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Mutations in Metastatic Melanoma Tissue." *Cancer Research* 2003; 63: 3955-3957.

Abstracts

Gorden A, Yang R, Still CD, Gerhard GS, Shuldiner AR. 2014. "Liver steatosis grade is associated with expression levels of NCAN locus genes." Abstract for Digestive Diseases Week 2014, Chicago, IL.

Chen K, Lu W, Cheng Y-C, Ma K, Chu X, Yang R, **Gorden A**, Gerhard GS, Quon MJ, Mitchell BD, Shuldiner AR, Fu M. 2013. "Functional Assessment of Genetic Variants Associated with Lp(a) levels on chromosome 6q25-26." Abstract for American Society of Human Genetics Annual Meeting 2013, Boston, MA.

Gorden A, Ryan K, Yerges-Armstrong L, Speliotes EK, Still CD, Gerhard GS, Shuldiner AR. 2011. "Genetic Variation in the NCAN Locus is Associated with Increased Risk for Hepatic Inflammation and Fibrosis in Nonalcoholic Fatty Liver Disease." Abstract for parallel session presentation, The 62nd Annual Liver Meeting 2011, San Francisco, CA.

Calvisi, DF, Ladu S, **Gorden A**, Farina M, Lee JS, Conner EA, Schroeder I, Factor VM,

Thorgeirsson SS. 2006. Molecular pathogenesis of human hepatocellular carcinoma: Mechanistic and prognostic significance of aberrant methylation. Abstract for poster presentation, American Association for Cancer Research 96th Annual Meeting, Bethesda, MD.

Howell CD, Thompson AJ, Ryan K, Zambeeli S, **Gorden A**, Fried M, Afdhal NH, McHutchison JG, Shianna KV, Mitchell B, Goldstein DB, Shuldiner A. 2010. IL28B genetic variation associated with early viral kinetics and SVR in HCV

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Julius Friedenwald Lectureship

"Genetic variation at *NCAN* locus is Associated with Inflammation and Fibrosis in Non-alcoholic Fatty Liver Disease in Morbid Obesity."

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ABSTRACT

Dissertation Title:

Fine-Mapping the *NCAN* Locus: Using Genetic Variations to Understand Non-Alcoholic Fatty Liver Disease

Alexis Gorden, Doctor of Philosophy, 2014

Dissertation Directed by:

Alan Shuldiner, MD, Professor, Department of Medicine

To extend the findings of the GOLD Consortium GWAS meta-analysis, we studied a large population of bariatric surgery patients with intra-operatively obtained liver biopsies. Single nucleotide polymorphism rs2228603 in *NCAN* was associated with liver steatosis, hepatocyte ballooning and inflammation. Because *NCAN* is not highly expressed in liver, the rs2228603 in *NCAN* is likely marking the involvement of a nearby gene. Therefore, to determine if expression of any of the genes in the *NCAN* locus varies based on liver steatosis and SNP rs2228603 genotype, patients with extremes of hepatic steatosis who had been grouped based on *NCAN* SNP rs2228603 genotype were compared. For two of the genes in the *NCAN* locus, *GATAD2A* and *TM6SF2*, lower mRNA levels were associated with liver steatosis in patients with the *NCAN* rs2228603 CC genotype while higher levels were associated

with liver steatosis in patients with the CT genotype. These findings suggest that *NCAN* SNP rs2228603 may affect the way in which levels of the nearby genes *GATAD2A* and *TM6SF2* are associated with liver steatosis. To further evaluate the role of *TM6SF2*, a gene of unknown function, we performed gene silencing with morpholino oligonucleotides in the zebrafish model to demonstrate that knockdown of *Tm6sf2* reproduced the liver steatosis phenotype and simultaneously increased hepatic expression of *Fabp6*.

Fine-Mapping the NCAN Locus: Using Genetic Variations to
Understand Non-Alcoholic Fatty Liver Disease

by
Alexis Gorden

Dissertation submitted to the faculty of the Graduate School of the
University of Maryland, Baltimore in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
2014

DEDICATION

I dedicate my thesis
to my loving and supportive parents
Bernard and Adel Gorden.

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ABBREVIATIONS

ALT	alanine aminotransferase
AST	aspartate aminotransferase
BMI	body mass index
ChREBP	carbohydrate-responsive element-binding protein
CNS	central nervous system
CSPG	chondroitin sulfate proteoglycan
CT	computed tomography
DNA	deoxyribonucleic acid
eQTL	expression quantitative trait locus
FA	fatty acid
FABP	fatty acid binding protein
GGT	gamma-glutamyl transferase
GWAS	genome wide association study
HA	hyaluronan
HDL	high density lipoprotein
LDL	low density lipoprotein
MO	morpholino oligonucleotide
mRNA	messenger ribose nucleic acid
MRI	magnetic resonance imaging
MRS	magnetic resonance spectroscopy
NAFLD	non-alcoholic fatty liver disease
NASH	non-alcoholic steatohepatitis

NEFA	non-esterified fatty acid
NuRD	nucleosome remodeling deacetylase
PCR	polymerase chain reaction
PI	phosphatidyl inositol
PPAR	peroxisomal proliferator-activated receptor
PPRE	peroxisomal proliferator response element
qRT	quantitative reverse transcriptase
RNA	ribonucleic acid
RNAi	RNA interference
ROS	reactive oxygen species
SAM	S-adenosyl methionine
SD	standard deviation
SEM	standard error of the mean
SNP	single nucleotide polymorphism
SREBP-1c	sterol regulatory element-binding protein 1c
TZD	Thiazolidinedione
US	ultrasound
VLDL	very low density lipoprotein
WAT	white adipose tissue

CHAPTER 1: GENERAL BACKGROUND

Non-Alcoholic Fatty Liver Disease

Epidemiology

Nonalcoholic fatty liver disease (NAFLD) is characterized by hepatic steatosis as determined by imaging or liver histology. NAFLD ranges from simple steatosis (liver fat without hepatocyte injury or fibrosis) to steatohepatitis (lobular inflammation, hepatocyte injury and ballooning) to various degrees of liver fibrosis.¹ Non-alcoholic steatohepatitis (NASH) with fibrosis has a worse prognosis than NASH without fibrosis.² Characteristics associated with increased liver fibrosis include diabetes, increased body mass index (BMI), cigarette smoking, and rising serum transaminases.^{2,3} NAFLD patients with advanced fibrosis and cirrhosis are at the highest risk for hepatocellular carcinoma.⁴ Long-term liver-related prognosis in NASH is correlated with severity of steatohepatitis and, in particular, fibrosis. Compared with the general population, patients with simple steatosis have a good long-term prognosis and do not have an

increased overall mortality. In contrast, patients with NASH have a three-fold increase in liver-related mortality.^{5,6} However, it remains difficult to predict which patients with simple steatosis will progress to more advanced disease.

