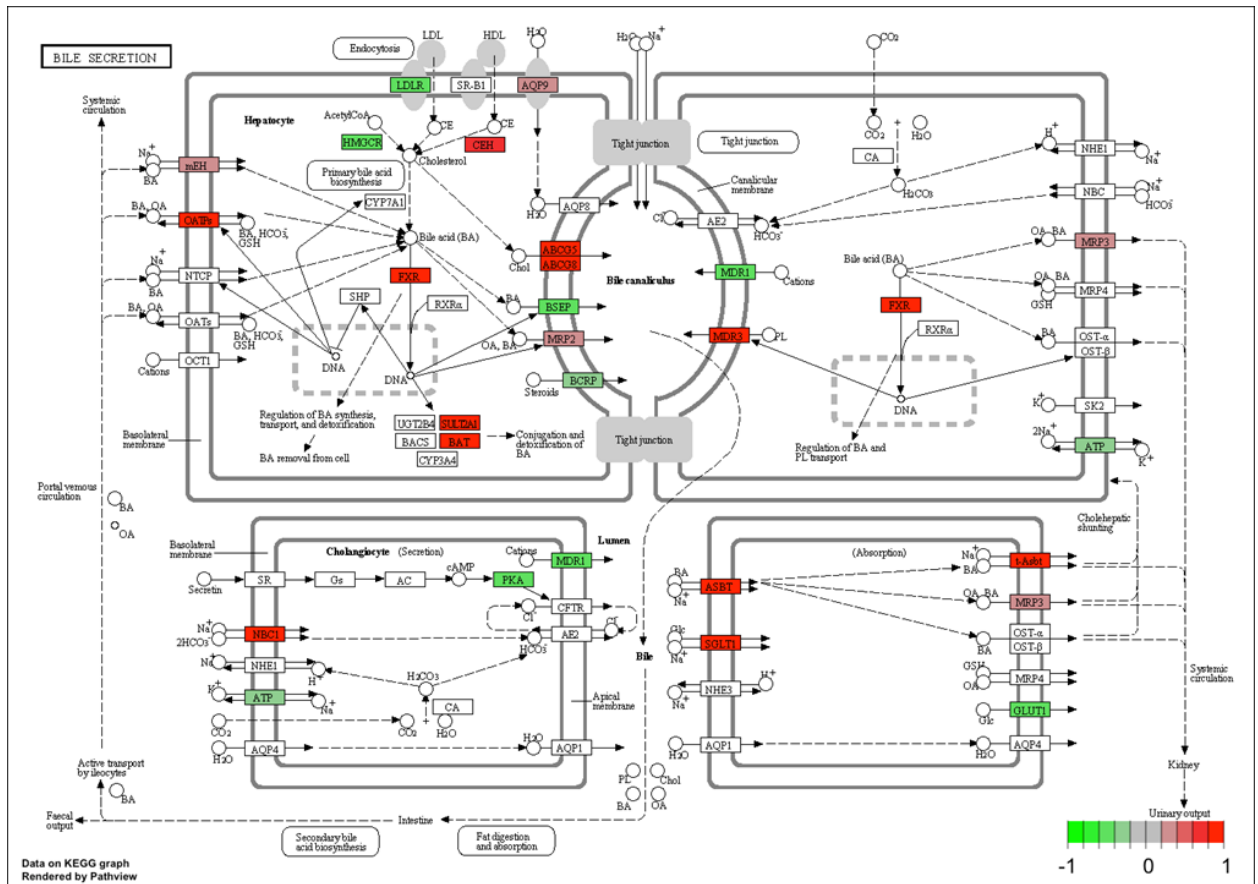
**Figure 11. KEGG Pathways that are regulated by S1PR2.** S1PR2 overexpression regulated genes in the PPAR signaling pathway. Genes highlighted in green and red indicate downregulation and upregulation, respectively.



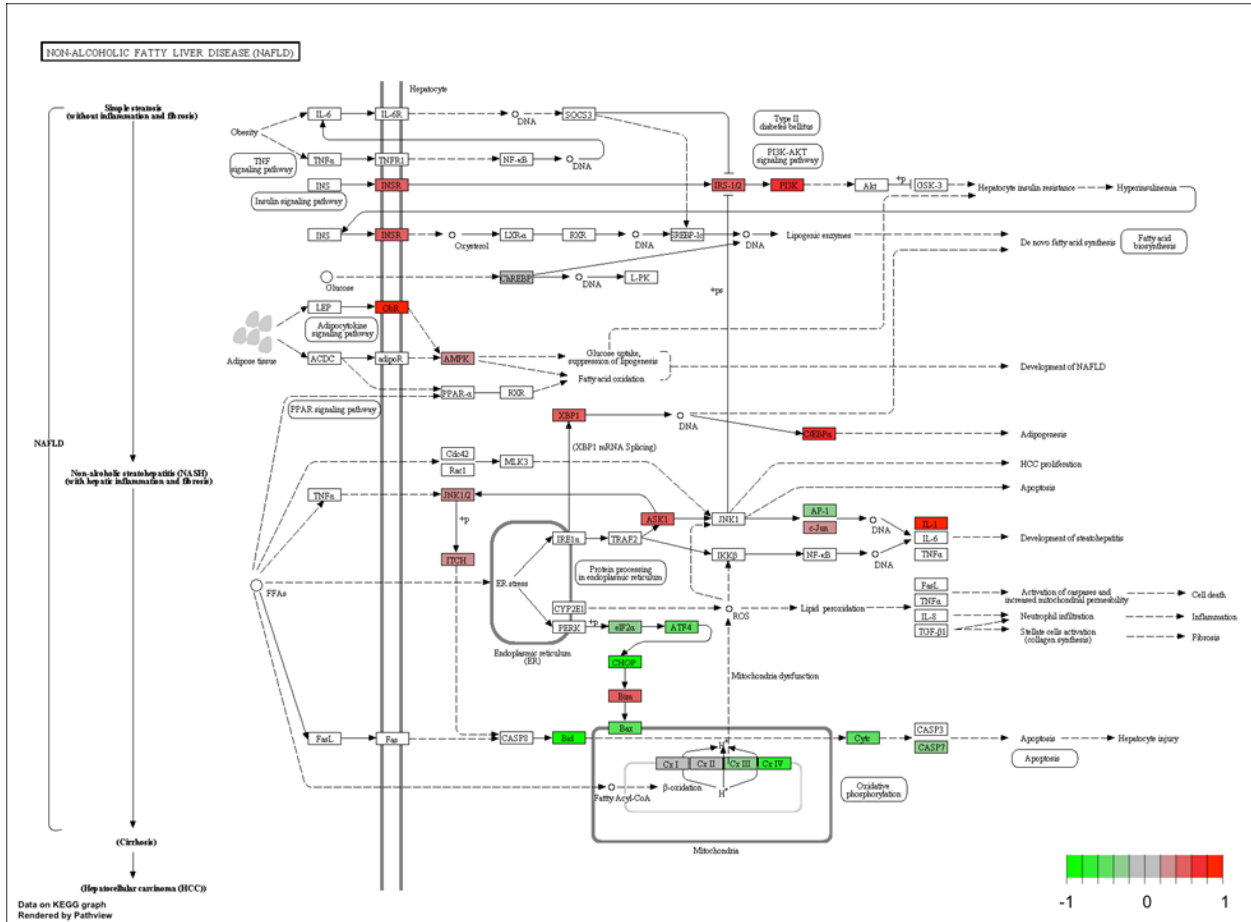
**Figure 11. KEGG Pathways that are regulated by S1PR2.** S1PR2 overexpression regulated genes in the MAPK signaling pathway. Genes highlighted in green and red indicate downregulation and upregulation, respectively.



**Figure 11. KEGG Pathways that are regulated by S1PR2.** S1PR2 overexpression regulated genes in the AMPK signaling pathway. Genes highlighted in green and red indicate downregulation and upregulation, respectively.



**Figure 11. KEGG Pathways that are regulated by S1PR2.** S1PR2 overexpression regulated genes in the bile acid secretion pathway. Genes highlighted in green and red indicate downregulation and upregulation, respectively.



**Figure 11. KEGG Pathways that are regulated by S1PR2.** S1PR2 overexpression regulated genes that have been implicated in non-alcoholic fatty liver disease. Genes highlighted in green and red indicate downregulation and upregulation, respectively.



**Figure 12. REVIGO gene ontology clustering of biological processes based on semantic similarity.** RNA sequencing data of genes and their p-values are entered through the REVIGO web server and an algorithm sorts out similar biological processes based on semantic similarities. Color-coded clusters represent p-values entered.

AMPK signaling		MAPK signaling		Sterol Synthesis	
Gene symbol	log2 fold change	Gene symbol	log2 fold change	Gene symbol	log2 fold change
G6pc	3.10	Fgfr2	1.39	Hmgcs2	3.16
Pck1	2.76	Fgf1	1.35	Nr1h4	1.33
Ppp4r4	1.19	Tgfb3	0.95	Cyp7b1	1.19
Fbp1	1.11	Il1a	0.97	Cyp26b1	1.19
Creb3l3	0.89	Il1r1	0.91	Cyp26a1	0.54
Lepr	0.85	Il1b	0.72	Apob	0.42

**Figure 13. Top 6 most significant genes in the AMPK signaling, MAPK signaling, and sterol synthetic pathways.** Six genes were selected based on the most significant p-value. The gene symbol and log2 fold change are shown in each pathway.

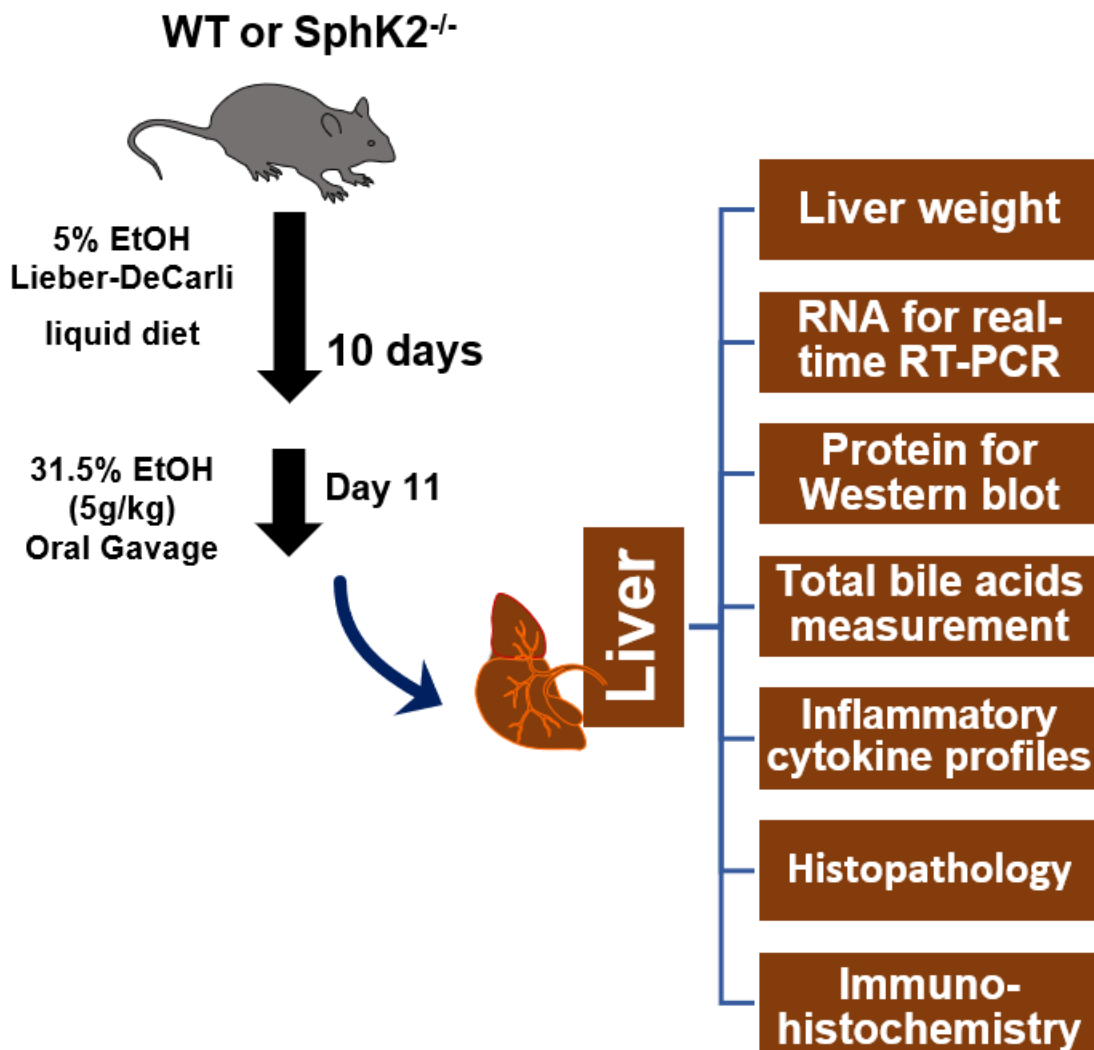


research. We adopted the NIAAA mouse model of chronic and binge alcohol feeding for our studies because this pattern of drinking is typical of human patients presenting with ALD (Figure 14). Both wild type and SphK2<sup>-/-</sup> mice of the C57BL background were fed a 5% alcohol liquid diet (Lieber-DeCarli) for 10 days followed by a binge *via* 31.5% alcohol gavage (body weight in grams x 20  $\mu$ L) on day 11. After 6 hours following gavage, mice were euthanized and liver tissue was harvested to investigate the effects of alcohol on hepatic lipid accumulation. Various downstream biochemical assays were performed as shown in Figure 14.

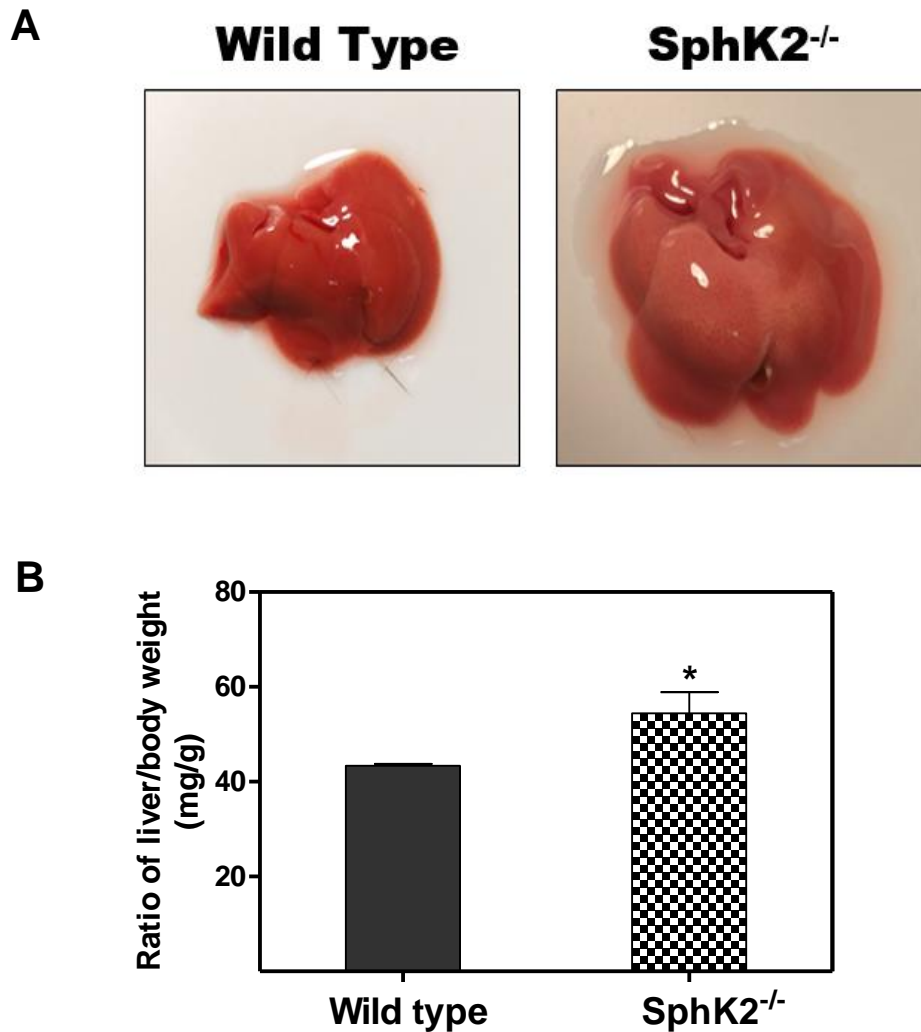
### **SphK2<sup>-/-</sup> mice on an alcohol diet develop overt hepatic steatosis**

As shown in Figure 15, SphK2<sup>-/-</sup> mice had enlarged livers and were pale in color compared to the wild type. The liver index was calculated based on the weight of the liver (mg) divided by body weight (g). SphK2<sup>-/-</sup> mice had a relatively higher liver weight as indicated by an increase in the liver index. Mice maintained their relative starting weights at the end of the alcohol feed. Both wild type and SphK2<sup>-/-</sup> mice consumed the same amount of alcohol diet on average over the course of the 10-day treatment. H&E and Oil Red O staining further showed a marked increase in lipid accumulation in SphK2<sup>-/-</sup> mice compared to wild type (Figure 16). H&E staining showed evidence of macrovesicular steatosis as demonstrated by large white vacuoles. Oil Red O stains neutral lipids red and is more abundant in SphK2<sup>-/-</sup> mouse livers. These results are strikingly similar to our previous data revealing SphK2<sup>-/-</sup> mice on a high-fat diet develop increased fatty liver compared to wild type (data not shown).

### **Effect of alcohol in SphK2<sup>-/-</sup> mouse livers on mRNA expression of key genes in lipid metabolism.**

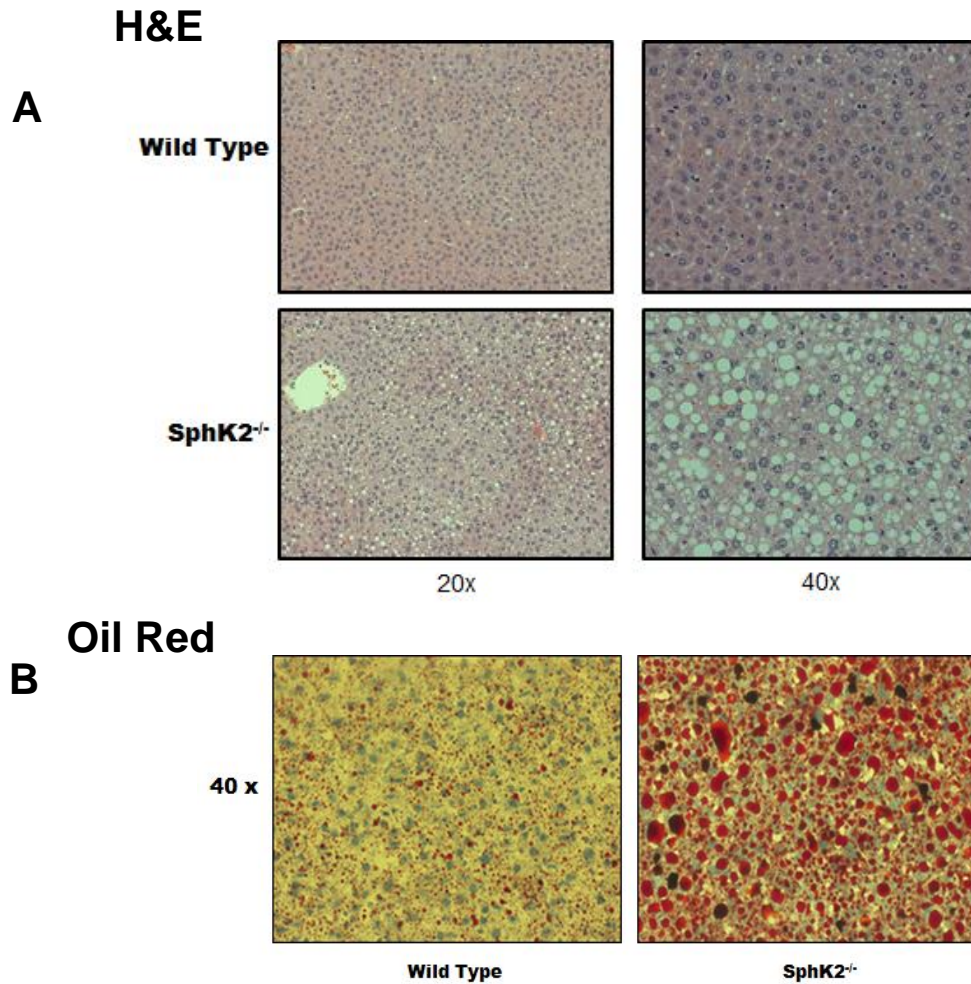


**Figure 14. Schematic diagram of the acute on chronic alcohol model (the NIAAA model) of alcohol feeding and protocol for analyzing liver tissue.** Wild Type and SphK2<sup>-/-</sup> mice are fed a 5% alcohol Lieber-DeCarli diet for 10 days followed by a single dose of alcohol (5g/kg) *via* oral gavage on day 11. Subsequent biochemical analyses were performed as illustrated.



**Figure 15. Liver images and weights of wild type and SphK2<sup>-/-</sup> mice on the NIAAA model.**

**A)** Representative images of wild type and SphK2<sup>-/-</sup> mouse (male and female, 19 weeks old) livers **B)** Liver index based on the weight of the liver (mg) divided by body weight (g). Results are represented as mean  $\pm$  SE from each group (n = 5). Statistical significance relative to the wild type control group, \*P < 0.05.



**Figure 16. H&E and Oil Red O staining of wild type and SphK2<sup>-/-</sup> mouse livers on the NIAAA model.** Wild type and SphK2<sup>-/-</sup> mouse (male and female, 19 weeks old) livers were preserved in 3% formaldehyde and sectioned to be stained with **A)** H&E **B)** Frozen sections of the livers were stained with Oil Red O to identify lipid accumulation in the liver tissue with hematoxylin used to stain the nuclei. Representative images are shown.

We have previously shown that S1PR2<sup>-/-</sup> and SphK2<sup>-/-</sup> mice have marked downregulation of hepatic lipid genes. We wanted to determine whether alcohol would cause a similar downregulation of these genes. We used quantitative Real Time-PCR to analyze the relative mRNA expression levels of genes that have been shown to play a key role in lipid metabolism. The genes were normalized to a housekeeping gene *Gapdh* as an internal control. As shown in Figure 17, various key lipid metabolism genes are downregulated with *Fxrα* and *Cyp7b1* being statistically significant.

### **Alcohol induces the expression of SphK2 in mouse primary hepatocytes**

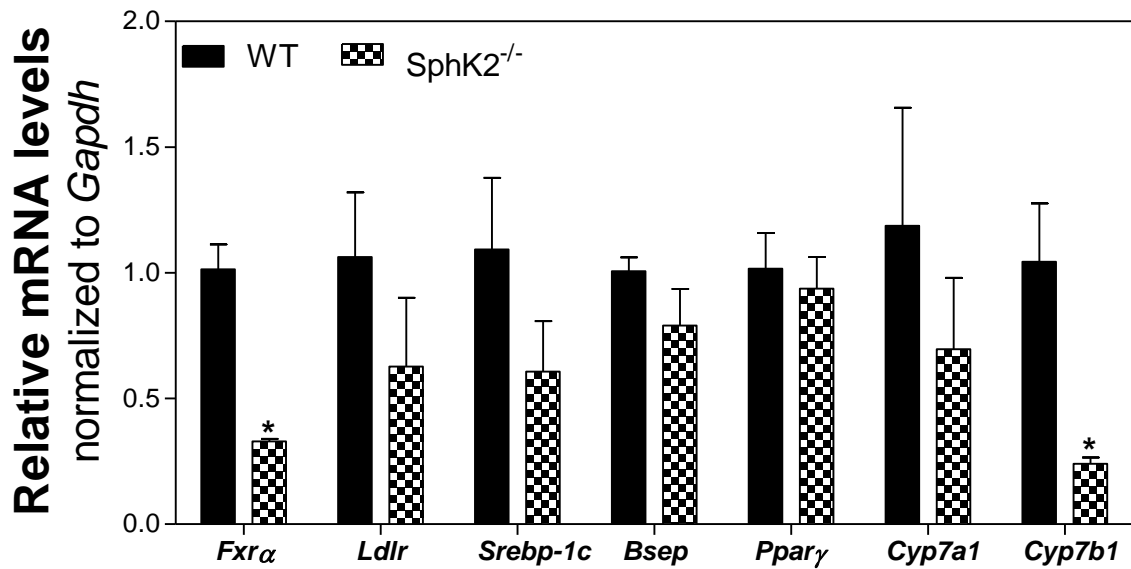
To test the hypothesis that SphK2 is upregulated in hepatocytes when challenged with alcohol, we obtained mouse primary hepatocytes from wild type mice. Mouse primary hepatocytes were treated with varying concentrations of alcohol (0, 50, 100 mM) for 6 hours. Relative mRNA and protein levels were obtained using qRT-PCR and western blotting, respectively (Figure 18).

### **Characterization of the SphK2<sup>-/-</sup> mouse liver on a chronic 60-day alcohol diet**

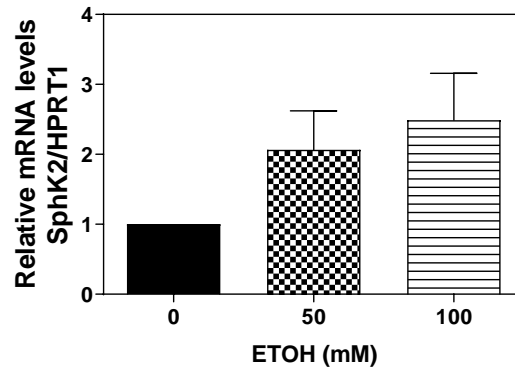
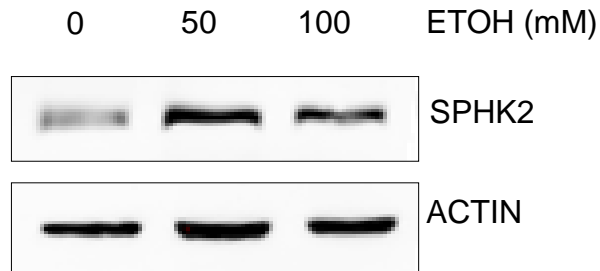
To determine the effects of prolonged alcohol consumption on SphK2<sup>-/-</sup> mice, we fed wild type and SphK2<sup>-/-</sup> mice control Lieber-DeCarli or Lieber-DeCarli 5% alcohol diet for 60 days. The mice were sacrificed for liver and intestine tissues followed by various biochemical assays as illustrated in Figure 19.

### **Effect of 60-day alcohol feeding on wild type and SphK2<sup>-/-</sup> mouse livers**

As shown in Figure 20, SphK2<sup>-/-</sup> mice had enlarged livers and were pale in color



**Figure 17. Effect of alcohol on the mRNA expression of key genes in lipid metabolism in SphK2<sup>-/-</sup> mouse livers from the NIAAA model.** Total RNA was isolated from wild type and SphK2<sup>-/-</sup> mouse livers (male, 19 weeks old) on an alcohol diet. Quantitative RT-PCR was used to determine the relative mRNA expression levels using *Gapdh* as an internal control. Results are represented as mean  $\pm$  SE from each group (n = 3). \*p < 0.05, statistical significance relative to WT.

**A****B**

**Figure 18. Effect of alcohol on SphK2 expression in hepatocytes and macrophages.**

Mouse primary hepatocytes were treated with alcohol (0-100 mM) for 6 hours. (A) Total RNA was isolated and mRNA levels of SphK2 were determined using quantitative RT-PCR. *Hprt1* was used as an internal control (B) Total protein lysates were prepared and protein levels of SphK2 were determined by western blot analysis. Results are represented as mean  $\pm$  SE from each group (n = 3). Statistical significance relative to no treatment, \*P < 0.05.

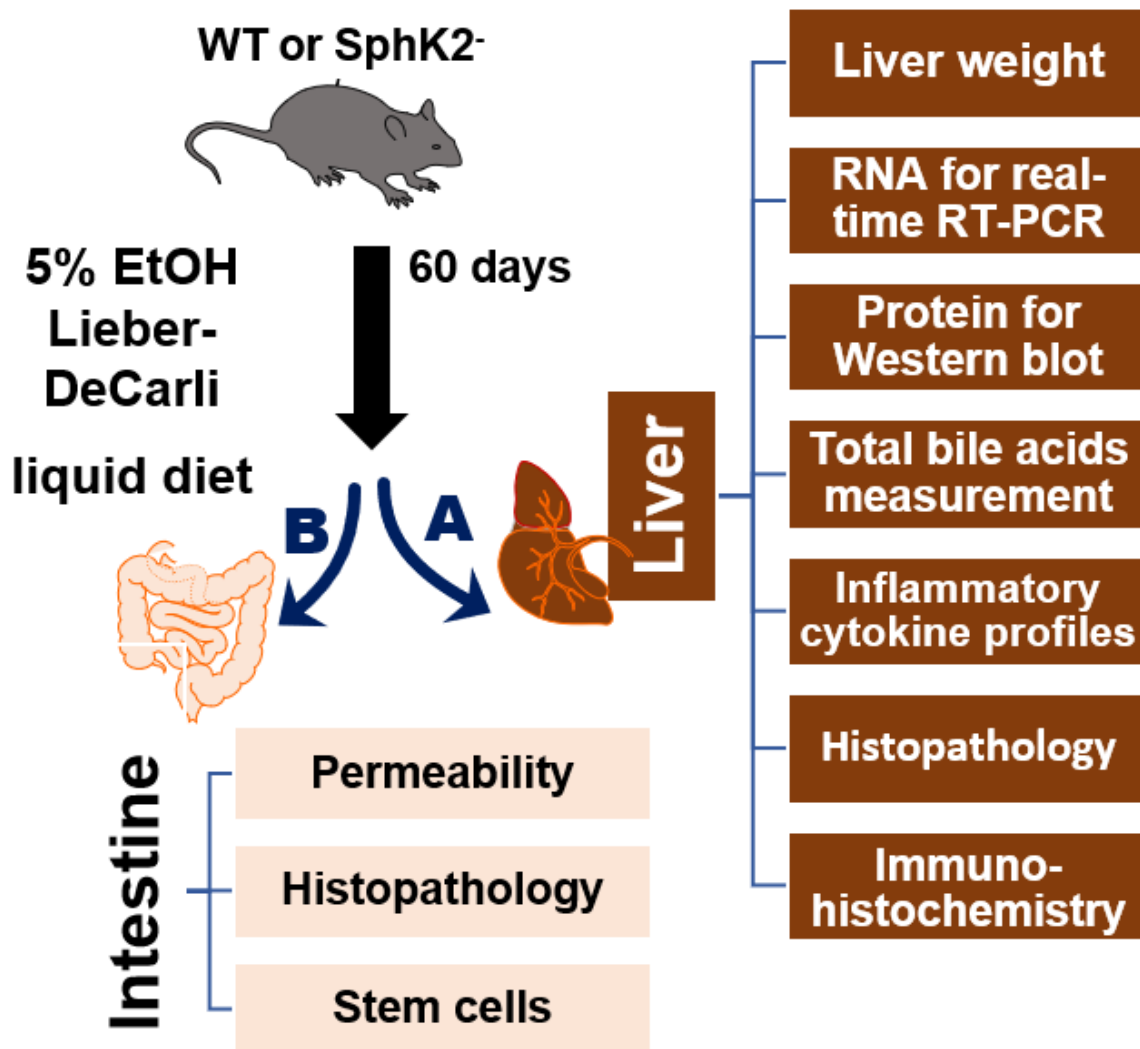
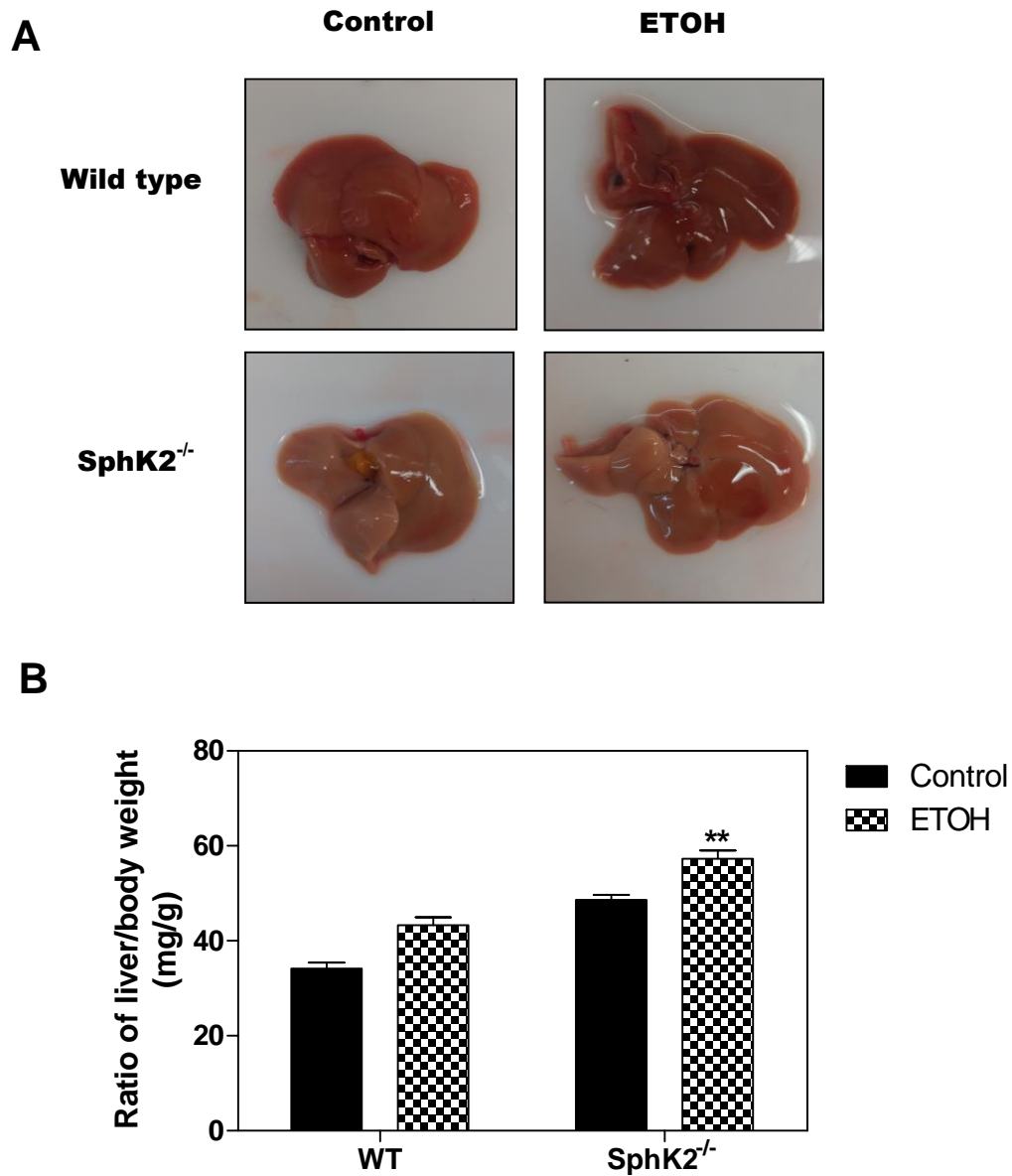


Figure 19. Schematic diagram of the 60-day chronic alcohol feeding model and protocol for analyzing liver and intestine tissue.