NAFLD is considered the hepatic manifestation of the metabolic syndrome, which is defined by the presence of three of the following: visceral obesity, elevated fasting plasma glucose, hypertension, hypertriglyceridemia or low serum levels of high-density lipoprotein (HDL).⁷ The global prevalence of NAFLD ranges from 2.8 to 46%.^{8,9} It is the most common cause of chronic liver disease in the United States and its prevalence has coincided with the rise in obesity and diabetes. The National Health and Nutrition Examination Surveys (NHANES) revealed that the prevalence of NAFLD in the United States increased from 5.5 to 11% from 1988 to 2008.¹⁰ However, determining the true incidence and prevalence of NAFLD, specifically NASH, is a challenge due to the asymptomatic presentation of the disease and the lack of accurate, noninvasive diagnostic tests. The evaluation of patients undergoing bariatric surgery confirmed the high prevalence rates among obese patients in which the prevalence of NAFLD and NASH is 91% and 37%, respectively.¹¹ However, while NAFLD is strongly

associated with obesity, it can occur in non-obese patients and obesity does not guarantee NAFLD.

Clinical Manifestations & Diagnosis

Patients with NAFLD are usually asymptomatic upon presentation, although some may describe vague, nonspecific symptoms like fatigue, nausea, and abdominal pain. Patients are often first diagnosed by mild elevations of the serum transaminases and/or radiographic evidence of liver steatosis. With advanced fibrosis, there may also be elevated serum alkaline phosphatase and gamma-glutamyl transpeptidase levels and there may be an increase in the AST level as well as the AST to ALT ratio.¹² In general, while very high aminotransferase levels suggest the presence of NASH, there is poor correlation between plasma liver enzymes levels and histology.^{13,14}

NASH is unequivocally diagnosed by liver biopsy with the identification of macrovesicular steatosis, hepatocyte ballooning degeneration, and lobular inflammation.¹⁵ However, because liver biopsy is an invasive and risky procedure, imaging is the first modality used to diagnose NAFLD. MRS is the gold standard with a high sensitivity and specificity for steatosis without involving radiation and allowing quantification of liver steatosis. However,

it is expensive and of limited availability. Therefore, ultrasound is typically the first imaging modality used, although it is less sensitive for detecting liver steatosis compared to both CT and MRS.¹⁶ Recently, transient elastography has been used for assessing liver fibrosis, although a high BMI reduces the accuracy of the study.¹⁷

Treatment

Since NAFLD is the hepatic manifestation of the metabolic syndrome, its management has focused on treating comorbidities such as obesity, insulin resistance/diabetes, and dyslipidemia. Lifestyle modifications, like weight loss with dietary changes and exercise, are the first-line in NAFLD management.^{18,19} A weight loss of 3-7% with lifestyle intervention has been associated with decreased liver steatosis on imaging and liver histology.²⁰⁻²³ However, it appears that intensive intervention is necessary to achieve sufficient weight loss to improve liver histology.²⁴

Currently there is no medical therapy approved by the Food and Drug Administration for NASH. The use of medications is being researched with emphasis on drugs that target dyslipidemia and insulin resistance as well as oxidative stress, inflammatory cytokines, apoptosis and

other pathways that may play a role in NAFLD progression to hepatic fibrosis. Weight loss agents have been investigated, including Orlistat, an enteric lipase inhibitor, and Sibutramine, a serotonin and norepinephrine reuptake inhibitor.^{25,26}

Diabetic medications have also been studied. Metformin has not been proven to be effective in improving liver histology in NASH so it is not currently recommended for treatment.¹⁸ Thiazolidinediones (TZDs), which are peroxisomal proliferator-activated receptor (PPAR)- α agonists, decrease hepatic lipogenesis, increase insulin sensitivity, and stimulate hepatic fatty acid oxidation.²⁷ However trials of TZDs for the treatment of NASH have shown that while they reduce hepatic steatosis, inflammation and hepatocyte ballooning, they do not consistently improve liver fibrosis.²⁸⁻³¹ Incretin mimetics like exenatide are the most recent diabetic medications that may improve NASH. Animal studies of exenatide have shown improved insulin sensitivity and hepatic steatosis along with decreasing fat accumulation in the liver by inhibition of hepatic lipogenesis.³² In human case studies, exenatide decreased serum aminotransferases and hepatic steatosis.^{33,34}

In the past, lipid-lowering agents, particularly statins, were avoided in patients with liver disease for

fear of causing transaminitis due to liver injury. However, a review of case studies concluded that these agents are all safe for use in patient with NAFLD and may actually improve liver steatosis.³⁵ Nevertheless, because studies in NASH populations have been limited to pilot trials that showed only modest histologic improvement, guidelines support the use of statins in patients with NAFLD only for the treatment of dyslipidemia.^{18,36}

Since oxidative stress is considered a potential cause of steatohepatitis, antioxidant agents have been studied for the treatment of NAFLD. Vitamin E, the most commonly studied agent, has shown significant improvements in hepatic steatosis and lobular inflammation (but no change in fibrosis) and is currently considered the first-line medical therapy for NASH.^{18,31} However, it should be noted that Vitamin E has been reported to increase cardiovascular risk, all-cause mortality, and prostate cancer.^{37,38} Other antioxidants such as betaine and N-acetyl-cysteine have yet to definitively show NAFLD improvement so they cannot be recommended at this time.^{39,40}

Pathogenesis of Hepatic Steatosis

The hallmark of NAFLD is triglyceride (TG) accumulation in the cytoplasm of hepatocytes as a result of an imbalance between lipid acquisition and removal. Under normal physiological conditions, the steady state concentration of hepatic TG is low because the liver does not function as a storage depot for fat. However, there is considerable trafficking of both TG and fatty acid (FA) into and out of the liver in response to feeding. Dietary FAs are absorbed from the small intestine, assembled into TGs, and incorporated into chylomicrons that enter the plasma as TG-rich chylomicrons. Approximately 70% of the FAs from these chylomicrons are delivered to peripheral adipose tissue, with the remainder taken up by the liver.⁴¹ In the setting of excess carbohydrates, FAs are also synthesized de novo within the liver, where these FAs may be converted into other lipid species that can be packaged into VLDL particles and secreted into the plasma.⁴² Hepatic FAs that are not incorporated into VLDL particles can be oxidized in the liver by mitochondria.⁴² (Figure 1)

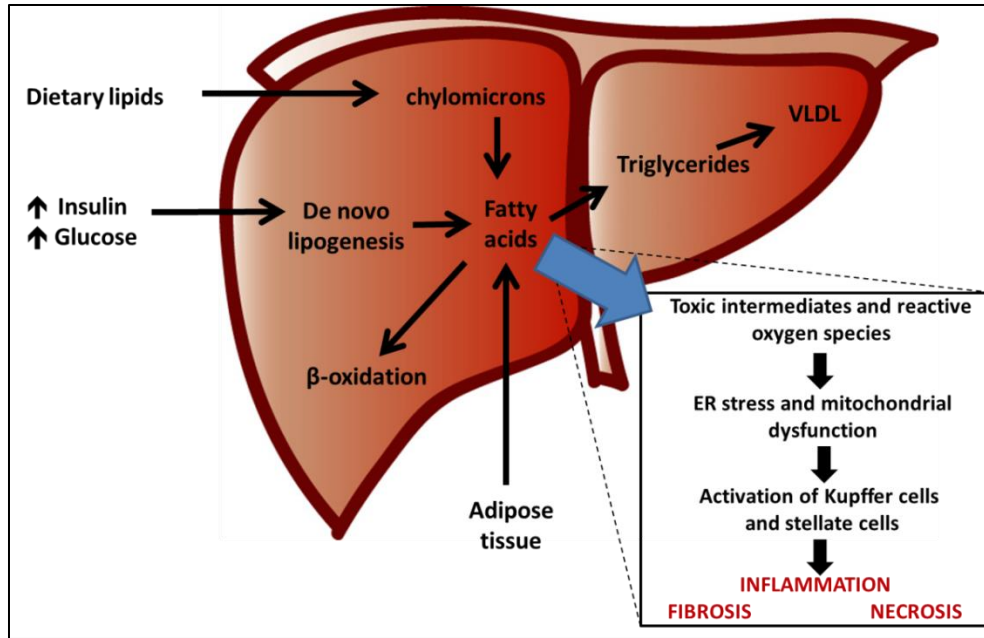


Figure 1: NAFLD Pathogenesis

Fatty Acid Uptake

Fatty acid uptake into the liver contributes to the steady state balance of hepatic TGs but is also an underlying mechanism of hepatic TG accumulation. The rate of FA uptake from the plasma into the liver depends on the plasma FA concentration and the liver's capacity for FA uptake, specifically the number and activity of transporter proteins on the hepatocyte sinusoidal plasma membrane.⁴³ After entry into cells, FAs are rapidly activated by conversion to fatty acyl-CoAs. Fatty acid transport proteins (FATPs) and CD36 (also known as fatty acid translocase, FAT) are necessary for the transmembrane process. The importance of FA uptake in NAFLD was shown

when it was demonstrated that the majority of hepatic TGs in humans are derived from plasma non-esterified fatty acid (NEFA).⁴¹ In NAFLD subjects, the increase in plasma NEFAs is attributable to increased FA release from adipose tissue.^{44,45} Peripheral insulin resistance in NAFLD patient also contributes to increased rates of FA release from adipose tissue.⁴⁶ A potential pathogenic role is suggested by the observation that elevated hepatic *CD36* expression has been observed in NAFLD and appears to increase hepatic uptake of NEFA.^{47,48} Furthermore, hepatic overexpression of *Cd36* in mice increases hepatic TG content,⁴⁹ while adenovirus-mediated knockdown of *Fatp2* or *Fatp5* reduced hepatic TG accumulation in mice fed a high fat diet.^{50,51}

Triglyceride Synthesis

The NEFAs that are incorporated into TGs within the liver are either derived from the plasma or synthesized de novo from glucose. In de novo lipogenesis, which produces 26% of hepatic TGs⁴¹, glucose is converted to acetyl-CoA, which is then converted to malonyl-CoA by ACC. (Figure 2) Lipogenesis is controlled primarily at the transcriptional level.^{52,53} Postprandially, plasma glucose and insulin concentrations rise and activate ChREBP and SREBP1c, respectively.⁵³ SREBP1c is a transcription factor that

promotes expression of lipogenic genes like *FAS*, *ACC*, and *SCD1*.^{52,54} (Figure 2) ChREBP, binds to the promoter of lipogenic genes and also triggers expression of liver-type pyruvate kinase, thus providing more substrate for FA synthesis.^{55,56} Free FA then gets assembled into TG which is packaged to form VLDL particles that are secreted into the plasma.⁴²

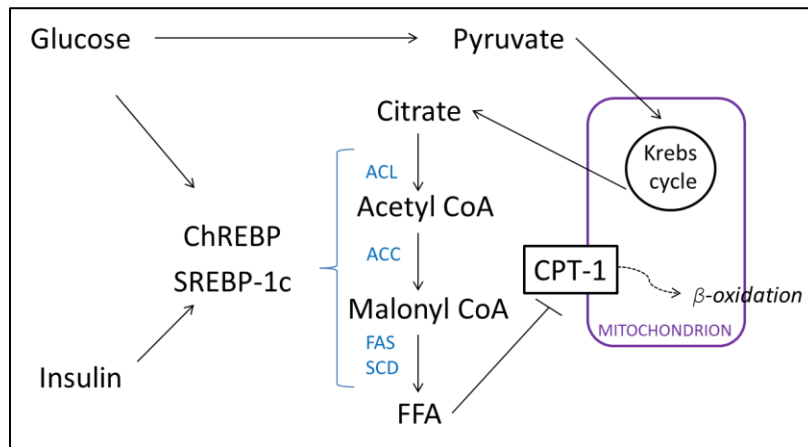


Figure 2: Hepatic De Novo Lipogenesis

Fatty Acid Oxidation

The steady state balance of hepatic TGs is also controlled by the consumption of FAs by mitochondrial β -oxidation.⁵⁷ Translocation of fatty acyl-CoAs across the outer mitochondrial membrane is controlled by the activity of CPT1. (Figure 2) In the post-prandial state, β -

oxidation in the liver is suppressed due to the anti-lipolytic effect of insulin on white adipose tissue (which reduces the flux of NEFA to the liver) and the direct control by glucose and insulin of the rate of FA entry into the mitochondria.⁵⁸ Insulin facilitates de novo lipogenesis through upregulation and activation of SREBP1c as well as through induction of ACC. Malonyl-CoA inhibits the activity of CPT1 by decreasing FA availability for entry into mitochondria, thus decreasing the rate of β -oxidation. (Figure 2) Thus malonyl-CoA is the molecular switch between fatty acid synthesis and oxidation.⁵⁹ However, studies in human NAFLD patients have yield mixed results with respect to alterations in rates of FA oxidation.⁶⁰ In some, impaired ATP production has been described in NAFLD patients.^{61,62} Others have reported evidence for increased rates of FA oxidation in NAFLD^{63,64} suggesting that increased mitochondrial activity could promote oxidative stress within the liver and contribute to the development of NASH.⁶⁵

VLDL Secretion

The liver secretes TGs in the form of VLDL particles for delivery to peripheral tissues. VLDL is a lipoprotein with a hydrophobic core composed mainly of TGs and

cholesterol esters. Each VLDL particle is stabilized by a single molecule of apoB 100, which is a long polypeptide lipidated with TGs within the endoplasmic reticulum (ER) lumen while it is being translated and translocated across the ER membrane.⁶⁶ Lipidation of apoB 100 is facilitated by microsomal triglyceride transfer protein (MTTP), an ER resident protein that has both apoB 100 binding and lipid transfer domains.⁶⁷ Nascent VLDL particles are transported from ER to Golgi; during this process, the maturing VLDL particles are further lipidated by MTTP.⁶⁸