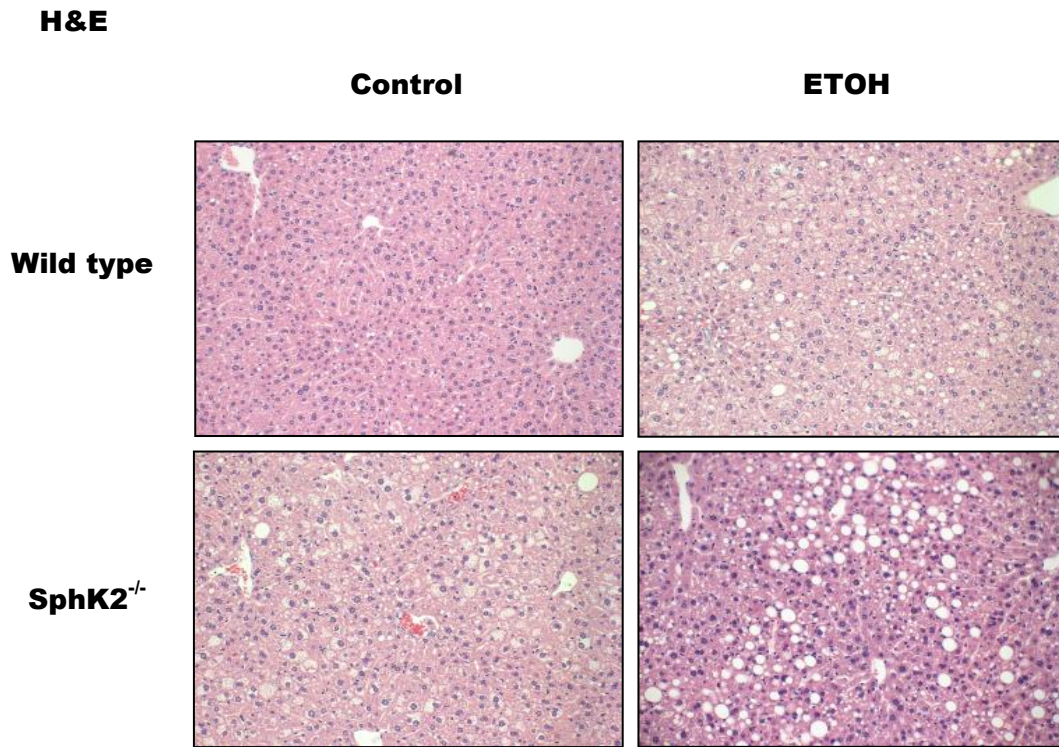


**Figure 20. Liver images and weights of wild type and SphK2<sup>-/-</sup> mice on a 60-day alcohol diet. A)** Representative images of wild type and SphK2<sup>-/-</sup> mouse (male and female, 20-week-old) livers **B)** Liver index based on the weight of the liver (mg) divided by body weight (g). Results are represented as mean ± SE from each group (n = 5). Statistical significance relative to the control group, \*\*P < 0.01.

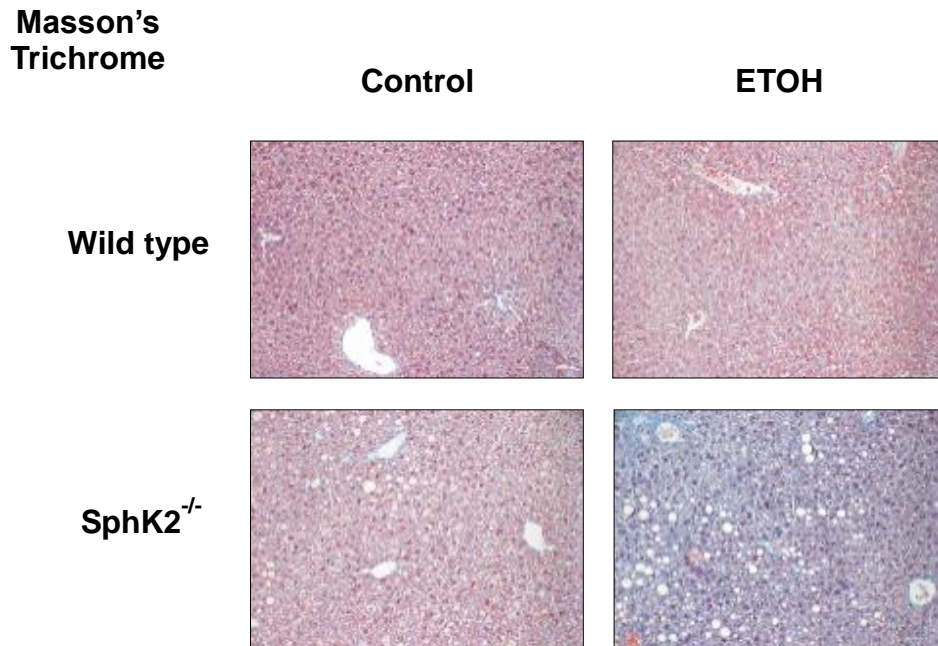
compared to wild type. In addition, mice fed an alcohol diet had larger livers than their control fed counterpart. The liver index was calculated based on the weight of the liver (mg) divided by body weight (g). SphK2<sup>-/-</sup> mice had a relatively higher liver weight as indicated by an increase in liver index than wild type mice. Mice maintained their relative starting weights at the end of the alcohol feed. Both wild type and SphK2<sup>-/-</sup> mice consumed the same amount of alcohol diet on average over the course of the 60-day treatment. H&E staining showed a marked increase in lipid accumulation in SphK2<sup>-/-</sup> mice compared to wild type (Figure 21). Compared to the NIAAA model shown in Figure 16, SphK2<sup>-/-</sup> mice on the 60-day alcohol diet exhibited a greater degree of hepatic steatosis as evident by the increased in clear vacuole sizes on H&E staining. However, Masson's trichrome staining did not reveal any evidence of liver fibrosis (Figure 22). Oil Red O staining confirms the extensive hepatic steatosis observed in H&E staining through the visualization of neutral fats stained in red (Figure 23).

#### **Effect of alcohol in SphK2<sup>-/-</sup> mouse livers on mRNA expression of key genes in lipid metabolism.**

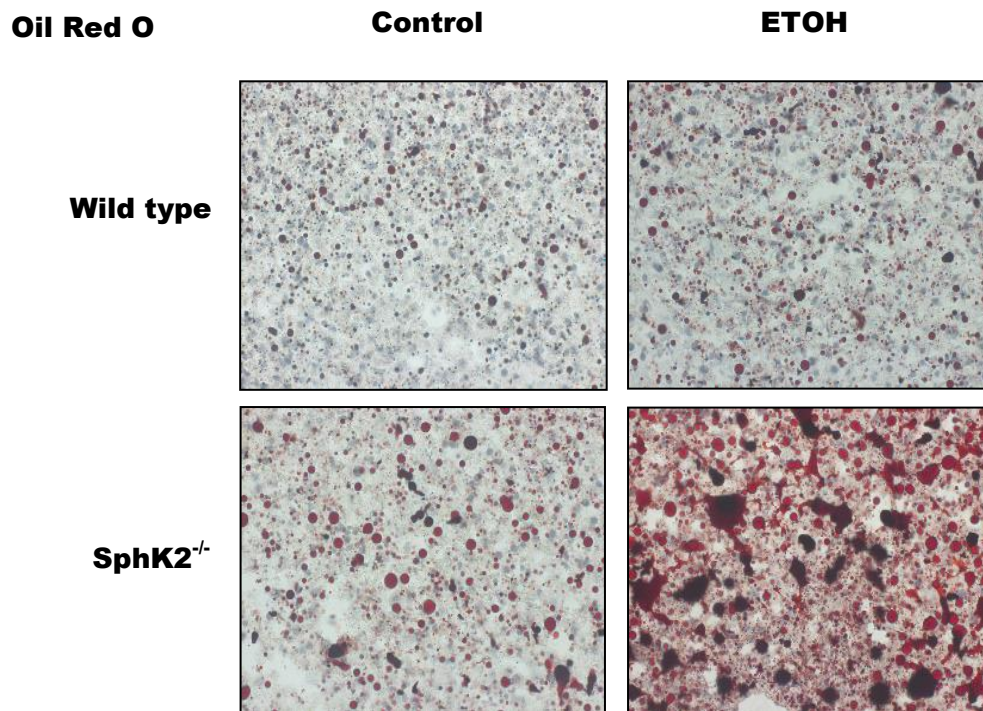
To determine the effect of alcohol on wild type and SphK2<sup>-/-</sup> mouse livers, we analyzed the relative expression of several key genes in bile acid synthetic and lipid metabolism gene (*Cyp7a1*, *Cyp7b1*, *Fas*, *Acc-1*) (Figure 24). SphK2<sup>-/-</sup> mice on an alcohol diet had a 2-fold reduction in the expression of the key genes involved in bile acid metabolism. Both wild type and SphK2<sup>-/-</sup> mice on an alcohol diet had higher expression levels of lipid metabolism genes compared to the control diet.



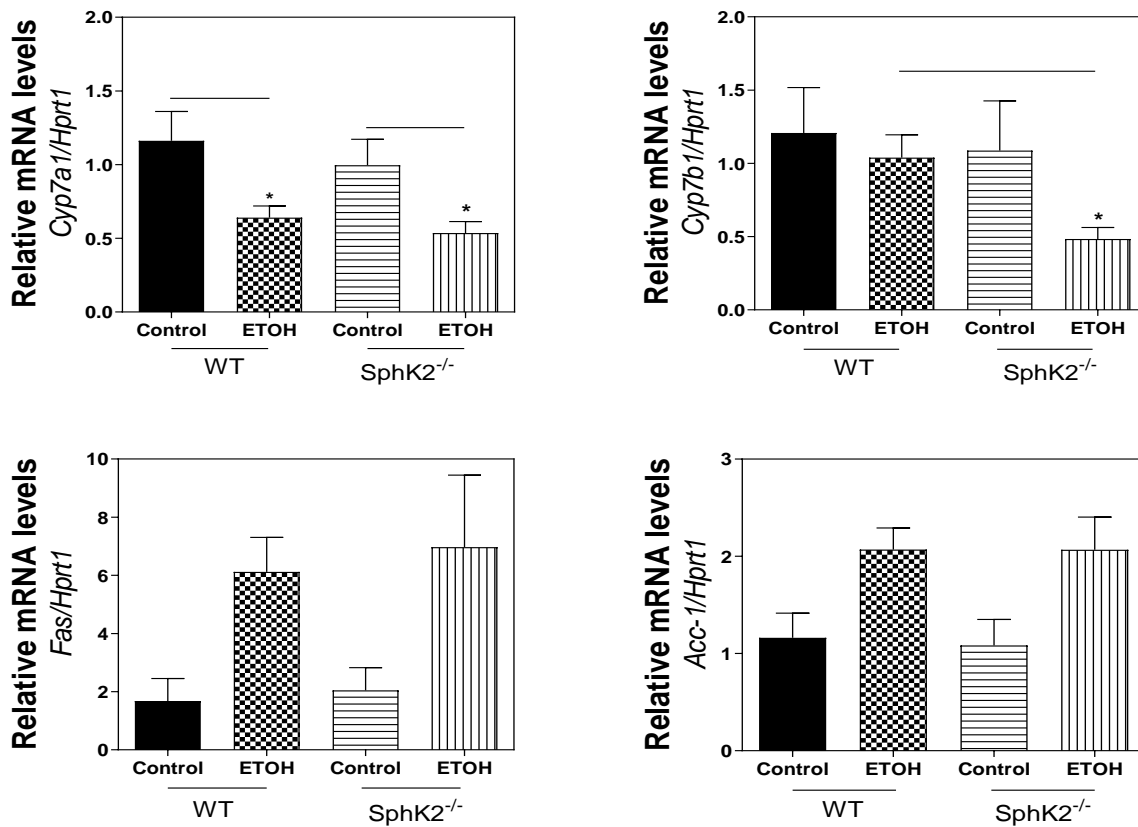
**Figure 21. H&E staining of livers of wild type and SphK2<sup>-/-</sup> mice on a 60-day alcohol diet.** Wild type and SphK2<sup>-/-</sup> mouse (male and female, 20-week-old) livers were preserved in 3% formaldehyde and sectioned to be stained with H&E. Images were taken on an Olympus microscope at 20x magnification. Representative images of each treatment are shown.



**Figure 22. Masson's Trichrome Staining on livers of wild type and SphK2<sup>-/-</sup> mice on a 60-day alcohol diet.** Wild type and SphK2<sup>-/-</sup> mouse (male and female, 20-week-old) livers were preserved in 3% formaldehyde and sectioned to be stained with Masson's Trichrome staining for the detection of liver fibrosis. Images were taken on an Olympus microscope at 20x magnification. Representative images of each treatment are shown.



**Figure 23. Oil Red O staining on livers of wild type and SphK2<sup>-/-</sup> mice on a 60-day alcohol diet.** Frozen sections of the mouse (male and female, 20-week-old) livers were stained with Oil Red O to identify lipid accumulation in the liver tissue with hematoxylin used to stain the nuclei. Representative Images were taken on an Olympus microscope at 20x magnification. Representative images of each treatment are shown.



**Figure 24. Effect of 60-day alcohol feeding on hepatic lipid metabolism genes in SphK2<sup>-/-</sup> mice.** Total RNA was isolated from wild type and SphK2<sup>-/-</sup> mouse livers (male and female, 20-week-old) on an alcohol diet. Quantitative RT-PCR was used to determine the relative mRNA expression levels using *Hprt1* as an internal control. Results are represented as mean ± SE from each group (n = 7). \*p < 0.05.

### III. Discussion

Previous studies from our collaborators and laboratory have demonstrated that S1P plays an important role by inhibiting HDAC1/2 in cancer cells [152]. We hypothesized that removing the inhibition of gene transcription could be a potential mechanism by which the cell allows the transcription of key genes in various pathways. However, the exact physiological function of S1P and the role it plays in the liver is not well understood. Concurrently, bile acids have recently been discovered to have potent signaling functions and bind to ligands to activate various cellular functions. Taken together, various liver studies were performed that led to the discovery that conjugated bile acids activate the S1PR2 along with the ERK1/2 and AKT pathways [251, 252]. ERK1/2, also known as classical MAP kinases, are responsible for mediating a diverse group of cellular functions including cell adhesion, cell cycle progression, cell migration, cell survival, differentiation, metabolism, proliferation, and transcription [253]. The role of ERK1/2 signaling in hepatocytes and on lipid metabolism has been studied extensively including mediating hepatic lipid metabolism through regulating fatty acid composition [254-256]. The AKT pathway plays a key role in insulin signaling and has been implicated in various diseases including cancer and type 2 diabetes [257, 258]. Moreover, AKT affects hepatic metabolism through regulating glucose and lipid metabolism [259-261].

Recently, our laboratory made a crucial discovery that increased levels of conjugated bile acids or the overexpression of S1PR2 lead to the upregulation of SphK2 but not SphK1 [183]. The upregulation of SphK2 generates high levels of S1P which in turn, could inhibit HDAC1/2 and promote the transcription of key genes in hepatic lipid metabolism. More importantly, a striking observation was made when SphK2<sup>-/-</sup> mice on a two-week high-fat diet developed overt fatty liver compared to wild type [183]. We previously postulated that the physiologic role of SphK2 is to tightly regulate key genes and pathways in hepatic lipid

metabolism. Disruption of SphK2 could lead to a downregulation of key genes responsible for lipid homeostasis and transport. This observation has been substantiated by experiments showing downregulation of key genes in hepatic lipid metabolism in SphK2<sup>-/-</sup> mouse livers [183]. This led to our proposed model where the activation of S1PR2 by conjugated bile acids leads to the upregulation of SphK2. Nuclear S1P would inhibit HDAC1/2 to allow the transcription of genes in sterol metabolism (Figure 10). Interestingly, another group published data in the same year supporting the role of SphK2 in ameliorating hepatic steatosis and insulin resistance [184]. The mechanism proposed in this study is the SphK2 activation is driven by ER stress *via* the upregulation of ATF4. However, we did not see any significant changes in the gene expression levels of ER stress-related genes between wild type and SphK2<sup>-/-</sup> mice on an alcohol diet (data not shown). One possibility is that alcohol-induced significant ER stress and activation of UPR genes in the wild type mice beyond baseline levels and we could not observe additional gene upregulation in SphK2<sup>-/-</sup> mice. Nonetheless, these findings are consistent with the notion that SphK2 has a physiologic role in attenuating hepatic steatosis when imposed with cellular challenges.

With preliminary studies showing that the activation of S1PR2 and SphK2 leads to the upregulation of several key genes in hepatic lipid metabolism, we took on a holistic unbiased approach to examining the key pathways and genes that S1PR2 regulates. Biological systems are complex and converge on various cellular pathways. Therefore, we sought to uncover potential novel pathways that were overlooked in addition to supporting our initial hypothesis that S1PR2 regulates key genes in hepatic lipid metabolism. We performed an RNA sequencing experiment on hepatocytes that were overexpressed with S1PR2 to look at the transcriptome level of S1PR2 regulation. We then took this large data set of genes that are either upregulated or downregulated by S1PR2 and mapped it using KEGG pathway analysis.



Interestingly, various pathways including lipid metabolism, bile acid synthesis, sphingolipid metabolism and genes affecting NAFLD are regulated in part by S1PR2. In the PPAR signaling pathway, genes involving fatty acid transport is upregulated along with genes that are responsible for ketogenesis, lipogenesis, fatty acid transport, fatty acid oxidation, and gluconeogenesis. Moreover, a couple of genes namely Apo-AII and ApoCIII involved in lipid transport are downregulated. AMPK signaling pathway is also heavily influenced by S1PR2 overexpression. Several genes involved in gluconeogenesis (*G6pase*, *Pepck*) are upregulated along with genes involved in free fatty acid metabolism (*Cpt1*, *Acc*). Most notably is the dramatic upregulation of long-chain fatty acid oxidation genes such as *Cpt1*, which is consistent with the hepatic lipid accumulation we observed in S1PR2<sup>-/-</sup> and Sphk2<sup>-/-</sup> mice. These results suggest that S1PR2 is a regulator of various pathways that govern lipid metabolism in hepatocytes. It is also interesting to note that S1PR2 affects bile acid synthesis. Shown in the KEGG pathway is the upregulation of bile acid transporters (*Abcg5*, *Abcg8*, *Mdr3*) and downregulation of *Bsep* and *Mdr1*. There is a downregulation of cholesterol receptors enzymes such as *Ldlr* and *Hmgcr*. This would make physiologic sense since the upregulation of S1PR2 corresponds with an increase in cellular bile acids and these bile acids need to be transported between hepatocytes and the bile duct canaliculi. FXR, a master regulator of bile acid synthesis, is upregulated in S1PR2 overexpression. This is perhaps a form of cellular negative feedback loop system to decrease the synthesis of bile acids when bile acids are in a state of abundance. FXR interacts with SHP to downregulate *Cyp7a1*, the rate-limiting step to bile acid synthesis from cholesterol [262].