Upon maturation, VLDL particles are released from the liver into the plasma. The VLDL secretion rate appears dependent not only on the availability of hepatic TGs but also on the liver's overall capacity for VLDL assembly. Impaired VLDL assembly and secretion result in excessive lipid accumulation in the liver. Hepatic steatosis has been reported in subjects with hypobetalipoproteinemia or abetalipoproteinemia,^{69,70} and similar observations have been seen in liver-specific MTTP knockout mice⁷¹ while mice with liver-specific overexpression of MTTP have VLDL overproduction with elevated plasma TG levels.⁷² NAFLD is characterized by overproduction of VLDL particles, which reflects enhanced de novo lipogenesis plus lipolysis of intrahepatic and intra-abdominal fat.⁴⁵ In these subjects,

lipid availability for VLDL assembly is increased and this is combined with the failure of insulin to suppress VLDL production. Due to the increased availability of TGs, apoB 100 is not degraded, and MTTP expression is increased.⁷³ Although chronic exposure to insulin drives VLDL overproduction⁷³, augmented VLDL secretion does not compensate the TG overproduction in the liver, therefore steady state hepatic TG concentrations remain increased. Moreover, apoB 100 secretion is not increased in NAFLD, suggesting that apoB 100 production limits the liver's capacity to export hepatic TGs.⁴⁵ Prolonged exposure of the liver to NEFAs promotes excessive ER stress, and this leads to degradation of apoB 100, thus decreasing TG secretion and worsening steatosis.⁷³

Steatohepatitis

The biological mechanisms underlying the progression of fatty liver disease from simple steatosis to steatohepatitis remain poorly understood. The pathogenesis of the NAFLD spectrum was first described by the "two-hit hypothesis."⁷⁴ The first hit refers to the development of hepatic steatosis due to dysregulation of fatty acid metabolism, which is largely determined by insulin

resistance. The second hit was thought to trigger the progression from simple steatosis to hepatocyte inflammation, ballooning and fibrosis, but the factors involved are poorly characterized at this time. It is likely that multiple pathways involving oxidative stress, inflammatory cytokines, cellular autodigestion and endotoxins leading to apoptosis play a role in NASH pathogenesis.^{41,75,76} Furthermore, genetic factors likely promote hepatic steatosis, inflammation and fibrogenesis. Insulin resistance is a key component of NAFLD that favors high rates of free FA flux to the liver from increased adipose tissue lipolysis.^{45,77} Whole body insulin resistance and the associated hyperinsulinemia results in an increase in hepatic lipogenic gene expression, predominantly mediated by sterol regulatory element-binding protein (SREBP)-1c and carbohydrate response element binding protein (ChREBP), thus triggering increased hepatic TG synthesis.^{41,78} In the presence of high rates of TG synthesis and saturated VLDL export, hepatic mitochondrial tricarboxylic acid cycle activity continues unchecked and has the potential to overload the mitochondrial electron transport chain and thus increase the production of reactive oxygen species (ROS).⁷⁹ Hepatocellular lipid accumulation and high ROS production results in a cascade

of events leading to higher rates of lipid peroxidation, cytotoxic aldehyde formation and inflammatory cytokine production.⁷⁸ Free fatty acid overload of the mitochondria also causes diversion of a small portion of free fatty acids (FFAs) from the normal oxidative routes (beta-oxidation and TCA cycle) to synthesis of toxic lipid intermediates.^{80,81}

Genetic Variants Underlying NAFLD

Genetic variation in several genes has been associated with NAFLD. *MTTP* regulates synthesis, storage and export of hepatic TG and is critical for VLDL synthesis and secretion.⁸² The -493 G/T *MTTP* SNP is associated with elevated serum ALT levels⁸³, which serves as a marker for steatohepatitis. Furthermore, patients with NASH have a higher frequency of the G allele and the G/G genotype compared with controls.⁸³ The G allele of this SNP is also associated with decreased transcription of the *MTTP* gene and is associated with increased accumulation of liver TG.⁸⁴ The G/G genotype was associated with more severe steatosis and more advanced stages of NASH compared to the G/T genotype.⁸⁴ Finally, reduced methylation of the *MTTP* promoter has been shown to decrease liver steatosis in rats fed a high fat diet.⁸⁵

Although expressed primarily in adipose tissue, *Adiponutrin/patatin-like phospholipase domain-containing 3* (PNPLA3) is also present in the liver.⁸⁶ Its protein product, has both lipolytic and lipogenic activity.^{87,88} PNPLA3 gene expression is regulated by ChREBP and SREBP, implicating its function in glucose and lipid metabolism. PNPLA3 expression is suppressed by fasting and induced by a carbohydrate rich diet.⁸⁹ Thus, PNPLA3 may be involved in TG synthesis and storage during times of energy excess. However, it is unclear whether variant PNPLA3 would liberate more free fatty acids to be taken up by the liver. Alternatively, if the main function of PNPLA3 is to regulate lipolysis, its inactivity would favor TG accumulation, which is desirable in adipose tissue but potentiates abnormal lipid storage in the liver. The PNPLA3 SNP I148M is associated with increased hepatic fat and inflammation⁹⁰ and plays a strong role in NAFLD disease severity.⁹¹ Experimental approaches show that while I148M substitution does not alter the subcellular distribution of PNPLA3 between membranes and lipid droplets, it does abolish TG hydrolysis; thus, this variant PNPLA3 appears to increase hepatocellular TG content.⁸⁶

The Genetics of Obesity-related Liver Disease (GOLD) Consortium determined the genetic basis of NAFLD by

performing a genome-wide association (GWA) meta-analysis between CT hepatic steatosis and ~2.4 million SNPs in over 7,000 individuals from 4 cohorts. They identified three variants near *PNPLA3*, *NCAN* and *PPP1R3B* that associated with CT diagnosed hepatic steatosis and showed that variants near *NCAN*, *GCKR*, *LYPLAL1*, and *PNPLA3* were associated with histological features of NAFLD.⁹² These findings suggest that there NAFLD pathogenesis is influenced by several metabolic pathways.

CHAPTER 2: OBJECTIVES

The purpose of this project was to determine the role of the *NCAN* locus in NAFLD pathogenesis. The central hypothesis was that *NCAN* SNP rs2228603 is a marker for a gene in the locus that plays a role in NAFLD progression. To test this hypothesis we proposed three specific aims:

- Aim 1 was to replicate the association of rs2228603 with NAFLD that was identified by the GOLD Consortium. We hypothesized that the SNP genotype is associated with liver steatosis. We planned to determine this by genotyping rs2228603 in an independent cohort of morbidly obese patients.
- Aim 2 was to quantify hepatic expression levels of genes in the *NCAN* locus near the NAFLD-associated variant rs2228603. We hypothesized that liver expression of the putative gene(s) would differ between obese individuals with and without NAFLD. We planned to determine this by measuring hepatic mRNA levels of several *NCAN* locus genes to determine if gene expression levels are associated with liver steatosis.

- Aim 3 was to use functional studies to reproduce the NAFLD phenotype in an animal model. We hypothesized that silencing the putative *NCAN* locus gene will produce liver steatosis. We planned to determine this by using morpholino technology in zebrafish to establish if putative gene knockdown produces hepatic steatosis.

Identification of the causative variant(s)/gene(s) is likely to provide novel insights into NAFLD pathogenesis and discover new molecular targets for prevention and treatment of this increasingly prevalent disease.