One of the challenges of modern genetic and molecular studies is the interpreting of large data sets. With the advent of DNA microarrays and RNA sequencing, it is possible to look at a snapshot of what is happening at the cellular level all at once. However, with large data sets includes a risk of high false discovery rates and redundancy of terms that could be categorized

together [263]. To address the latter, a web server called REVIGO aims to solve the issue of redundancy of biological terms and gene ontology (GO) [264]. By using a clustering algorithm that summarizes a long list of GO terms, it sorts them out by semantic similarity into similar biological processes. The final result is a multidimensional analysis of biological processes that are grouped based on similar semantics to solve the issue of over inflation of the number of perceived biologically relevant results. This tends to happen when analyzing a parent-child relationship for a biological process. For example, a positive result for biosynthetic processes will fully encompass a positive result for the lipid biosynthetic process. Using REVIGO revealed that S1PR2 governed mostly genes that are responsible for cellular metabolism and stress response. This is consistent with our previous data and KEGG pathway analysis.

We have shown that SphK2<sup>-/-</sup> mice on a two-week high-fat diet developed overt fatty liver compared to wild type. However, the role of alcohol on liver injury in SphK2<sup>-/-</sup> mice has not been studied and was the aim of this study. We have shown that SphK2<sup>-/-</sup> mice on an alcohol diet rapidly develop overt fatty liver compared to wild type. These results are very similar to the results we obtained with SphK2<sup>-/-</sup> mice on a two-week high-fat diet. The physiologic function of the liver is to act as a master regulator of nutrient metabolism. Both a high fat, high caloric intake, and alcohol consumption are associated with an energy surplus. These macronutrients are processed in the liver and converge on similar or overlapping pathways that lead to lipogenesis in the mitochondria [265, 266]. Bile acid and cholesterol metabolism have been implicated in fatty liver disease [267]. We suspect that some of the key bile acid synthetic genes are altered in hepatic steatosis compared to normal livers. In an agreement, our results show that SphK2<sup>-/-</sup> mice on an alcohol diet have changes in the expression of *Cyp7a1* and *Cyp7b1*. More notably is the repression of *Cyp7b1* in SphK2<sup>-/-</sup> mice on an alcohol diet. Recent studies demonstrated that chronic downregulation of *Cyp7b1* leads to the activation of the inflammasome which is a key step in the progression from hepatic steatosis to steatohepatitis

[268]. It is believed that the overexpression of the protein STARD1, a regulator of cholesterol transport in the mitochondria, is mediating the repression of *Cyp7b1*. Future experiments are needed to confirm this in *SphK2<sup>-/-</sup>* mice on an alcohol diet.

## Chapter 4: SphK2 Deficiency Promotes Alcohol-induced Liver Injury and Inflammation

### I. Rationale

Alcohol can injure hepatocytes and cause the release of danger-associated molecular patterns (DAMPs). DAMPs are released from dying cells and induce an inflammatory response [269]. In addition, hepatocytes produce pro-inflammatory mediators such as cytokines and chemokines to mediate inflammation. Hepatocytes are known to release IL-1 $\beta$ , IL-18, IL-6 and TNF- $\alpha$  [270]. Macrophages are also known to play a crucial role in mediating liver injury and inflammation. Resident macrophages known as Kupffer cells are sensitized to LPS after exposure to alcohol [271]. Moreover, circulating monocytes migrate to the liver after injury and mount an immune response that causes further injury and inflammation. Evidence suggests that the initial phase of injury that is mediated by macrophages is the polarization of macrophages towards favoring the M1 subtype [248].

In this study, we initially investigate the extent of liver injury by performing a liver function test to detect alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels. A rise in these enzymes is a hallmark of liver injury. After determining a relative increase in liver injury caused by alcohol in SphK2<sup>-/-</sup> mouse livers, we sought to determine the mechanism of injury. Interestingly, various stressors and stimuli can induce an inflammatory response. ER stress is known to be caused by the misfolding of protein when hepatocytes are exposed to alcohol [272]. ER stress has been linked to the inflammatory process and the activation of the inflammasome also mediates a pro-inflammatory response [273]. The goal of this study is to determine the potential causes of inflammation by analyzing the various inflammatory mediators and macrophage markers.

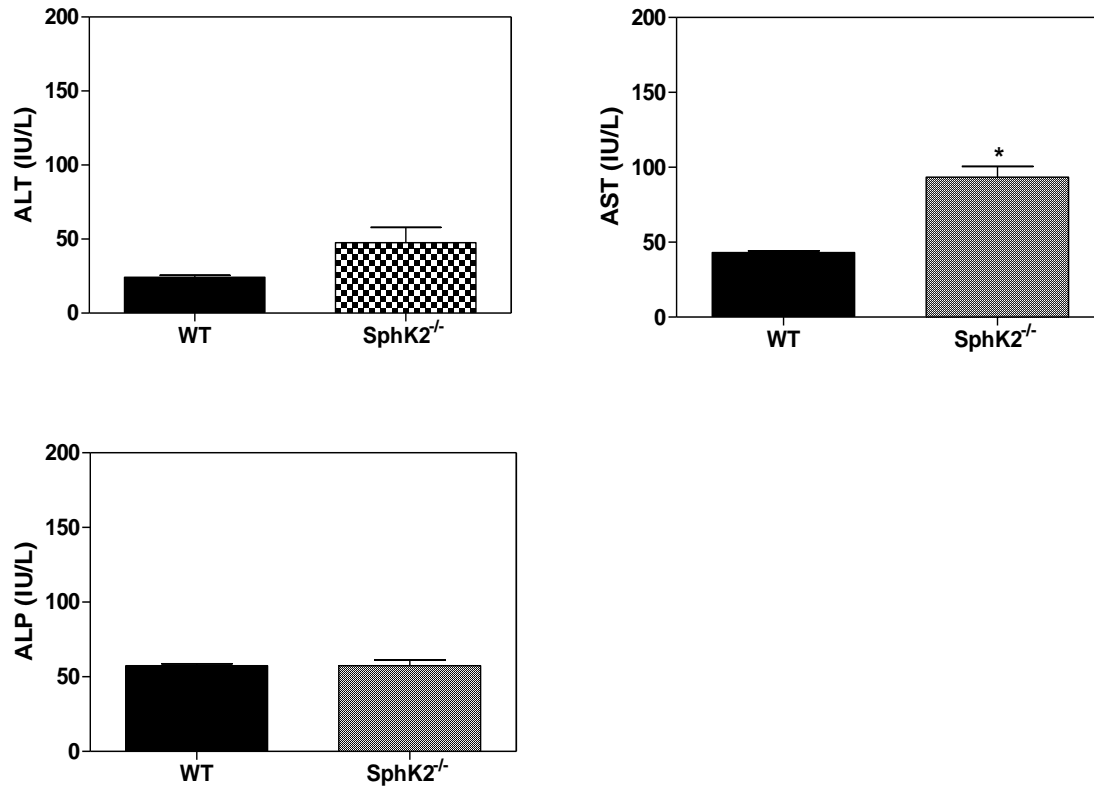
## II. Results

### **SphK2<sup>-/-</sup> potentiates alcohol-induced liver injury**

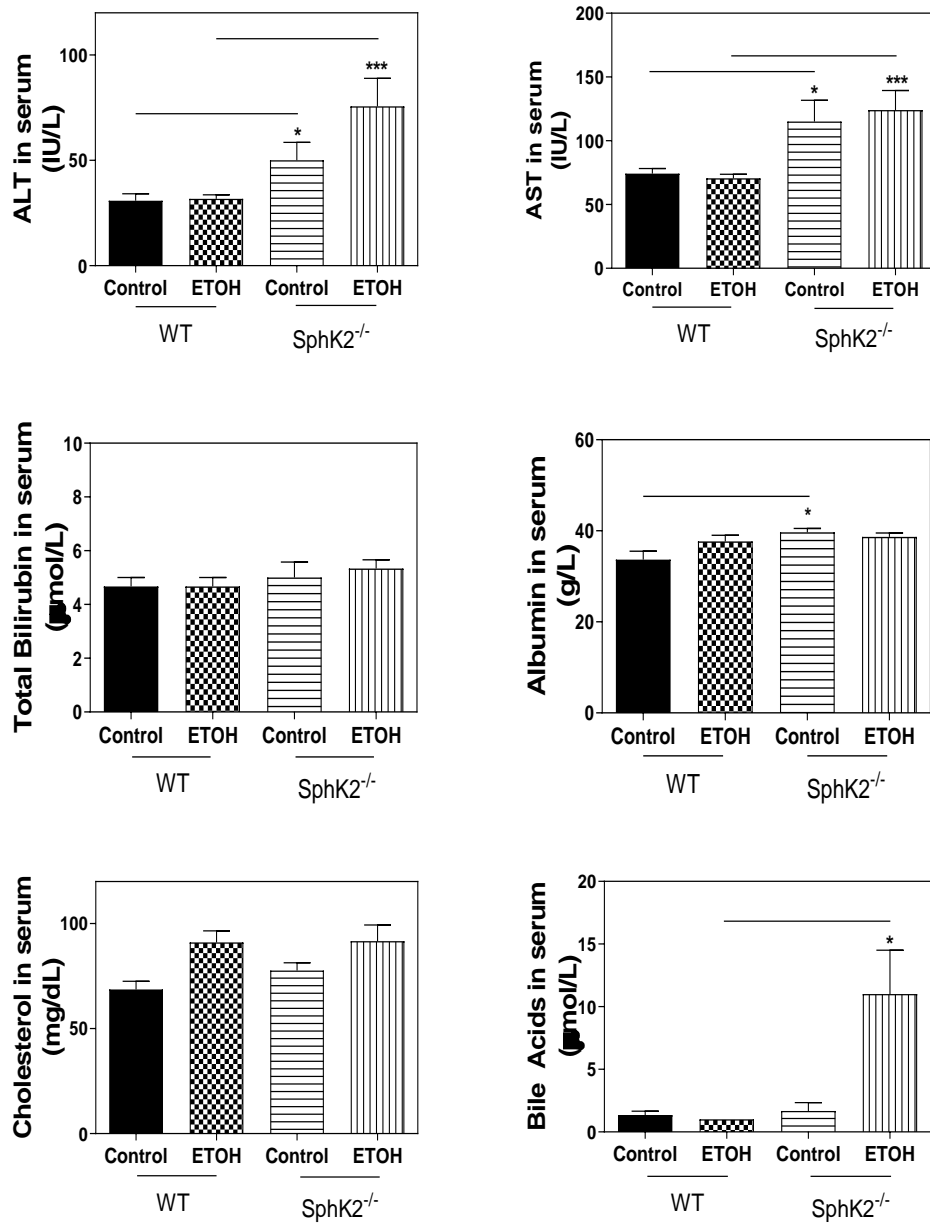
To determine the severity of liver injury in wild type and SphK2<sup>-/-</sup> mice on an alcohol diet, we first obtained blood serum from the inferior vena cava on the day of sacrifice from the NIAAA mouse model of alcohol feeding. Levels of ALT, AST and alkaline phosphatase (ALP) were measured. SphK2<sup>-/-</sup> mice on an alcohol diet had greater relative levels of ALT and AST compared to wild type suggesting increased liver injury (Figure 25). A more comprehensive liver function test was performed on mice from the 60-day chronic alcohol feeding. The wild type and SphK2<sup>-/-</sup> mice were on either a control or alcohol diet. We looked at relative levels of ALT, AST, serum total bile acids, albumin, cholesterol and total bilirubin. Results show that there is an increase in ALT, AST and serum total bile acids in SphK2<sup>-/-</sup> mice on an alcohol diet than wild type (Figure 26).

### **SphK2<sup>-/-</sup> promotes inflammation alcohol-induced liver injury**

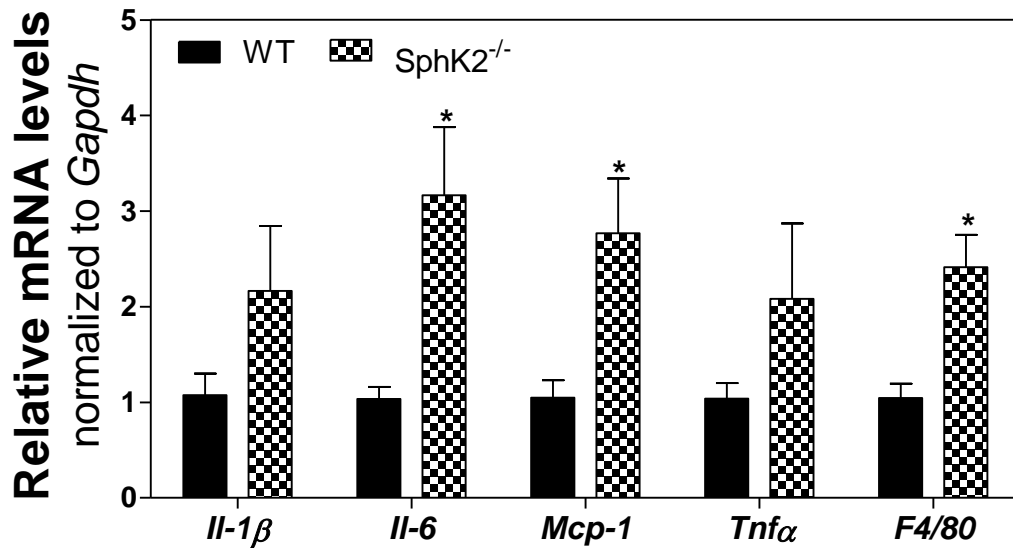
To investigate the inflammatory response in SphK2<sup>-/-</sup> mouse livers, we initially extracted total RNA from the whole liver from mice belonging to the NIAAA model. The relative mRNA expression levels of key genes that mediate inflammation were determined (*Il-1 $\beta$* , *Il-6*, *Mcp-1*, *Tnf- $\alpha$*  and *F4/80*) and *Gapdh* was used as an internal control (Figure 27). All pro-inflammatory mediators were upregulated in SphK2<sup>-/-</sup> mouse livers compared to wild type. In addition, *Cd11b*, a marker for macrophages, was upregulated in male SphK2<sup>-/-</sup> mouse livers compared to wild type (Figure 28). Next, we looked at the inflammatory cytokine profile of wild type and SphK2<sup>-/-</sup> mouse livers on a control and alcohol diet from the 60-day alcohol feeding. qRT-PCR results show that inflammatory cytokines (*F4/80*, *Tnf- $\alpha$* , *Il-1 $\beta$* ) were upregulated in SphK2<sup>-/-</sup> mice on



**Figure 25. ALT, AST and ALP serum levels for the NIAAA mouse model of alcohol feeding.** Serum from mice (male and female, 14 weeks old) was obtained on the day of sacrifice by taking whole blood from the inferior vena cava. Whole blood was allowed to coagulate for 2 hours and subsequently centrifuged to obtain serum. Results were obtained from the clinical pathology laboratory of VA Medical Center (Richmond, VA).

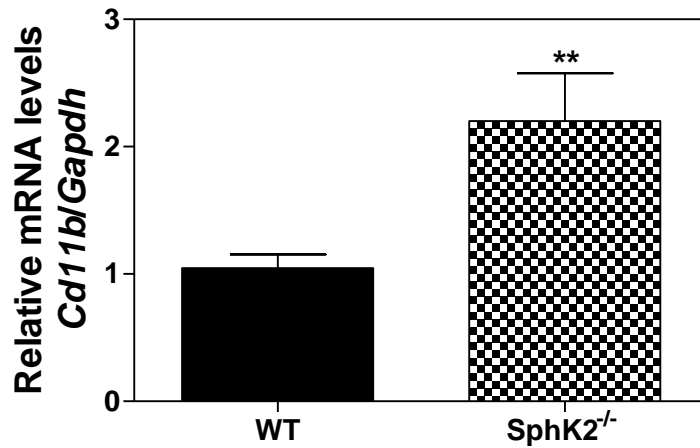


**Figure 26. Liver Function Test for the 60-day alcohol feeding.** Serum from mice (male and female, 20-week-old) was obtained on the day of sacrifice by taking whole blood from the inferior vena cava. Whole blood was allowed to coagulate for 2 hours and subsequently centrifuged to obtain serum. Results were obtained using VetScan Mammalian Liver Profile purchased from Abaxis. Each bar represents  $\pm$  S.E. (n = 3). \*p < 0.05; \*\*p < 0.01; \*\*\*p < 0.001.



**Figure 27. Effect of SphK2 on alcohol diet-induced hepatic inflammation in the NIAAA model.** Total RNA was isolated from wild type and SphK2<sup>-/-</sup> mouse livers (female, 14-week-old) on an alcohol diet. Quantitative RT-PCR was used to determine the relative mRNA expression levels of inflammatory cytokines (*Il-1β*, *Il-6*, *Mcp-1*, and *Tnf-α*) and macrophage recruitment marker *F4/80*. *Gapdh* was used for internal control. Each bar represents ± S.E. (n = 5). \*p < 0.05, statistical significance relative to WT.





**Figure 28. Effect of *SphK2*<sup>-/-</sup> on *Cd11b* expression.** Total RNA was isolated from wild type and *SphK2*<sup>-/-</sup> mouse livers (male, 14-week-old) on an alcohol diet. Quantitative RT-PCR was used to determine the relative mRNA expression levels of *Cd11b* normalized to *Gapdh* as an internal control. Each bar represents  $\pm$  S.E. (n = 5). \*\*p < 0.01, statistical significance relative to WT.

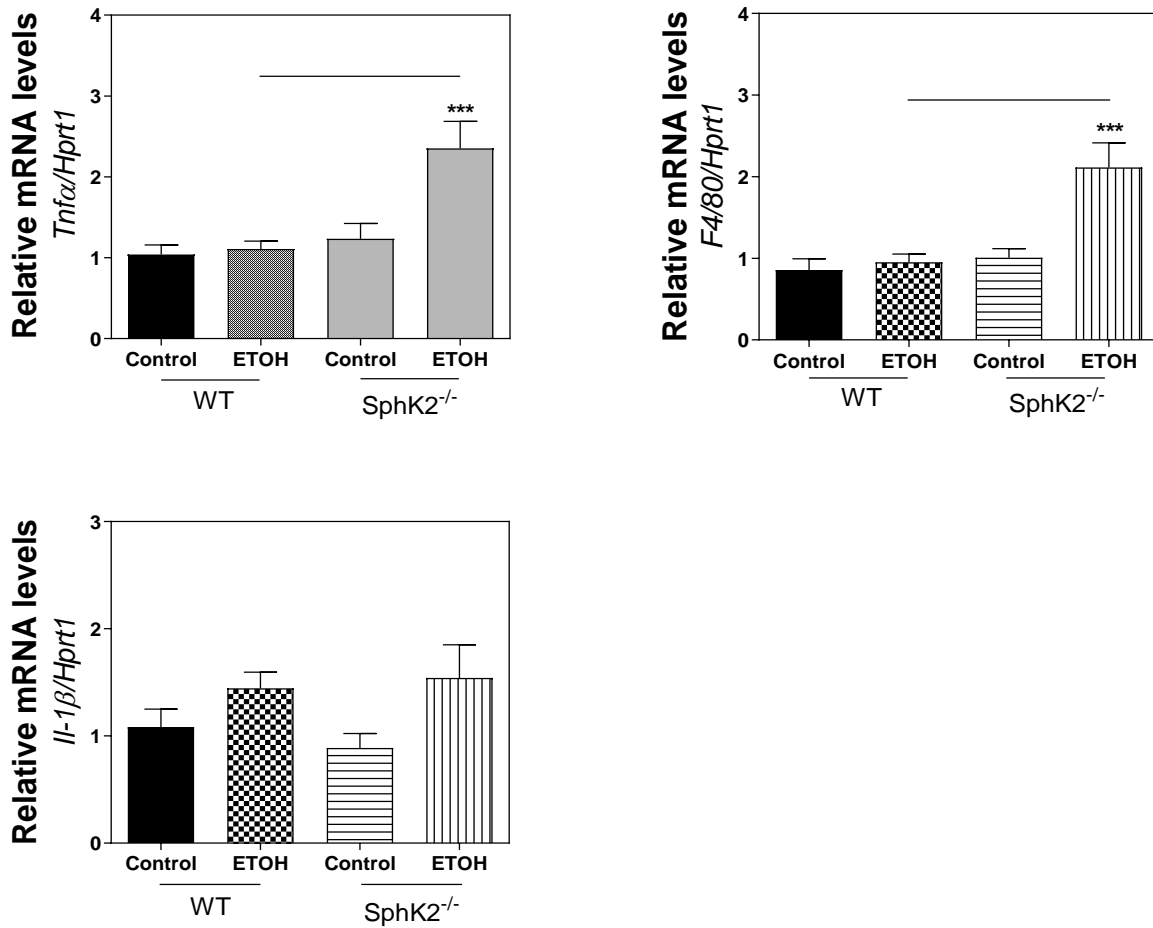
an alcohol diet (Figure 29). CD11B and F4/80 are macrophage recruitment markers indicative of an inflammatory process. To substantiate the results obtained from qRT-PCR, we used immunofluorescence to detect the presence of CD11B and F4/80 in the liver tissues of mice on the 60-day alcohol feeding. Results show that SphK2<sup>-/-</sup> mice on an alcohol diet had more CD11B (green) and F4/80 (red) compared to the other control groups. DAPI (blue) was used to stain nuclei and served as a control (Figure 30).

### **Effect of SphK2<sup>-/-</sup> on the expression of IL-22**

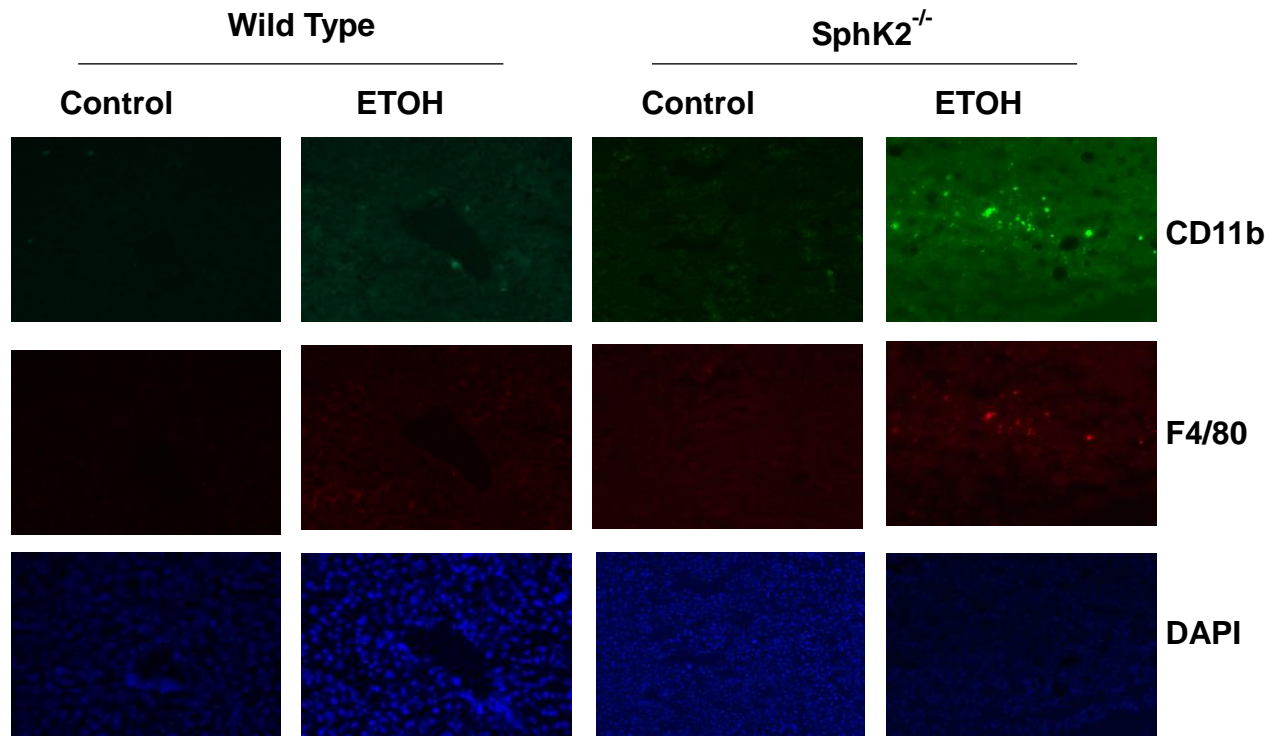
IL-22 has been shown to play an important role in tissue repair and ameliorating liver injury by binding to its receptors IL-22R1 and IL-10R2. To determine whether the increased severity of liver injury and inflammation observed in SphK2<sup>-/-</sup> mice on an alcohol diet may be due to a change in the expression of hepatoprotective cytokines and its receptors, we measured the relative mRNA levels of *Il-22*, *Il-22r1* and *Il-10r2* using qRT-PCR. SphK2<sup>-/-</sup> mice on an alcohol diet exhibited no change in *Il-22* expression when compared to control fed while wild type mice on an alcohol diet exhibited roughly a 2-fold increase in *Il-22* expression compared to control diet. We see a modestly similar trend in the expression levels of *Il-22r1* while no significant differences are observed in the expression of *Il-10r2* between the mouse groups (Figure 31).

### **Effect of alcohol on fibrogenic genes in the livers of SphK2<sup>-/-</sup> mice**

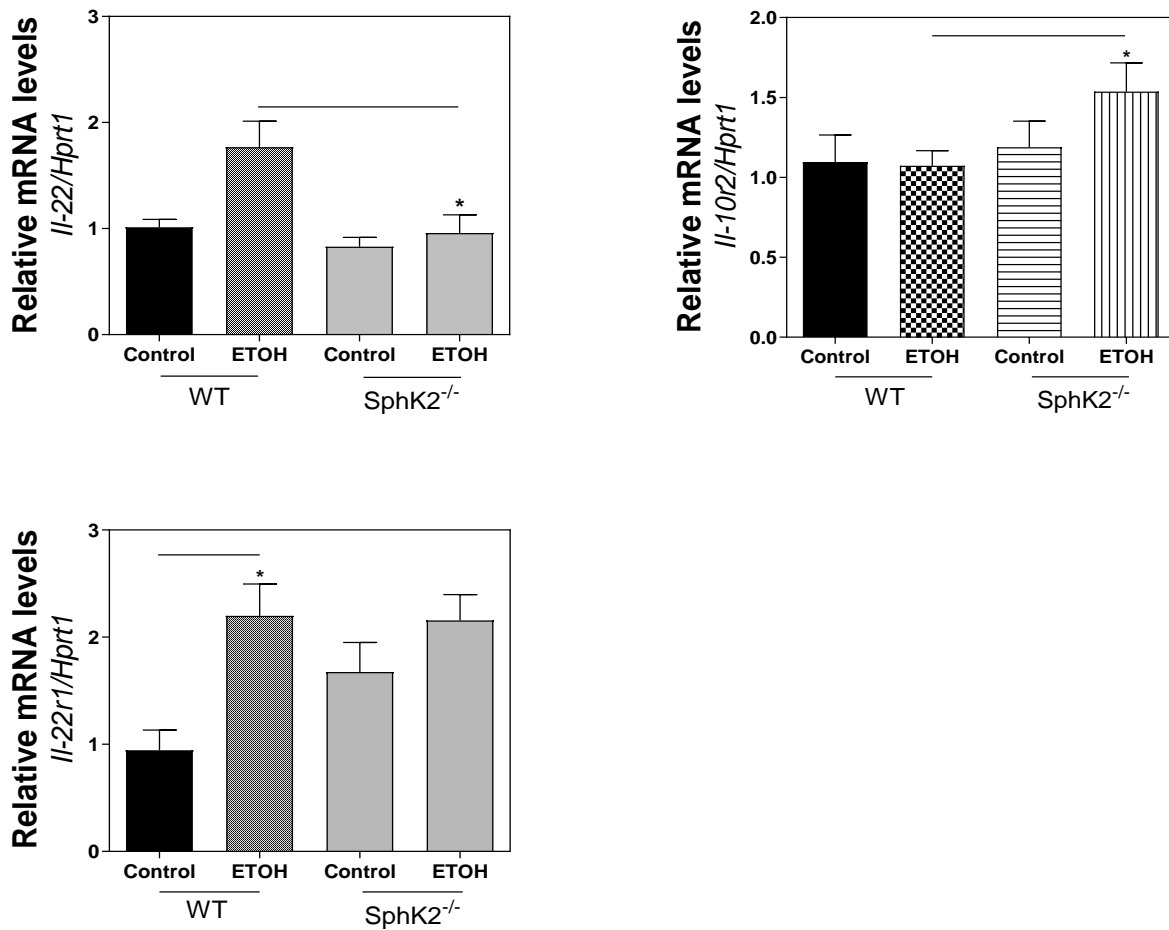
Although Masson's Trichrome staining did not show evidence of liver fibrosis, we wanted to determine whether there was evidence of an environment that would favor the beginning of fibrogenesis when mice were fed an alcohol diet. First, we examined the wild type and SphK2<sup>-/-</sup> mouse livers from the NIAAA model. We performed qRT-PCR to detect relative mRNA levels of



**Figure 29. Effect of SphK2 on alcohol diet-induced hepatic inflammation in the 60-day chronic alcohol model.** Total RNA was isolated from wild type and SphK2<sup>-/-</sup> mouse livers (male and female, 20-week-old) on an alcohol diet. Quantitative RT-PCR was used to determine the relative mRNA expression levels of inflammatory cytokines (*F4/80*, *Tnf-α*, *Il-1β*). *Hprt1* was used for internal control. Each bar represents  $\pm$  S.E. (n = 7). \*p < 0.05; \*\*p < 0.01; \*\*\*p < 0.001.



**Figure 30. Immunofluorescence of macrophage markers in SphK2<sup>-/-</sup> mouse livers.** Wild type and SphK2<sup>-/-</sup> mouse (male and female, 20-week-old) livers were frozen sectioned and stained with CD11B (green) and F4/80 (red). DAPI (blue) was used to stain nuclei. Representative images are shown.



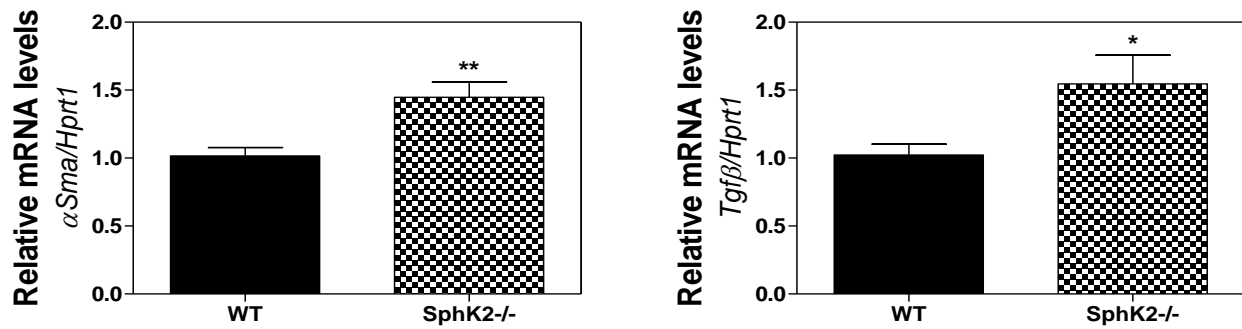
**Figure 31. Effect of *SphK2*<sup>-/-</sup> on the expression of hepatic IL-22 and its receptors.** Total RNA was isolated from wild type and *SphK2*<sup>-/-</sup> mouse livers (male and female, 20-week-old) on a 60-day alcohol diet. Quantitative RT-PCR was used to determine the relative mRNA expression levels of *Il-22* and its receptors (*Il-22r1*, *Il-10r2*). *Hprt1* was used for internal control. Each bar represents  $\pm$  S.E. (n = 7). \*p < 0.05.

genes that would be upregulated in liver fibrosis (*αSma* and *Tgfβ*) (Figure 32). Next, we performed qRT-PCR to detect relative mRNA levels of TGFβ, fibronectin, and H19 on the livers of wild type and SphK2<sup>-/-</sup> mice on the 60-day alcohol diet. Results show that SphK2<sup>-/-</sup> mice on an alcohol diet have higher levels of these fibrogenic genes compared to other treated groups (Figure 33).

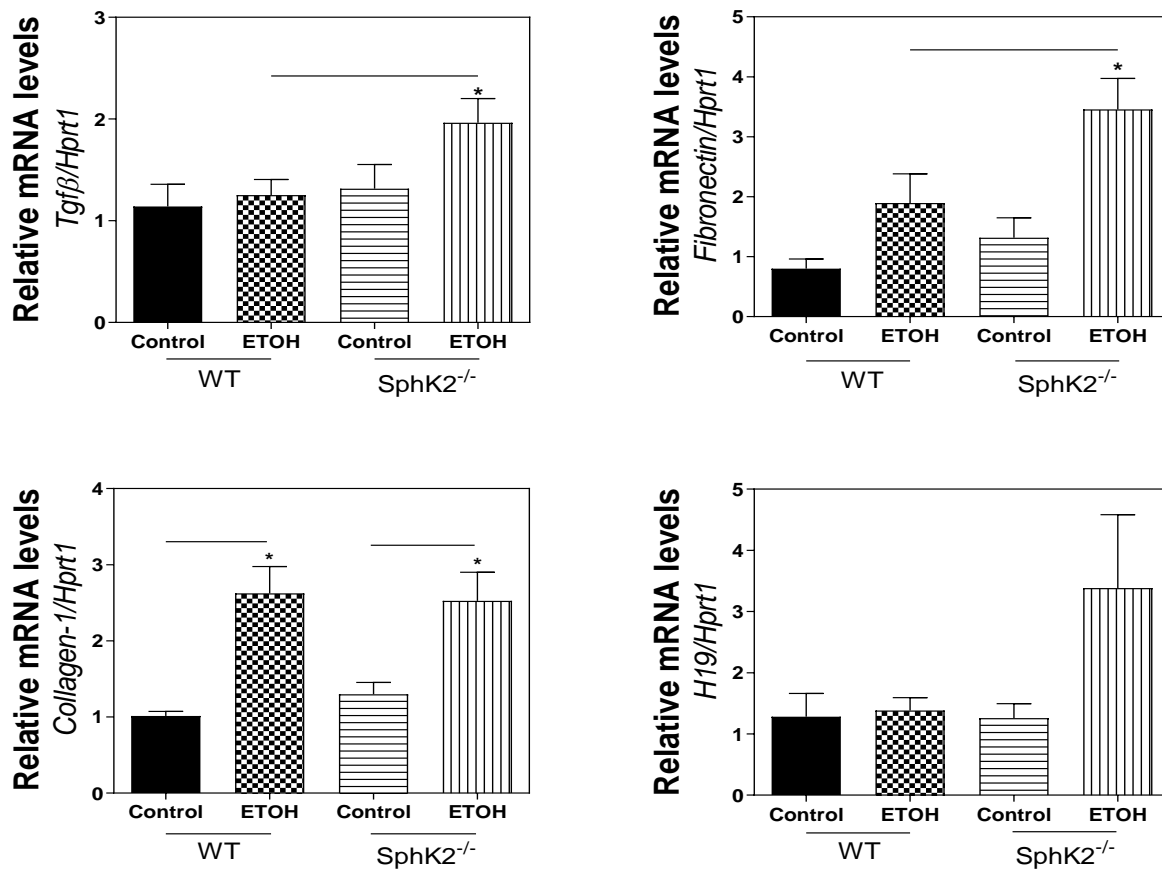
### **Effect of alcohol on the expression of SphK2 in cultured primary mouse hepatocytes and inflammatory mediators in RAW264.7 macrophage cells**

Previous studies have shown that the up-regulation of SphK2 can attenuate hepatic steatosis in the NAFLD mouse model. To investigate whether alcohol could induce the expression of SphK2, we isolated primary mouse hepatocytes and treated the cells with alcohol. Real-time PCR and Western blot analysis revealed that both mRNA and protein levels of SphK2 were up-regulated in hepatocytes treated with alcohol for 6 hours (Figure 34).