CHAPTER 3: REPLICATION & EXTENSION

Introduction

Obesity is a driving force for the metabolic syndrome, which is a combination of cardiovascular risk factors that include visceral obesity, hypertension, high serum triglycerides, low serum high-density lipoprotein (HDL) cholesterol, and glucose intolerance or diabetes.⁹³ In a substantial number of obese individuals with metabolic syndrome, fatty infiltration of the liver (hepatosteatosis) is observed. With the current epidemic of obesity, NAFLD and its progression is the leading cause of liver dysfunction and failure.^{78,94-96}

The prevalence rate of NAFLD increases with increasing BMI and abdominal fat accumulation is an independent predictor of hepatic steatosis.⁹⁷ An analysis of liver histology samples obtained from liver donors, automobile crash victims, autopsy findings, and clinical liver biopsies revealed a prevalence of steatosis and steatohepatitis of approximately 15% and 3%, respectively, in non-obese persons, 65% and 20%, respectively, in persons with class I and II obesity (BMI 30.0-39.9 kg/m²), and 85% and 40%, respectively, in extremely obese patients (BMI ≥40

kg/m²).⁹⁸ Stranges et al (2004) evaluated the relationship between central fat accumulation (based on abdominal height) relative weight (based on BMI) and liver function tests (ALT, AST and gamma glutamyl transferase (GGT)) in adult patients free from underlying hepatic disease. Several linear regression models were used; liver enzymes were the dependent variables, abdominal height and BMI were independent variables, and covariates included age, race, education, smoking, drinking. Abdominal height was the most powerful independent predictor of ALT and of GGT.⁹⁶ These findings suggest a role for central adiposity independent from BMI in predicting increased liver enzyme levels.

Since bariatric surgery is the most effective available weight loss therapy there has been speculation that rapid and significant weight loss may worsen NAFLD by increasing hepatic inflammation and fibrosis.⁹⁹ Surgical series data reveal that weight loss from bariatric surgery actually decreases steatosis, inflammation and fibrosis.^{100,101} Furthermore, weight loss from bariatric surgery has beneficial effects on liver metabolism, specifically decreased hepatic glucose production, decreased hepatic VLDL-TG secretion, and decreased hepatic gene expression of factors that induce hepatic inflammation

and fibrogenesis.¹⁰² Thus, bariatric surgery-induced weight loss appears to be beneficial for NAFLD patients with morbid obesity by normalizing metabolic abnormalities associated with NAFLD pathogenesis and by preventing the progression of hepatic inflammation and fibrosis.

Despite the link between obesity and NAFLD, it is unknown why only a fraction of obese individuals develop hepatic steatosis and only a subset of those patients progress to NASH, fibrosis, and cirrhosis. Recently, genetic factors have been implicated. In a genome-wide association study, a nonsynonymous variation I148M (rs738409) in the *PNPLA3* gene was found to be associated with NAFLD.⁹⁰ This variant has also been associated with increased serum AST and ALT levels as well as histological evidence of NASH.^{103,104} This is the most characterized NAFLD gene and has been studied specifically in the obese. Initial findings in cohorts with a mean BMI of 30 also suggest that it is associated with elevated liver enzymes.¹⁰⁵ To determine whether this variant alters the susceptibility of morbidly obese subjects to develop liver injury, Romeo et al (2010) carried out genotyping for the I148M variant in 678 obese patients with a mean BMI of 41. Indices of liver injury (ALT and AST) were significantly higher in carriers of the 148M allele.¹⁰³ Glucose tolerance

and insulin sensitivity were similar in all three genotypes, suggesting that the patients with the 148M allele have increased indices of liver damage independent from insulin resistance. Giudice et al (2011) showed that the association of PNPLA3 variants with liver enzymes in childhood obesity is driven by the interaction with abdominal fat. In a large group of obese children they analyzed the role of the interaction of factors such as BMI, WC and IR to determine the association between the I148M SNP and ALT levels. Children carrying the 148M allele showed higher AST and ALT levels and children homozygous for 148M showed a stronger correlation between ALT and WC than those carry the other genotypes.¹⁰⁶ This showed for the first time that the magnitude of the association of PNPLA3 with liver enzymes is driven by the size of abdominal fat. Santoro et al (2010) genotyped the *PNPLA3* SNP in a group of 85 obese children and measured hepatic fat content by magnetic resonance imaging (MRI). A subset of patients underwent subcutaneous fat biopsy. The prevalence of the G allele was higher in subjects showing hepatic steatosis and carriers of the G allele showed smaller adipocytes than those with the CC genotype.¹⁰⁷ However, subjects with the G allele had similar rates of hepatic glucose production, peripheral glucose disposal and

glycerol turnover as the CC homozygotes. Thus, the *PNPLA3* SNP confers susceptibility to hepatic steatosis in obese children without increasing the level of hepatic and peripheral insulin resistance while the G allele is associated with morphological changes in adipocyte size. Guichelaar et al (2013) hypothesized that the impact of the *PNPLA3* G allele may be exacerbated in patients with morbid obesity. They investigated the interactions of rs738409 with a variety of NAFLD characteristics in patients with complicated morbid obesity. *PNPLA3* GG genotype correlated with serum ALT and AST as well as the presence of NASH, and multivariate analysis showed that the rs738409 G allele remained an independent risk factor for NASH.¹⁰⁸

Several genes have been implicated in NAFLD in the obese. The Genetics of Obesity-related Liver Disease (GOLD) Consortium, involving 7,176 subjects of European descent, performed a genome-wide association meta-analysis of NAFLD.⁹² In this study, three novel gene loci (*NCAN*, *GCKR*, and *LYPLAL1*) and the previously reported *PNPLA3* locus were found to be associated with increased liver fat content as measured by both electron beam computed tomography (CT) and liver biopsy. Another gene locus, *PPP1R3B*, was associated with increased liver fat content by electron beam CT, but not with histologically-

defined NAFLD. Interestingly, some of these variants were associated with distinct changes in serum lipid levels. Specifically, the T allele of rs2228603 in *NCAN* was associated with increased liver fat and, seemingly paradoxically, with lower serum TG and low density lipoprotein (LDL) cholesterol levels.⁹² These differences suggest that each gene locus may affect lipid metabolism and NAFLD through a distinct mechanism.

To further examine the role of these gene loci in NAFLD in the obese, we performed association studies of these variants in an independent cohort of severely obese patients who underwent bariatric surgery and from whom histologically well-characterized liver samples were obtained. Our aim was not simply to replicate the findings of the GOLD Consortium, but also to determine the relevance of these loci in extreme obesity, a condition in which both strong genetic and environmental factors contribute to the phenotype and its metabolic complications.