Immune cells play a crucial role in mediating inflammation in alcohol-induced liver injury. To investigate the effects of macrophages may have on the liver, we cultured RAW264.7 derived from mouse peritoneal macrophages. These cells were treated with vehicle (DMSO), alcohol (100 mM), LPS (25 ng/ml) or both alcohol (100 mM) and LPS (25 ng/ml) for 24 hours. Total RNA was isolated and quantitative RT-PCR was used to determine the relative mRNA expression levels of SphK2, *Tnf-α*, *Mcp-1*, and *Il-1β*. Results show that there is an increase in the expression of SphK2 mRNA when treated with alcohol or alcohol and LPS. *Tnf-α*, *Mcp-1* and *Il-1β* were also upregulated when treated with alcohol, LPS or both (Figure 35).

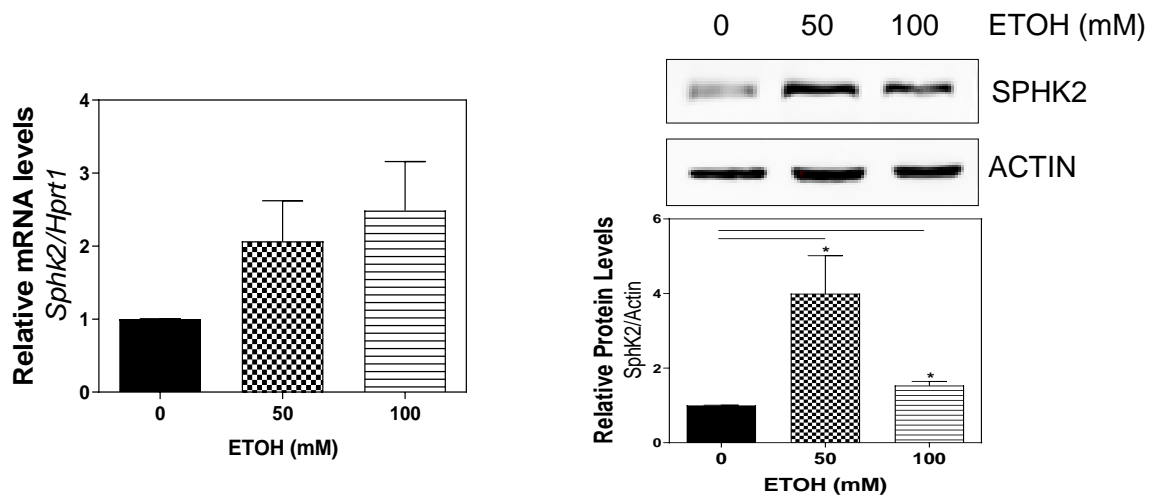


**Figure 32. Relative mRNA levels of fibrogenic genes in SphK2<sup>-/-</sup> mouse livers in the NIAAA model.** Total RNA was isolated from wild type and SphK2<sup>-/-</sup> mouse livers (male and female, 14-week-old) on an alcohol diet. Quantitative RT-PCR was used to determine the relative mRNA expression levels of inflammatory cytokines ( $\alpha Sma$  and  $Tgf\beta$ ). *Hprt1* was used for internal control. Each bar represents  $\pm$  S.E. (n = 9). \*p < 0.05 and \*\*p < 0.01, statistical significance relative to WT.

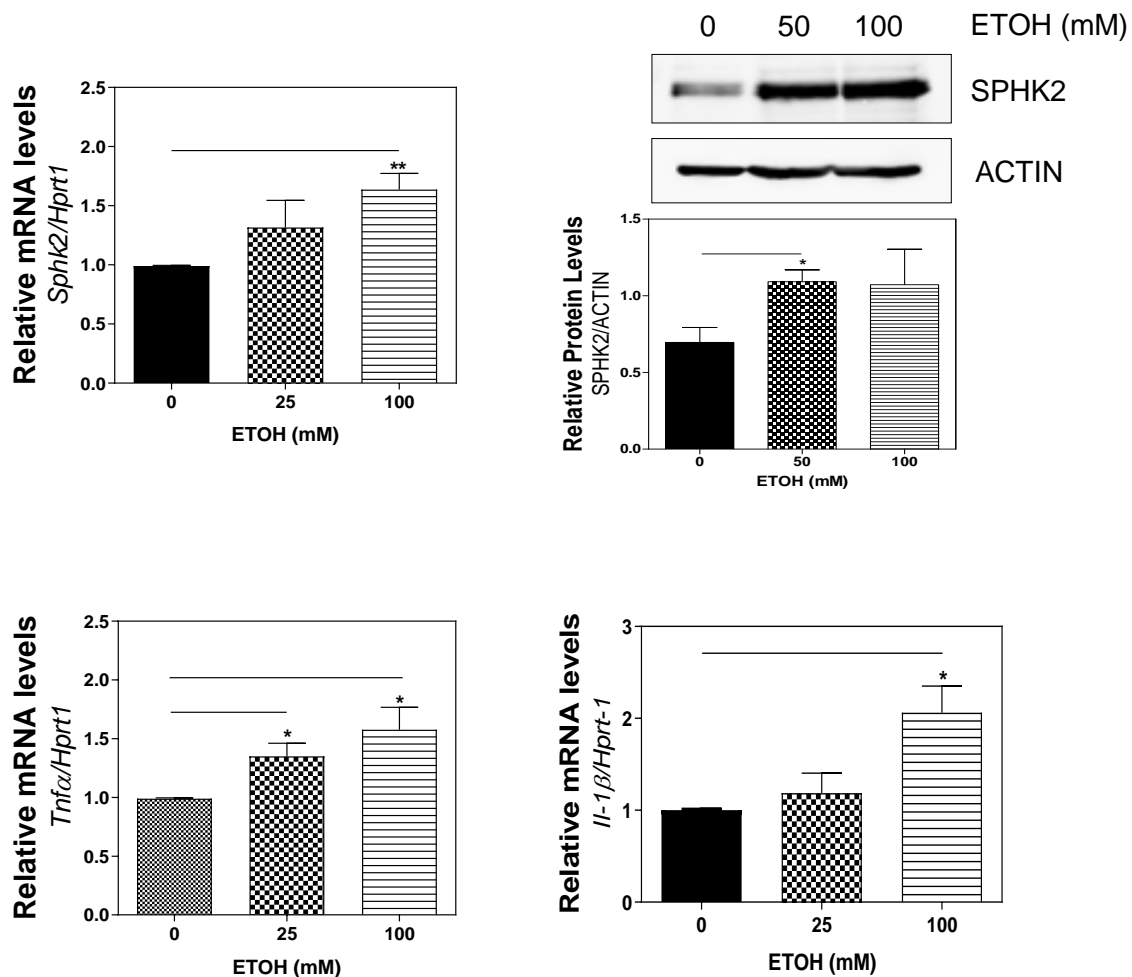


**Figure 33. Relative mRNA levels of fibrogenic genes in *SphK2*<sup>-/-</sup> mouse livers in the 60-day alcohol model.** Total RNA was isolated from wild type and *SphK2*<sup>-/-</sup> mouse livers (male and female, 20-week-old) on an alcohol diet. Quantitative RT-PCR was used to determine the relative mRNA expression levels of inflammatory cytokines (*Tgfβ*, fibronectin, H19). *Hprt1* was used for internal control. Each bar represents ± S.E. (n = 7). \*p < 0.05.





**Figure 34. Effect of alcohol on SphK2 expression in hepatocytes.** Mouse primary hepatocytes were treated with alcohol (0, 50, or 100mM) for 6 h. Total RNA was isolated, and the mRNA level of SphK2 was determined using quantitative RT-PCR and normalized to Hprt1 as an internal control. Total protein lysates were prepared, and the protein level of SPHK2 was determined by Western Blot analysis. Relative protein levels were determined by normalizing to loading control ACTIN. Results are represented as mean $\pm$ SE from each group (n = 3). Statistical significance relative to the no treatment group, \*P < 0.05.



**Figure 35. Effect of alcohol on the expression of key genes involved in inflammation in cultured RAW264.7 macrophage cells.** RAW 264.7 cells were treated with alcohol (0, 25 or 100mM) for 48 h. Total cellular RNA was isolated. Relative mRNA levels of SphK2 , Tnfα, Il-1β were determined using quantitative RT-PCR and normalized to Hprt1 as an internal control. RAW 264.7 cells were treated with alcohol (0, 50 or 100mM) for 6 h and total cellular protein was isolated. Relative protein levels of SPHK2 were determined and normalized to ACTIN. Results are represented as mean±SE from each group (n = 3). Statistical significance relative to the no treatment group, \*P < 0.05; \*\*P < 0.01.

### III. Discussion

Not only is inflammation a hallmark of liver injury, but studies have also shown that the inflammatory process plays a key role in the progression of ALD to advanced stages of ALD such as cirrhosis [248, 274, 275]. ALD patients that have liver inflammation such as AH are more likely to develop cirrhosis than simply hepatic steatosis without inflammation [276]. Alcohol damages hepatocytes and a rise in liver enzymes is indicative of liver injury. Liver injury leads to inflammation, and it is important to determine the levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) as these values are generally high in alcohol-induced liver injury [277]. Clinically, the values of liver enzymes generally predict different types of liver pathologies. For example, extremely high levels of ALT and AST are a sign of viral hepatitis or toxin-induced liver injury. A rise in alkaline phosphatase (ALP) is associated with the cholestatic injury. NAFLD and ALD are associated with modest increases in ALT and AST [278]. It is interesting to note that in human patients, a ratio of  $AST > ALT$  is associated with alcohol-induced liver injury. One should take caution when interpreting these values. While there is a positive correlation on the extent of the liver injury and elevated ALT levels, the absolute peak of ALT does not always correlate with the extent of hepatocyte damage [277]. Consistent with the idea that AST and ALT levels predict liver injury, we were able to show that  $SphK2^{-/-}$  mice on an alcohol diet exhibited higher levels of AST and ALT in blood serum compared to other groups.

Various inflammatory mediators are responsible for promoting liver inflammation. Circulating monocytes infiltrate the liver and polarize to pro-inflammatory macrophages that produce cytokines and chemokines (IL-1 $\beta$ , IL-6, TNF- $\alpha$ , MCP-1, F4/80, CD11B) [279]. As such, we saw an increase in these pro-inflammatory cytokines in the  $SphK2^{-/-}$  mouse livers on an alcohol diet compared to other groups. This would suggest that  $SphK2^{-/-}$  promote inflammation in the mouse livers when fed an alcohol diet. Although immune cells such as infiltrating

macrophages and resident liver macrophages like Kupffer cells produce the highest levels of these cytokines, hepatocytes are also capable of producing IL-6 and TNF- $\alpha$ . Hepatocytes can also activate the inflammasome to promote inflammation by secreting IL-1 $\beta$  and IL-18 [280, 281]. Moreover, MCP-1 has been shown to promote steatosis in the liver by increasing the accumulation of triglycerides through the liver X receptor [282]. MCP-1 is produced by immune cells and hepatocytes upon chronic alcohol exposure [283, 284]. Although we were able to demonstrate an increase in inflammation in the whole livers of SphK2<sup>-/-</sup> mice on an alcohol diet, it would be interesting to see which specific immune cells or cell types are producing which pro-inflammatory cytokine under alcohol-induced liver injury conditions. Furthermore, infiltrating macrophages could be polarized to either the M1 or M2 subtype [285]. M1 subtype favors a pro-inflammatory environment by secreting pro-inflammatory cytokines (IL-1, IL12, TNF- $\alpha$ , IFN  $\gamma$ ) and occurs mostly in the early phases of tissue injury. The M2 subtype is the resolution phase and favors tissue repair. Typical cytokines and chemokines that are produced by M2 macrophages are IL-10, MMPs, Arg1, VEG and TGF $\beta$  [286, 287]. Future experiments using acute versus chronic ALD models along with flow cytometry would be needed to address these questions.

Liver fibrosis marks the beginning of irreversible liver injury that could progress to liver failure as fibrotic tissue begins to take over healthy liver tissue and compromise liver function [288]. One of the challenges of studying liver fibrosis is that there are no effective mouse models that can reliably produce liver fibrosis. Part of the difficulty in recapitulating advanced ALD and liver fibrosis is even in human ALD patients, the majority of chronic alcohol users do not progress to advanced stages of ALD. Moreover, those patients who do develop advanced stages of ALD such as liver fibrosis and cirrhosis often takes almost two decades on average to develop [289]. It is difficult to develop a mouse model that can consistently produce fibrosis within a reasonable experimental time frame under these circumstances. Within this context, we

did not see any liver fibrosis in our SphK2<sup>-/-</sup> mice on an alcohol diet model when we performed a histological analysis of the livers using trichrome staining. However, using qRT-PCR we did see some genes that are responsible for promoting fibrogenesis (*αSma*, *Tgfβ*, fibronectin) upregulated in the SphK2<sup>-/-</sup> mice on an alcohol diet group (Figure 32 and Figure 33). In addition, H19 was upregulated and our laboratory previously published data supporting that noncoding RNA H19 correlates with liver fibrosis [290]. One possibility is that the early stages of fibrosis require the initiation of these fibrogenic genes and that extensive collagen and extracellular matrix deposition is not apparent yet in these SphK2<sup>-/-</sup> mice on a 60-day alcohol diet. Perhaps an even longer alcohol treatment option may yield higher degrees of fibrosis. However, the overall health of these mice may deteriorate, and we may see an increase in mortality under these prolonged alcohol feeding protocols.

## Chapter 5: The Gut-Liver Axis and the Role of SphK2 in Alcoholic Liver Disease

### I. Rationale

Studies have shown that alcohol disrupts the intestinal barrier and results in the leaky gut where bacterial endotoxins such as LPS reach the liver *via* the hepatic portal system and promote liver inflammation and injury [239]. The liver receives about 70% of blood from the intestines and encounters many antigens derived from bacteria from the gut [240]. In the liver, Kupffer cells are sensitized to LPS and promote hepatic inflammation [291]. This close communication between the gut and the liver in disease pathogenesis is termed the gut-liver axis.

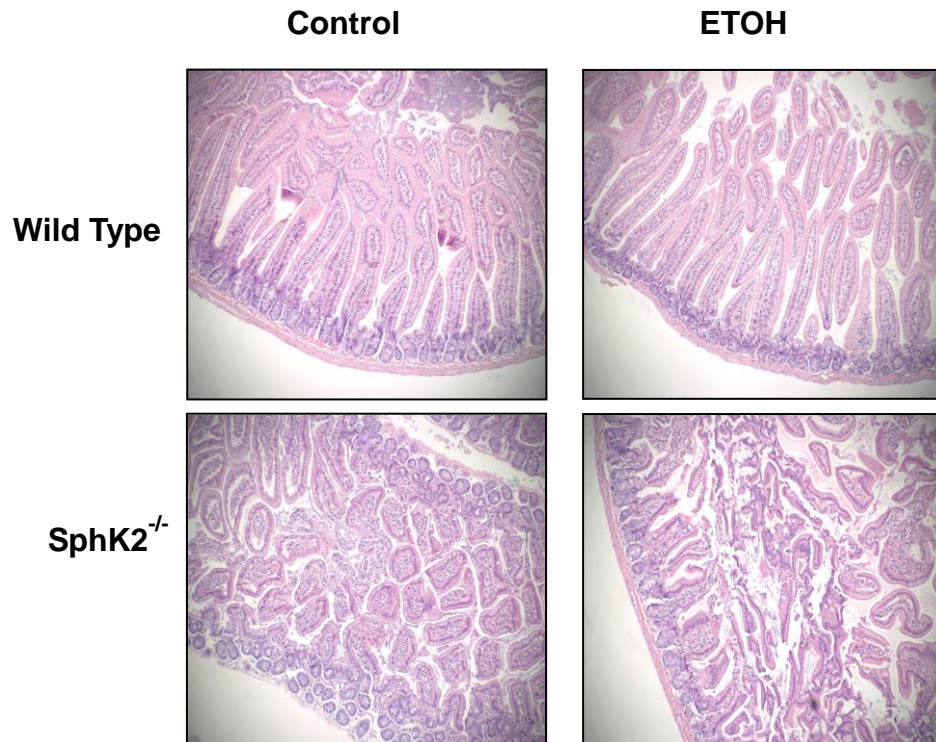
S1P has a functional role in stimulating intestinal epithelial stem cell growth [244]. Dysregulation in S1P signaling has been implicated in various gastrointestinal disorders including colorectal cancer [292]. In this current study, we hypothesize that SphK2 plays a role in stimulating intestinal epithelial stem cell growth and that disruption of SphK2 could potentiate alcohol-induced liver injury.

## II. Results

### **SphK2<sup>-/-</sup> mice on an alcohol diet have intestinal barrier dysfunction**

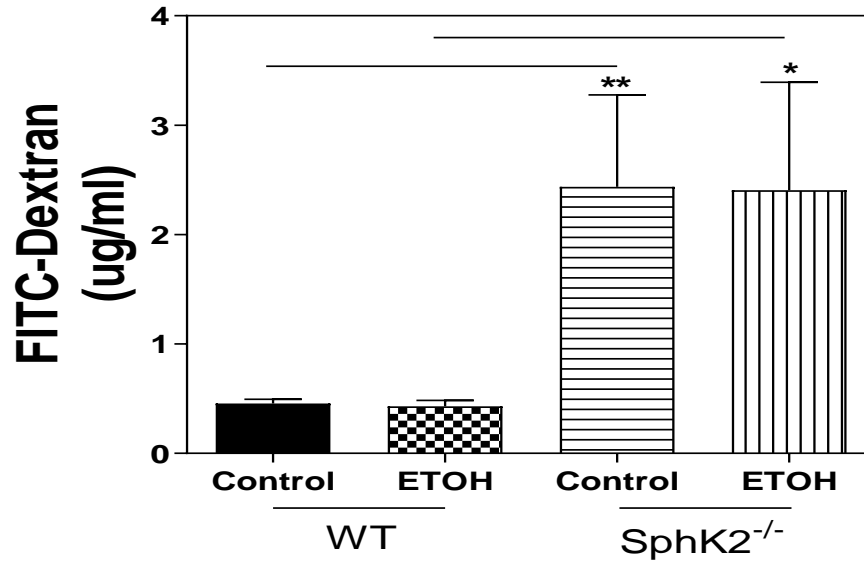
To investigate the effects of alcohol on SphK2<sup>-/-</sup> mouse intestines, wild type and SphK2<sup>-/-</sup> mice on an alcohol diet were sacrificed and the small intestines were processed for histology. H&E staining of the small intestines of wild type and SphK2<sup>-/-</sup> mice on a control or alcohol diet shows that there is significantly more intestinal barrier disruption in SphK2<sup>-/-</sup> mouse intestines on an alcohol diet than the other groups (Figure 36). SphK2<sup>-/-</sup> mice on a control diet have less severe intestinal disruption compared to its alcohol counterpart followed by wild type mice on an alcohol diet. Wild type mice on a control diet have normal small intestinal integrity.