Methods

Study Population and Phenotyping

The participants were 1,092 bariatric surgery patients (Geisinger Medical Center in Danville, Pennsylvania) with normal liver tissue or varying degrees of NAFLD, ranging from simple steatosis, to steatohepatitis, to cirrhosis (these subjects were not included in the original GOLD Consortium). (Figure 3) The protocol was approved by the Geisinger Clinic Institutional Review Board, and all subjects provided written informed consent. Prior to surgery, patients were extensively phenotyped, including a comprehensive medical history and physical examination, anthropometry, fasting serum glucose and lipid levels, liver and kidney function tests, and documentation of medication usage.¹⁰⁹

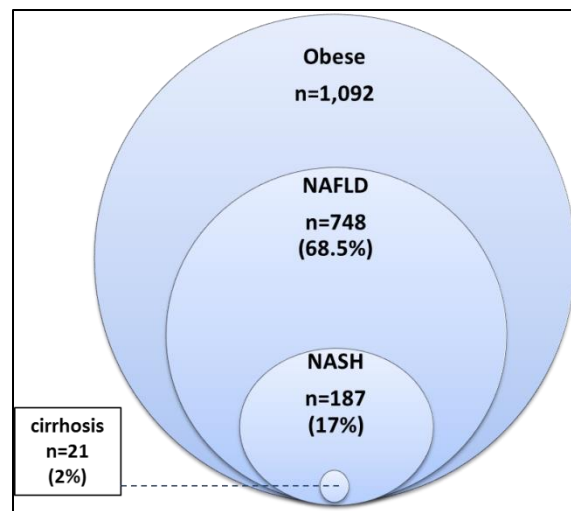


Figure 3: Spectrum of NAFLD in the Bariatric Cohort

Intra-operative liver biopsy specimens were formalin fixed and stained with hematoxylin and eosin for routine histology and Masson's trichrome for assessment of fibrosis.¹¹⁰ All specimens were read by experienced pathologists and graded according to standard NAFLD criteria.¹⁵ Steatosis was graded in severity from 0 (no steatosis) to 3 (severe steatosis). Scoring for lobular inflammation, hepatocyte ballooning, and perivenular fibrosis was dichotomized, with a score of 0 indicating absence of the feature and a score of 1 indicating any presence of the feature (ranging from mild to severe).

SNP Genotyping

Genomic DNA was isolated from peripheral blood leukocytes.¹¹¹ The five hepatic steatosis-associated SNPs from the GOLD Consortium meta-analysis were genotyped using TaqMan[®] SNP Genotyping Assays (Life Technologies, Carlsbad, CA). These included rs12137855 (C/T) on chromosome 1 near *LYPLAL1*, rs780094 (C/T) on chromosome 2 in *GCKR*, rs4240624 (A/G) on chromosome 8 near *PPP1R3B*, rs2228603 (C/T) on chromosome 19 in *NCAN*, and rs738409 (G/C) on chromosome 22

in *PNPLA3*. PCR amplification was performed on the GeneAMP PCR System 9700 thermal cycler (Applied Biosystems) under the following conditions: 10 min at 95°C, then 40 cycles of 15 sec at 92°C and 1 min at 60°C. Allelic discrimination was performed on the ABI Prism 7900 HT Sequence Detection System (SDS) and SDS Software according to the manufacturer's directions. All five SNPs passed genotyping quality control. The average genotype call rate was 96.7%. The genotype concordance rate of blind replicates was 99.2%. None of the SNP allele frequencies deviated significantly from the Hardy-Weinberg equilibrium.

Statistical Analysis

Statistical analyses were carried out using SAS version 9.2 (SAS Institute Inc., Cary, NC). For continuous traits (steatosis grade, and serum levels of total-, LDL- and HDL-cholesterol, and TG), associations between SNPs and phenotypes were assessed by linear regression. Dichotomous traits (lobular inflammation, ballooning, perivenular fibrosis and cirrhosis) were analyzed using logistic regression. Analyses were adjusted for age, sex, and use of lipid-lowering medication. Adjustment for lipid lowering

therapy was performed using as covariates whether the subject was or was not on statin therapy and was or was not on fibrate therapy (the vast majority of subjects on lipid-lowering therapy were taking statins). Regression analyses tested for an additive SNP association between the number of copies (0, 1 or 2) of the NAFLD-associated allele and the trait of interest. Due to the relatively low minor allele frequency of the T allele of rs2228603 in *NCAN* and the G allele of rs4240624 in *PPP1R3B* (7.4% and 8.3% respectively), the rare homozygous and heterozygous genotype groups were combined into a single group and compared to the common homozygous genotype for analysis. Since a small number of SNPs were genotyped, each with a high posterior probability of being true positives, a two-sided p-value < 0.05 was used as the threshold for statistical significance.

Results

Like most bariatric surgery cohorts¹¹²⁻¹¹⁴, the majority (80%) of our cohort was female. (Table 1) The average age was 46 years and the mean pre-operative BMI was 50. Although the BMI did not differ between patients with and without NAFLD ($p = 0.22$), those with NAFLD had greater waist circumferences ($p < 0.0001$), lower serum HDL-cholesterol levels ($p < 0.0001$) and, as shown in other studies¹¹⁵, higher serum TG levels ($p < 0.0001$). (Table 1) In addition, more patients in the NAFLD group had diabetes ($p = 0.0007$). About one third of all subjects were on statin or fibrate therapy. However, the percentage of subjects on lipid-regulating medication was not significantly different between subjects with and without NAFLD or among any of the genotypes examined. (Table 1)

	NAFLD + (n=748)	NAFLD - (n=344)	p-value
Female, N (%)	579 (77.4%)	296.0 (86.0%)	0.001
Age, years (mean \pm SD)	47 \pm 10.6	46 \pm 11.8	0.36
BMI, kg/m ² (mean \pm SD)	50.4 \pm 8.9	49.6 \pm 8.6	0.22
Waist Circumference, in (mean \pm SD)	54.2 \pm 6.7	52.4 \pm 6.2	<0.0001
Diabetes, N (%)	292 (39%)	93 (27%)	0.0007
Total cholesterol, mg/dl (mean \pm SD)	191.7 \pm 40.8	185.4 \pm 40.8	0.006
HDL-cholesterol, mg/dl (mean \pm SD)	46.3 \pm 10.6	50.9 \pm 12.1	<0.0001
LDL-cholesterol, mg/dl (mean \pm SD)	107.6 \pm 34.5	106.4 \pm 36.0	0.39
Triglycerides, mg/dl (mean \pm SD)	197.4 \pm 141.2	140.3 \pm 69.4	<0.0001
Statin Therapy, N (%)	228 (30%)	117 (34%)	0.07
Fibrate Therapy, N (%)	41 (5%)	20 (6%)	0.38
Statin or Fibrate Therapy, N (%)	250 (33%)	122 (35%)	0.14

Table 1. Bariatric Cohort Clinical Characteristics

Of all 1,092 obese subjects, 32% had no evidence of hepatic steatosis, 39% had grade 1 steatosis, 19% had grade 2, and 10% had grade 3.

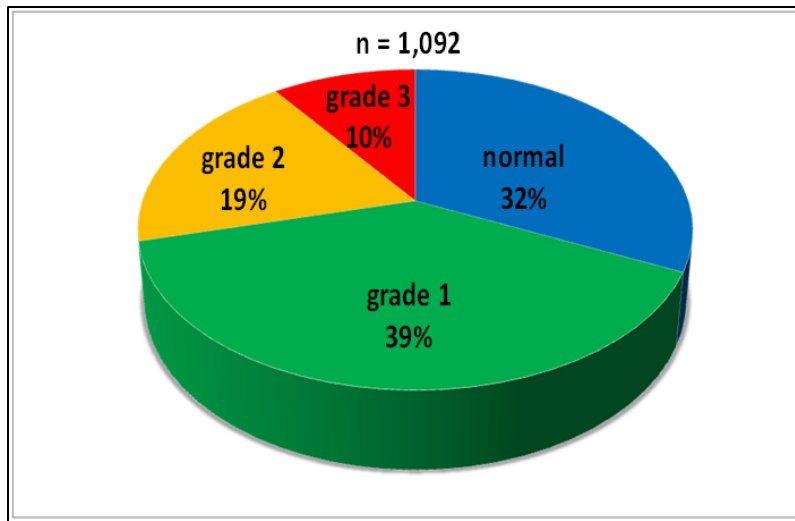


Figure 4: Liver Steatosis Grade in the Bariatric Cohort

Of the 748 patients with evidence of hepatic steatosis, 187 (25%) had at least one histological feature of steatohepatitis. The biopsies from these 187 NASH patients revealed lobular inflammation (mild: 24%, moderate: 7%, and severe: 1%), hepatocyte ballooning (mild: 17%, moderate: 8%, and severe: 0%) and perivenular fibrosis (mild: 11%, moderate: 5%, and severe: 3%). (Figure 5)

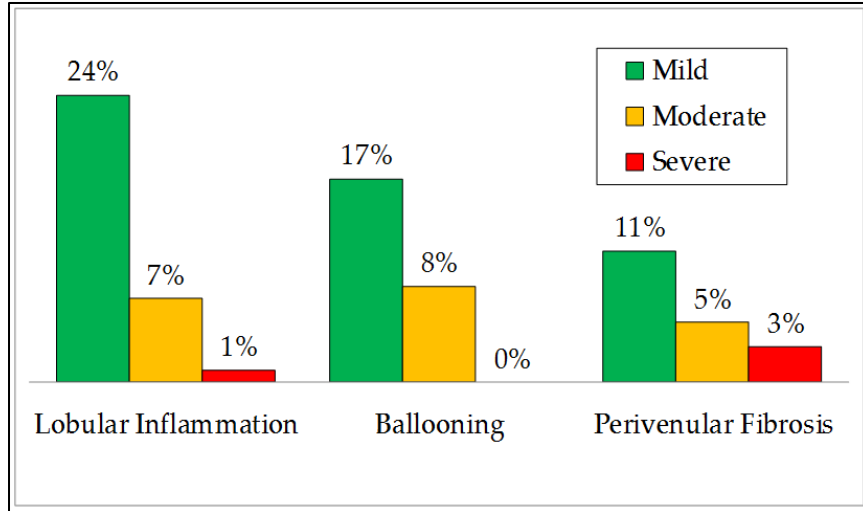


Figure 5: NAFLD Features in the Bariatric Cohort

Of the 187 NASH patients, 12% had cirrhosis.

Of the 5 SNPs associated with NAFLD in the GOLD Consortium meta-analysis, only rs780094[C] in *GCKR* was associated with higher weight ($p = 0.001$) and BMI ($p = 0.001$) in this bariatric cohort. SNPs rs780094[C] in *GCKR* and rs2228603[T] in *NCAN* were associated with increased waist circumference ($p = 0.02$ and 0.03 , respectively).

Except for rs12137855[C] near *LYPLAL1*, all of the SNPs were significantly associated with (or trended toward) increased steatosis in the same direction and with similar effect sizes as reported in the GOLD Consortium meta-analysis. Consistent with the results from the GOLD Consortium⁹², our data showed an association of *PNPLA3* SNP rs738409[G] with both hepatic steatosis ($p = 4.8 \times 10^{-8}$) and

hepatocyte ballooning ($p = 0.006$). (Figure 4, Table 2)

Similarly, as previously reported⁹², we found an association of the T allele of SNP rs2228603 in NCAN with liver steatosis ($p = 0.03$) as well as with lobular inflammation ($p = 0.02$) and perivenular fibrosis ($p = 0.002$). (Figures 6 & 7, Table 2)

SNP	780094			2228603		1213785			4240624		738409		
Effect allele	T			T		C			A		G		
Other allele	C			C		T			G		C		
Nearest gene	GCKR			NCAN		LYPLAL1			PPP1R3B		PNPLA3		
Genotypes	TT N=186	CT N=522	CC N=384	TT & CT N=155	CC N=928	CC N=696	CT N=325	TT N=39	AA N=883	AG & GG N=172	GG N=664	CG N=370	CC N=54
Steatosis (mean)	1.16	1.07	1.02	1.22	1.03	1.07	1.06	1.11	1.08	1.02	0.96	1.16	1.63
SE	0.07	0.04	0.05	0.07	0.03	0.04	0.05	0.15	0.03	0.07	0.03	0.05	0.13
p-value*	0.16			0.03		0.9			0.49		4.8x10 ⁻⁸		
Lobular inflammation % (> 0)	34.4	30.9	31.1	39.7	30.1	31.4	32.2	32.4	31.4	32.7	29.6	34.8	32.7
p-value*	0.54			0.02		0.96			0.66		0.16		
Ballooning% (> 0)	23.4	29.1	21.6	32.6	23.7	24.2	28.0	23.8	24.9	27.6	21.5	31.3	31.4
P-value*	0.42			0.06		0.54			0.55		0.006		
Perivenular fibrosis% (> 0)	20.0	18.8	18.8	27.8	17.2	19.2	18.0	21.6	19.1	17.3	17.1	22.0	21.2
p-value*	0.82			0.002		0.74			0.66		0.09		
Cirrhosis% (1)	2.8	2.5	1.6	4.0	2.1	2.5	1.9	2.7	2.0	4.2	1.7	3.3	1.9
p-value*	0.35			0.21		0.56			0.06		0.21		