Next, we wanted to confirm that there is indeed a leaky gut phenomenon in SphK2<sup>-/-</sup> mouse intestines. Prior to animal sacrifice, mice were orally given FITC-dextran 3 hours before obtaining blood serum. At the time of the liver and small intestine tissue harvest, we obtained blood serum from the inferior vena cava. Normally, dextran does not cross the intestinal wall but if the intestinal barrier is compromised FITC labeled dextran would be detected in the blood. Figure 37 shows that there is about a 4-fold increase in the amount of dextran present in the serum of SphK2<sup>-/-</sup> both in control- and alcohol-fed mice compared to wild type. However, there is no difference in dextran levels in the serum between the control and alcohol-fed SphK2<sup>-/-</sup> mouse groups. To substantiate the notion that SphK2 plays a role in promoting intestinal stem cell growth, we isolated intestinal crypts from wild type and SphK2<sup>-/-</sup> mice. The intestinal crypts were then grown on a Matrigel over 10 days. Images of the intestinal organoids were taken on days 2, 4 and 10. Results show that there is an attenuation of intestinal organoid growth in SphK2<sup>-/-</sup> compared to wild type (Figure 38). We hypothesized that there could be downregulation of tight gap junction genes in SphK2<sup>-/-</sup> mice. Compared to wild type, we see no difference in the

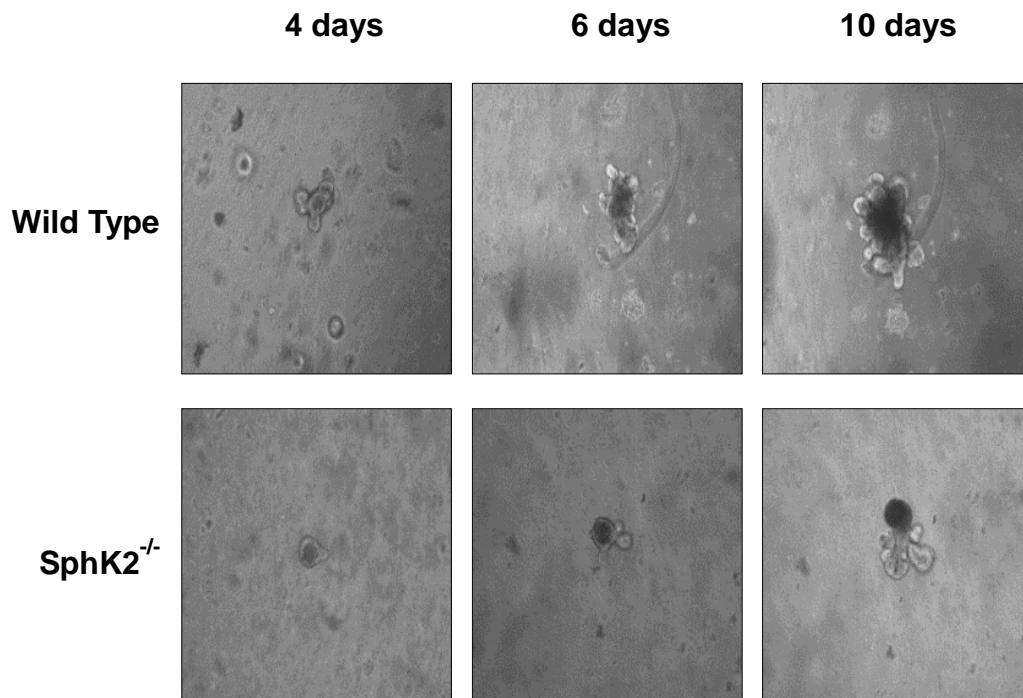


**Figure 36. Histological analysis of SphK2<sup>-/-</sup> mouse small intestines on a 60-day alcohol diet.** H&E staining of the proximal segment of wild type and SphK2<sup>-/-</sup> mouse (male and female, 20-week-old) small intestines on a 60-day control or alcohol diet. The images were taken on an Olympus microscope at 20x magnification. Representative images of each treatment are shown.





**Figure 37. FITC-Dextran Permeability Assay.** 60mg/100g of FITC-Dextran was administered to mice (male and female, 20-week-old) on a 60-day control or alcohol diet by oral gavage and blood samples were taken after 3 hours. The serum concentration of FITC-dextran was measured using Victor Multilabel Plate Counter (PerkinElmer, Waltham, MA) with an excitation wavelength of 490 nm and an emission wavelength of 530 nm. Each bar represents  $\pm$  S.E. (n = 6). \*p < 0.05; \*\*p < 0.01.



**Figure 38. Effect of SphK2<sup>-/-</sup> on intestinal organoid growth.** Intestinal crypts from WT and SphK2<sup>-/-</sup> mice were isolated and cultured in Matrigel with the supplement of EGF, Noggin and R-spondin-1 for 10 days. Representative images of intestinal organoids from different time points are shown.

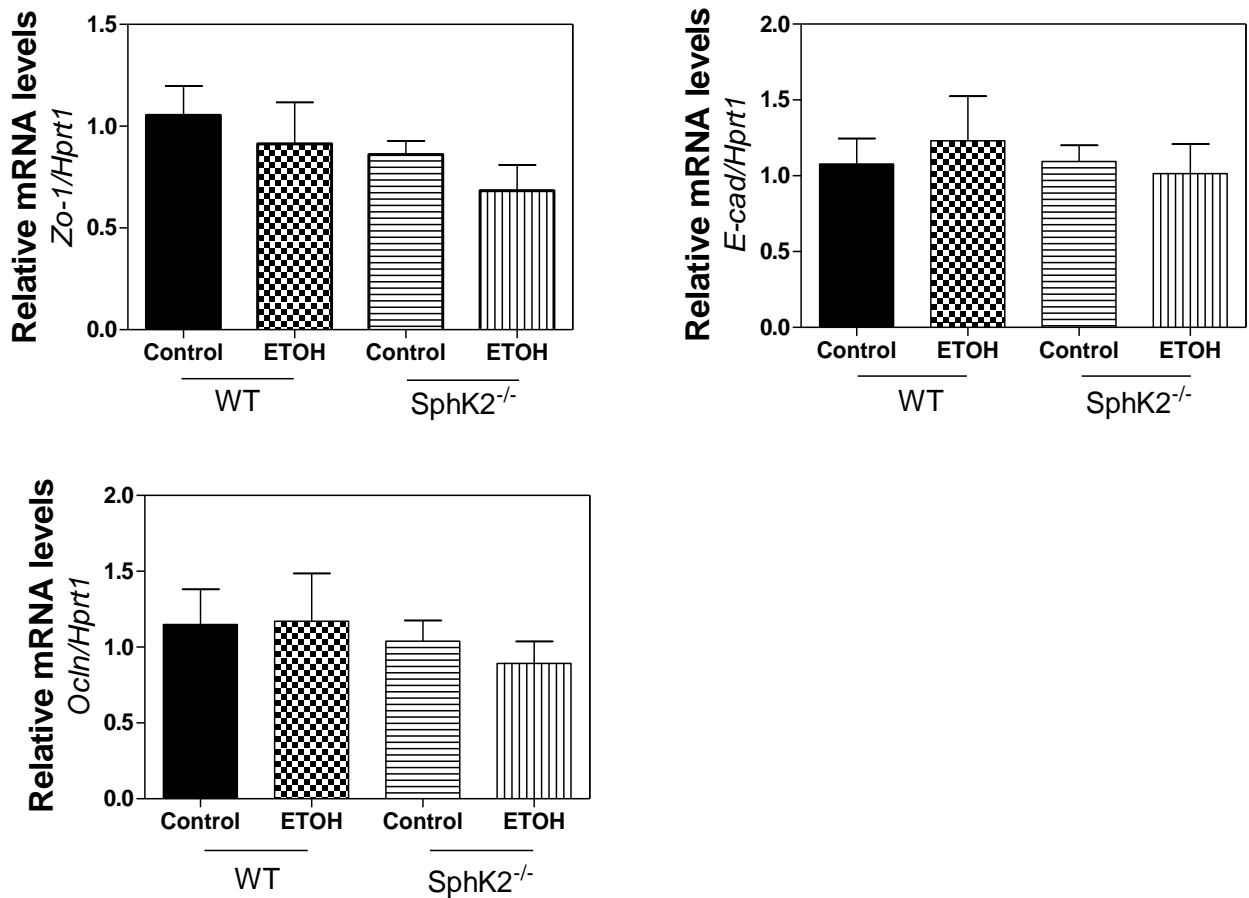
mRNA levels of several tight gap junction genes in SphK2<sup>-/-</sup> mice on the control or alcohol diet (Figure 39).

### **SphK2 protein expression levels are downregulated in the livers of human alcoholic liver disease and HCC**

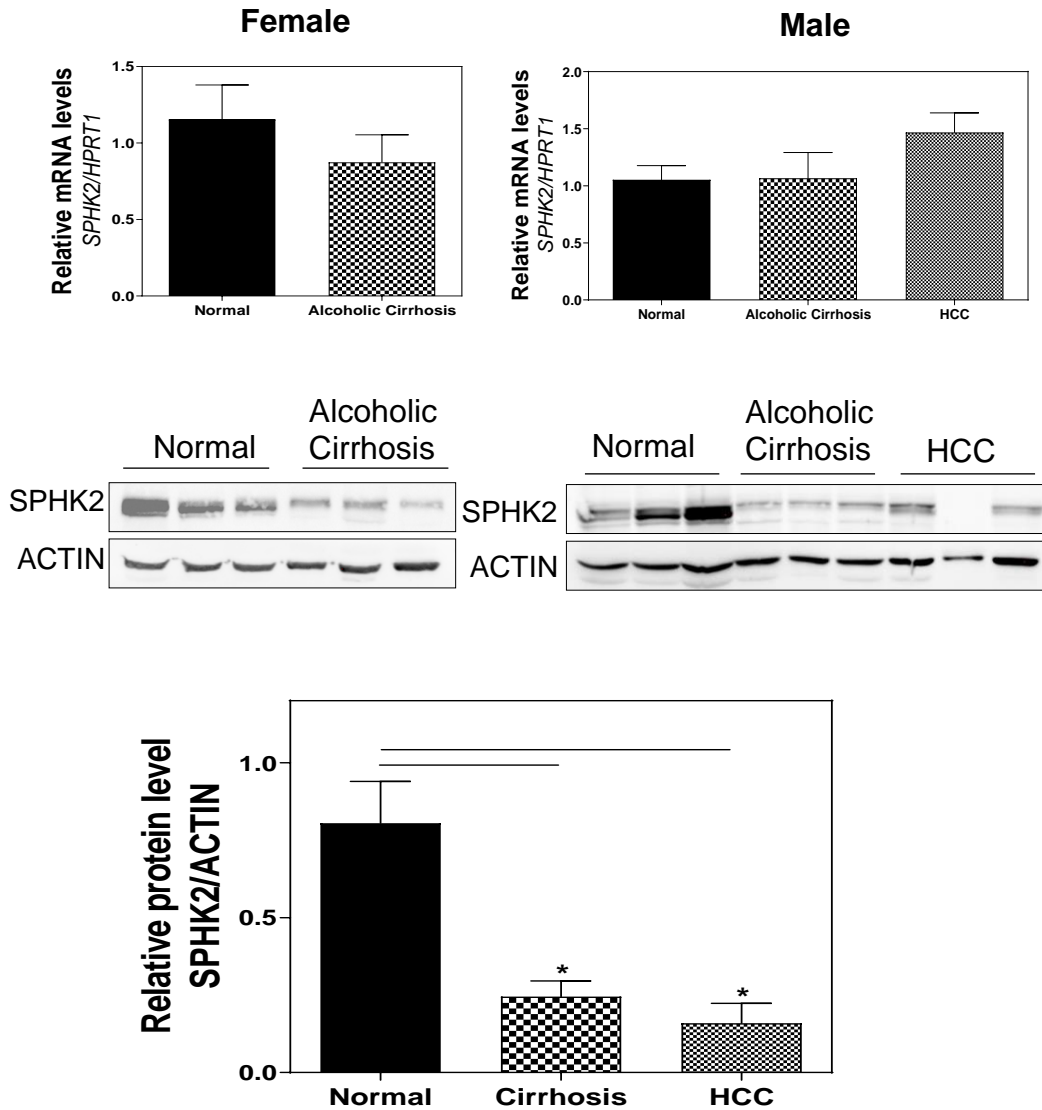
We wanted to determine whether our *in vitro* and *in vivo* results were translatable to human patients with ALD. Our central hypothesis is that SphK2 attenuates alcohol-induced liver injury and we reasoned that human patients with advanced stages of ALD have lower expression levels of SphK2. We took male and female patient livers that were diagnosed with alcoholic cirrhosis or hepatocellular carcinoma (HCC). The mRNA levels of SphK2 in alcoholic cirrhosis and HCC did not significantly change compared to normal controls. However, SphK2 protein expression levels were significantly downregulated in patients with alcoholic cirrhosis or HCC (Figure 40). Moreover, inflammatory mediators (IL-1 $\beta$ , IL-6, MCP-1, TNF- $\alpha$ , IL-22, IL-22R1) and bile acid synthetic and transporter genes (CYP7B1, NTCP, ABCG5, AGCG8) were up-regulated in alcoholic cirrhosis and HCC (Figure 41 and 42).

### **III. Discussion**

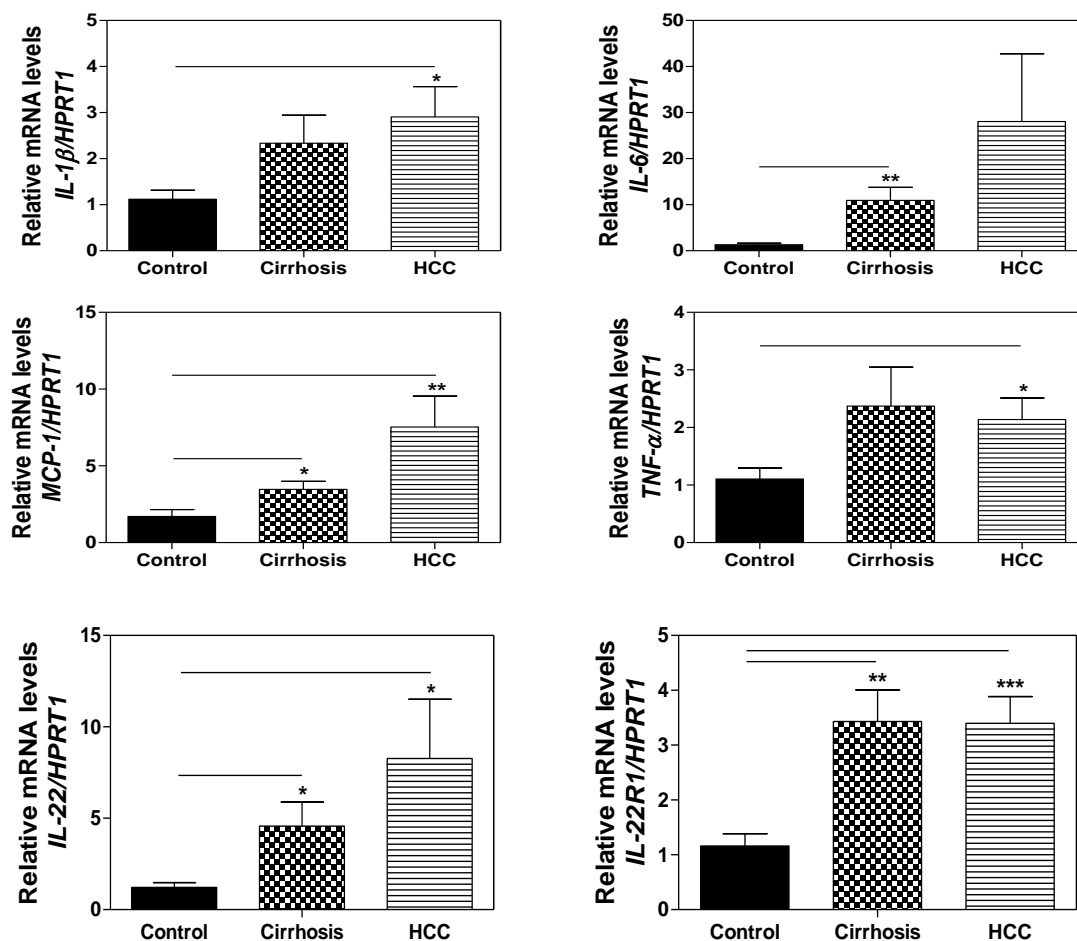
Consumption of alcohol causes damage to the mucosal lining of the intestinal wall and erodes the lamina propria through the loss of epithelial cells [293]. A unique function of the intestinal wall is to shield the liver from harmful substances that may trigger the immune system [294]. Disruption of this intestinal barrier exposes the liver to endogenous and exogenous antigens through the portal circulation that could initiate or perpetuate inflammation. Our results show that SphK2<sup>-/-</sup> disrupts intestinal integrity and is exacerbated on an alcohol diet. In addition, SphK2<sup>-/-</sup> attenuates the growth of intestinal organoids suggesting that SphK2 plays an important



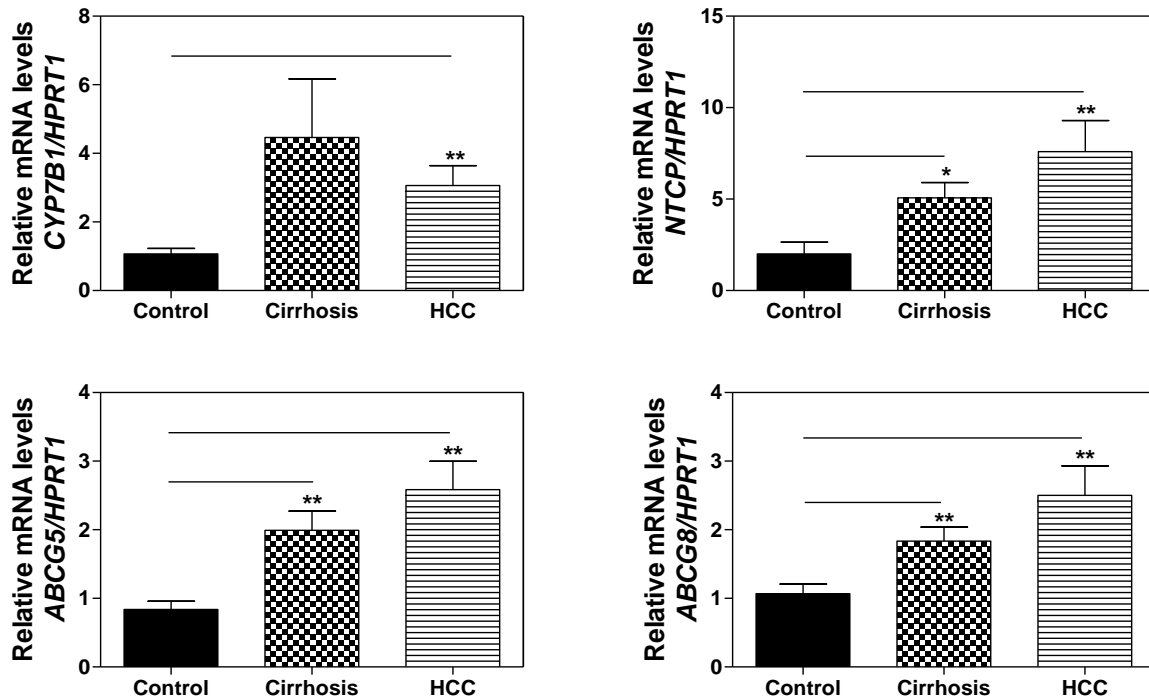
**Figure 39. Effect of SphK2<sup>-/-</sup> on gap junction genes.** Total RNA was isolated from wild type and SphK2<sup>-/-</sup> mouse livers (male and female, 20-week-old) on a 60-day alcohol diet. Quantitative RT-PCR was used to determine the relative mRNA expression levels of tight gap junction genes (*Zo-1*, *Occludin*, *E-cadherin*). *Hprt1* was used as an internal control. Each bar represents ± S.E. (n = 7).



**Figure 40. SphK2 expression in human alcoholic patients.** Protein lysates were isolated from whole livers of patients that were diagnosed with alcoholic cirrhosis or hepatocellular carcinoma. Normal livers were obtained from donors that did not have liver disease. Western blotting analysis was performed to determine the relative expression levels of SphK2 protein. Frozen liver tissues were obtained from gender- and age-matched ALD patients and normal controls from NIH-funded Liver Tissue Cell Distribution system, Dept. of Pediatrics – GI Division, University of Minnesota.



**Figure 41. Inflammatory markers in human alcoholic patients.** Total RNA was isolated from whole livers of patients that were diagnosed with alcoholic cirrhosis or hepatocellular carcinoma. Normal livers were obtained from donors that did not have liver disease. Quantitative RT-PCR was performed to determine the relative gene expression of inflammatory markers (IL-1 $\beta$ , IL-6, MCP-1, TNF- $\alpha$ , IL-22, IL-22R1). Frozen liver tissues were obtained from gender- and age-matched ALD patients and normal controls from NIH-funded Liver Tissue Cell Distribution system, Dept. of Pediatrics – GI Division, University of Minnesota.



**Figure 42. Bile acid synthetic and transporter genes in human alcoholic patients.** Total RNA was isolated from whole livers of patients that were diagnosed with alcoholic cirrhosis or hepatocellular carcinoma. Normal livers were obtained from donors that did not have liver disease. Quantitative RT-PCR was performed to determine the relative gene expression of bile acid synthetic and transporter genes (CYP7B1, NTCP, ABCG5, AGCG8). Frozen liver tissues were obtained from gender- and age-matched ALD patients and normal controls from NIH-funded Liver Tissue Cell Distribution system, Dept. of Pediatrics – GI Division, University of Minnesota.

role in the maintenance of intestinal stem cells. Previous studies demonstrated the importance of S1P in promoting intestinal growth [212]. Evidence suggests that S1P protects intestinal cells from apoptosis through the AKT signaling pathway [245]. Our studies show that SphK2<sup>-/-</sup> mice have increased gut permeability as shown by an increase in FITC-dextran in the serum. These results strongly support the hypothesis that SphK2 maintains intestinal integrity and that a deficiency in SphK2 could potentiate ALD. Tight junction proteins are junctional complexes that play an important role in preventing the leakage of solutes and cellular contents into extracellular space. They also hold cells together and form a protective barrier [295]. These tight junctions and cell adhesion proteins are disrupted by alcohol and acetaldehyde [296]. Moreover, these junction proteins such as occludin, zona occludens, E-cadherin and  $\beta$ -catenin could be redistributed by acetaldehyde [296]. Initially, we predicted that the mRNA expression of these genes could potentially be downregulated in SphK2<sup>-/-</sup> mice on an alcohol diet. However, we did not see any significant changes in mRNA expression levels. One explanation is that these proteins are not regulated at the mRNA transcript level but at the protein translational level. These proteins could alternatively be mislocated or redistributed rather than exhibiting expression level changes. A previous study demonstrated that CHOP deficiency reduced bile duct ligation-induced disruption of tight junctions. This study reported no changes in mRNA and protein levels of tight junctions but did observe a mislocation of tight junction proteins such as occludin [297].



## Chapter 6: Summary and Future Direction

ALD is one of the most common liver diseases worldwide [298]. However, there are currently no effective treatment options for ALD and liver transplantation remains as the only curative option for end-stage liver disease [299]. Despite significant advances in our understanding of alcohol-induced liver injury, the exact mechanisms of the disease remain largely unknown. Studies have shown that ALD is caused by a combination of oxidative stress, ER stress and inflammation [272]. In fact, the inflammatory process has been shown to be a key factor in driving the progression of ALD [300].

It was not until the past several decades have bile acids been discovered to be potent signaling molecules that activate various cellular responses [301]. Studies from our laboratory have shown that conjugated bile acids activate S1PR2-mediated activation of SphK2 to regulate hepatic lipid metabolism [183]. We were able to show that SphK2<sup>-/-</sup> mice on a two-week high fat diet develop overt fatty liver compared to wild type mice [183]. The goal of this study was to characterize the phenotype of SphK2<sup>-/-</sup> mice on an alcohol diet and to determine the underlying mechanisms by which SphK2<sup>-/-</sup> results in alcohol-induced liver injury.

We have adopted two alcohol drinking mouse models for our study. The chronic-binge model developed by Dr. Bin Gao at the NIAAA and the 60-day chronic alcohol feeding model [86]. Both of these models produced extensive steatosis and inflammation in SphK2<sup>-/-</sup> mice. Furthermore, we saw changes in the expression levels of hepatic lipid and bile acid metabolism genes along with an increase in pro-inflammatory mediators. These results suggest that SphK2 is important for maintaining liver homeostasis and protects against alcohol-induced liver injury. We hypothesize that the dysregulation of hepatic lipid metabolism leads to hepatic steatosis which in turn promotes the initiation of an inflammatory response that causes liver injury. The

chronic and prolonged inflammatory process will lead to liver fibrosis [247]. In addition, we also propose that SphK2<sup>-/-</sup> promotes leaky-gut syndrome by attenuating intestinal epithelial stem cell growth and is exacerbated by alcohol. We were able to demonstrate that SphK2<sup>-/-</sup> mouse intestines did not have normal histological architecture and that there was an increase in intestinal permeability in SphK2<sup>-/-</sup> mice on an alcohol diet. Figure 40 illustrates our proposed model in how SphK2 protects the liver from alcohol-induced injury. Finally, we were able to show that our *in vivo* experimental data has direct translational application by showing human patient livers diagnosed with alcoholic cirrhosis or hepatocellular carcinoma have lower expression levels of SphK2 compared to normal liver. This would support the idea that SphK2 acts as a hepatoprotective enzyme that generates nuclear S1P levels in response to cellular challenges such as alcohol.

Here we have demonstrated an important role of SphK2 in alcohol-induced liver injury in mice, but the exact mechanisms have not been fully elucidated. Further studies are warranted to determine which cellular pathways are directly impacted and which specific immune cells are contributing to the inflammatory process. ALD is a spectrum of disease encompassing hepatic steatosis, AH and liver fibrosis. It would not be difficult to imagine that changes in different pathways and genes may be the driving force for each of these different diseases on the spectrum. While macrophages may be largely responsible for the inflammatory process, research shows that hepatic stellate cells play a more significant role in promoting liver fibrosis [71].

The field of bile acids and the human gut microbiome is an exciting scientific discipline that has taken the interest of many scientists in the past decade. With the advent of large scale rRNA sequencing, it is possible to determine which bacterial genera or species are predominant in various diseases [302]. In the context of liver disease, we now understand that the liver

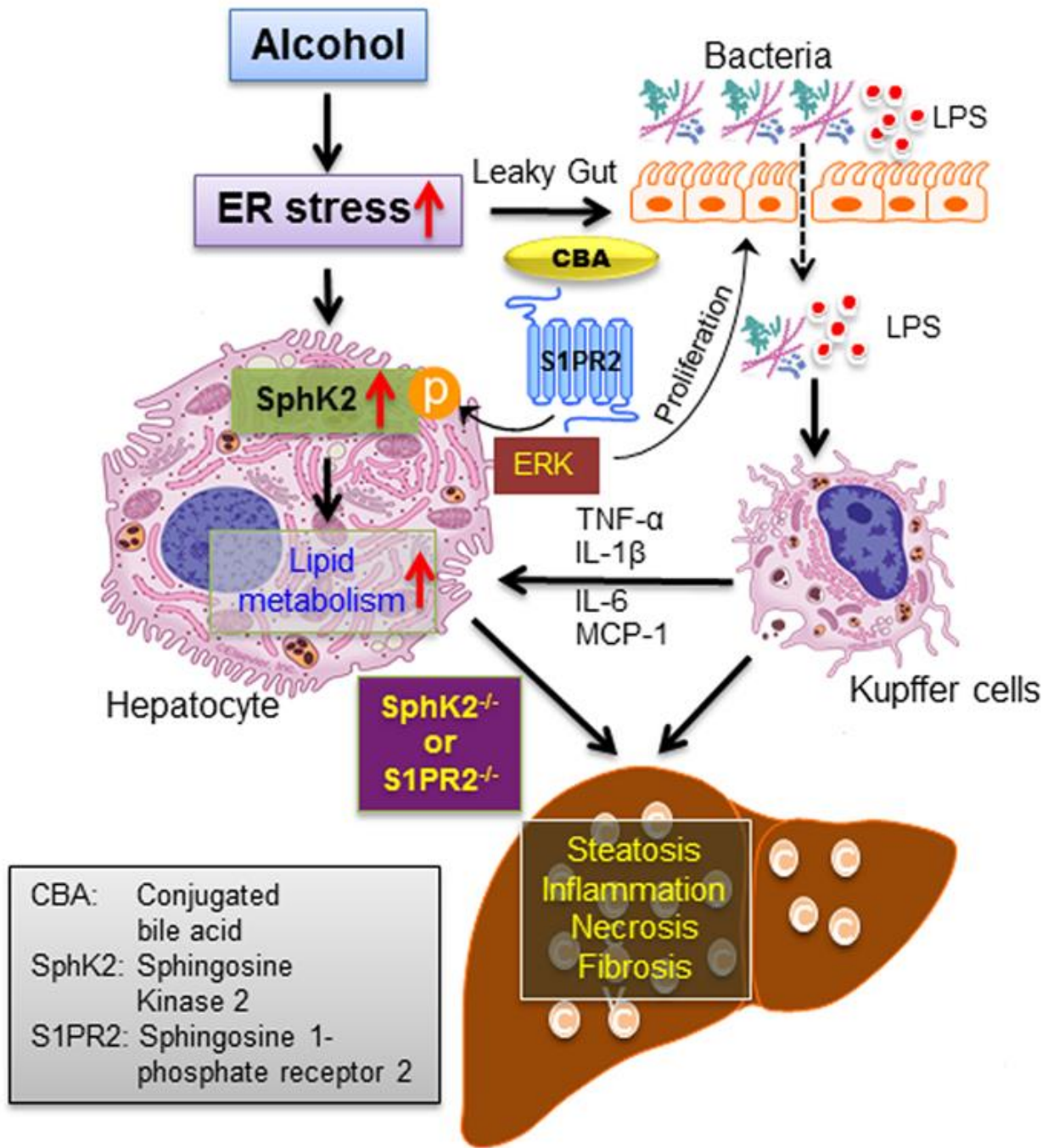


Figure 43. Schematic Diagram of Central Hypothesis.

regulates the distribution of various substances from the gut. Bile acids facilitate the communication between the gut and the liver by acting as ligands for nuclear receptors such as FXR [303]. It has been shown that a decrease in total fecal bile acids leads to an overgrowth of bacteria in the gut [304]. We have shown that there is an increase in total serum bile acids in SphK2<sup>-/-</sup> mice on an alcohol diet. A recent study showed that modulation of FXR and TGR5 attenuated alcohol-induced liver injury, steatosis, and inflammation [305]. It has been previously reported that alcohol alters the gut microbiome which may favor the growth of bacteria that produce potent toxins such as LPS [306]. Further studies on the change in the composition of the bile acid pool would provide further information on how bile acid changes lead to disease states. In summary, we have shown that SphK2 protects the liver from alcohol-induced liver injury. With our understanding of the role of bile acid signaling and SphK2-mediated activation of hepatic lipid metabolism, SphK2 may be a viable therapeutic target for ALD.

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## Vita

Eric Kwong was born on December 6th, 1988 in Pasadena, California. He attended the University of California, Irvine and graduated *cum laude* in 2011 with a Bachelor of Science in Biochemistry and Molecular Biology. He worked in the laboratory of Dr. Jefferson Chan at the University of California, Irvine before he matriculated in the Virginia Commonwealth University MD/PhD Program in 2014.

### Honors and Awards

2011	Phi Beta Kappa
2011	Departmental Honors in Biological Sciences, UC Irvine
2017	American Physiological Society Gastrointestinal and Liver Section Travel Award
2017	American Society for Investigative Pathology Liver Research Travel Award
2017	Ruth L. Kirschstein National Research Service Award F30 NIH/NIAAA
2018	Charles C. Clayton Award, VCU School of Medicine
2019	C. Kenneth and Dianne Wright Physician-Scientist Scholar

### Research Experience

2009 – 2014	Department of Pathology University of California, Irvine Laboratory of Jefferson Y. Chan, M.D., Ph.D. Topic: Cellular Stress Response and the Role of Nrf1 Transcription Factor
2016 – 2019	Department of Microbiology and Immunology Virginia Commonwealth University Laboratory of Huiping Zhou, Ph.D. Topic: The Role of S1PR2 and SphK2 in Alcoholic Liver Disease

### Publications

#### **Research Articles:**

Kwong EK, Kim K-M, Penalosa PJ, Chan JY. Characterization of Nrf1b, a Novel Isoform of the Nuclear Factor-Erythroid-2 Related Transcription Factor-1 That Activates Antioxidant Response Element-Regulated Genes. PLoS ONE. 7(10):e48404 (2012)

Biswas M, Kwong EK, Park E, Nagra P, Chan JY. Glycogen synthase kinase 3 regulates expression of nuclear factor-erythroid-2 related transcription factor-1 (Nrf1) and inhibits pro-survival function of Nrf1. Exp Cell Res. 319(13):1922-31 (2013)

Kwong E, Li Y, Hylemon PB, Zhou H. Bile acids and sphingosine-1-phosphate receptor 2 in hepatic lipid metabolism. Acta Pharmaceutica Sinica B. 5(2):151-7 (2015)

Kwong EK, Li X, Hylemon PB, Zhou H. Sphingosine Kinases/Sphingosine 1-Phosphate Signaling in Hepatic Lipid Metabolism. *Curr Pharmacol Rep.* 3(4):176-83 (2017)

Kwong EK and Zhou H. Sphingosine 1-Phosphate Signaling and the Gut-Liver Axis in Liver Diseases. *Liver Research* (2018)

Kwong EK, Liu R, Zhao D, Li X, Zhu W, Wang X, Gurley EC, Lai G, Liu J, Hylemon PB, Zhou H. The Role of Sphingosine Kinase 2 in Alcoholic Liver Disease. *Dig Liver Dis* (2019)

**Book Chapter:**

Hinton M, Li Y, Kwong E, Zhou H. ER Stress in Drug-Induced Liver Injury. Chapter 2 in *Cellular Injury in Liver Diseases, Cell Death in Biology and Diseases* (2017)

**Abstracts, Posters, and Presentations:**

Kwong EK and Chan JY. Identification and molecular characterization of Nrf1 protein isoforms. Abstract for poster presentation, 2011 University of California Undergraduate Research Symposium, Irvine, California, May 2011.

Kwong EK, Li X, Liu R, Hylemon PB, Zhou H. The role of sphingosine kinase 2 in alcoholic liver disease. Abstract for poster presentation, 2017 Experimental Biology Meeting, Chicago, Illinois, April 2017.

Kwong EK, Tao L, Li X, Liu R, Hylemon PB, Zhou H. The role of sphingosine kinase 2 in alcohol-induced hepatic inflammation and injury. Abstract for poster presentation, 2017 Pathobiology for Investigators, Students & Academicians, Pittsburgh, Pennsylvania, September 2017.

Sun L, Kwong EK, Hylemon PB, Zhou H. Sphingosine 1-phosphate receptor 2 plays a critical role in regulating macrophage recruitment and polarization in the cholestatic liver in MDR2<sup>-/-</sup> mice. Abstract for oral presentation, 2017 Liver Meeting, Washington DC, October 2017.

Kwong EK, Li X, Liu R, Hylemon PB, Zhou H. The Role of Sphingosine Kinase 2 in Chronic alcohol-induced Liver Injury and Disease. Abstract for poster presentation, 2018 Experimental Biology Meeting, San Diego, California, April 2018.

Kwong EK, Li X, Liu R, Hylemon PB, Zhou H. Sphingosine Kinase 2 Deficiency Promotes alcohol-induced Liver Injury and Fibrosis. Abstract for poster presentation, 2018 Liver Meeting, San Francisco, California, Nov 2018.