Table 2: Association of SNPs with NAFLD Features

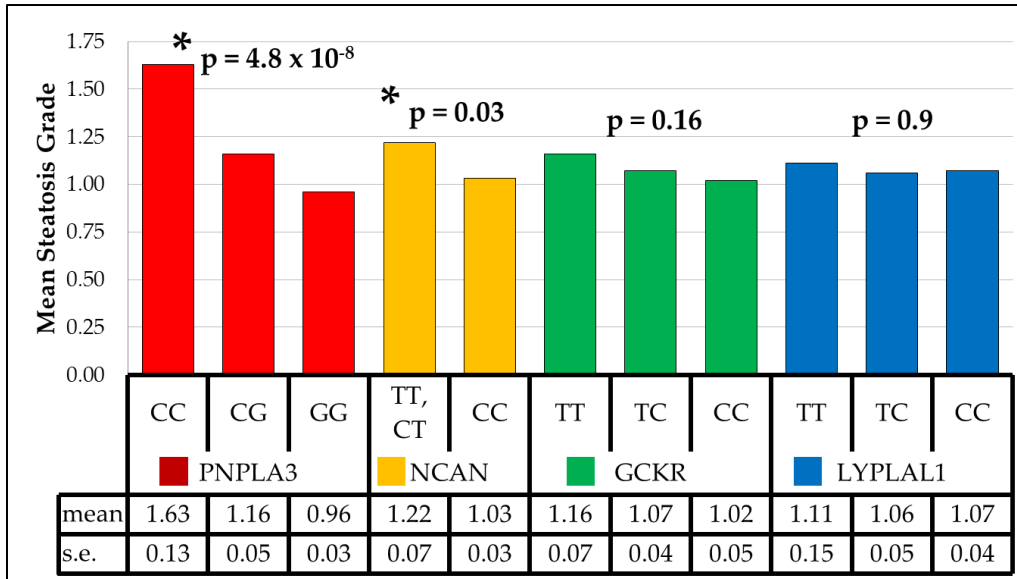


Figure 6: Association of rs2228603 with Hepatic Steatosis

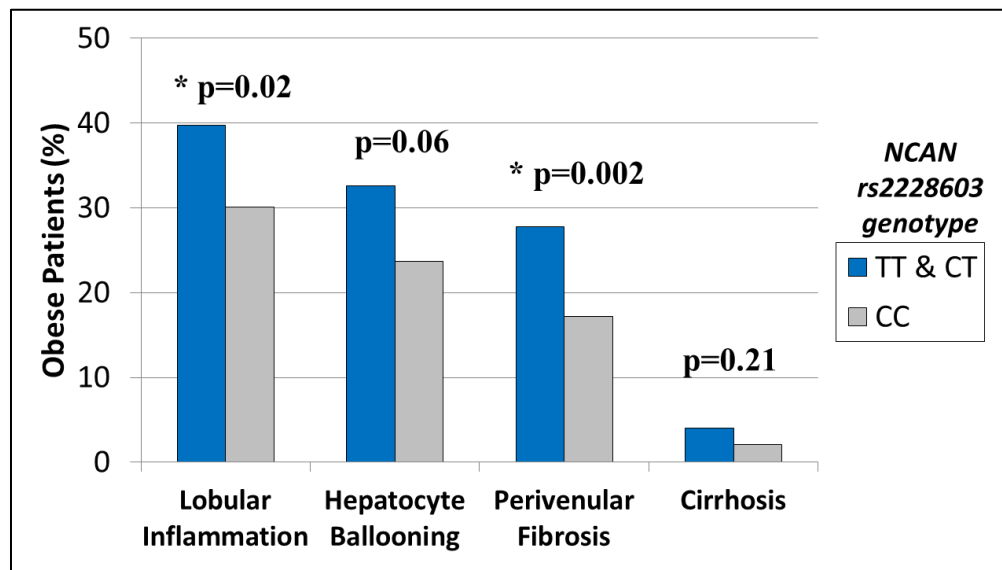


Figure 7: Association of rs2228603 with NAFLD Features

Though not statistically significant, rs2228603[T] also was associated with a trend toward increased hepatocyte

ballooning ($p = 0.06$; Figure 7; Table 2). These findings show that in extreme obesity, *PNPLA3* rs738409[G] and *NCAN* rs2228603[T] are associated with liver steatosis and an increased risk for progression from simple steatosis to NASH. By contrast, the variants in *LYPLAL1*, *GCKR* and *PPP1R3B* did not appear to show an increased risk for lobular inflammation, hepatocyte ballooning or perivenular fibrosis. Although there was only a trend toward statistical significance, the *PPP1R3B* SNP rs4240624[A] was the only variant associated with cirrhosis ($p = 0.06$). As observed in the GOLD Consortium study, the data from this bariatric surgery cohort revealed that some NAFLD-associated SNPs were associated with a distinct pattern of serum lipid levels (Table 3). These effects were most apparent in subjects with NAFLD. In NAFLD subjects, the NAFLD-associated G allele of rs738409 in *PNPLA3* was associated with lower total cholesterol ($p = 0.03$). (Table 3) Similarly, in NAFLD subjects, the NAFLD-associated T allele of rs2228603 in *NCAN* was associated with decreased serum total cholesterol ($p = 0.0002$), LDL-cholesterol ($p = 0.009$) and TG ($p = 0.004$). (Table 3) By contrast, as in the GOLD Consortium meta-analysis, the NAFLD-associated T allele of *GCKR* SNP rs780094 was associated with increased serum TG levels; this effect was most evident in subjects

with NAFLD ($p = 0.04$). (Table 3) Also consistent with the GOLD Consortium's findings, we did not find any significant association between either *LYPLAL1* SNP rs12137855 or *PPP1R3B* SNP rs4240624 and serum lipids.

SNP	780094			2228603		1213785			4240624		738409		
Effect allele	T			T		C			A		G		
Other allele	C			C		T			G		C		
Nearest gene	GCKR			NCAN		LYPLAL1			PPP1R3B		PNPLA3		
Genotypes	TT N=186	CT N=522	CC N=384	TT & CT N=155	CC N=928	CC N=696	CT N=325	TT N=99	AA N=883	AG & GG N=172	GG N=664	CG N=370	CC N=54
tchol, mg/dL (mean)	195	187	189	180	191	190	187	191	190	190	192	187	181
SE	3	2	2	3	1	2	2	6	1	3	2	2	6
p-value*	0.21			0.0002		0.65			0.96		0.03		
LDL, mg/dL (mean)	108	106	107	101	108	107	105	109	107	108	108	106	100
SE	3	2	2	3	1	1	2	5	1	3	1	2	5
p-value*	0.91			0.009		0.73			0.79		0.09		
HDL, mg/dL (mean)	48.6	47.3	47.6	46.7	47.8	47.9	47.1	49.2	47.7	48.3	48.3	46.7	46.9
SE	0.8	0.5	0.5	0.9	0.4	0.4	0.6	1.9	0.4	0.8	0.5	0.5	1.5
P-value*	0.27			0.32		1.00			0.60		0.05		
TG [§] , mg/dL (mean)	193	179	172	162	183	179	181	181	181	171	184	174	167
SE	9	6	7	7	4	4	8	28	4	8	6	5	13
p-value*	0.12			0.004		0.72			0.76		0.50		

Table 3: Association of SNPs with Serum Lipid Features

Discussion

Using an independent sample of 1,092 well-phenotyped morbidly obese subjects who underwent bariatric surgery and in whom liver histology was determined, we found that despite similar BMI values, subjects with NAFLD had a higher prevalence of diabetes, hyperlipidemia and a greater waist circumference than those without NAFLD.¹¹⁶ These data support that NAFLD is associated with insulin resistance and other features of the metabolic syndrome largely independent of obesity. Our association analyses of 5 NAFLD-associated genetic variants, initially identified by the GOLD Consortium genome-wide association meta-analysis, identified a non-synonymous variant in *PLPLA3* to be associated not only with the presence of steatosis but also with NASH, a more progressive and clinically ominous manifestation of NAFLD.¹¹⁶ These findings extend previously reported studies in the general population to patients with extreme obesity. The results are also consistent with other studies in which the G allele of *PLPLA3* rs738409 was not only associated with hepatic fat accumulation, but also with the degree of liver steatosis as evaluated by liver biopsy.¹¹⁷⁻¹¹⁹ In other studies, the same allele was associated with steatosis, portal inflammation, lobular

inflammation, Mallory-Denk bodies, NAFLD activity score, and fibrosis.^{90,103,104}

Furthermore, we replicated the GOLD Consortium's association of the T allele of rs2228603 in *NCAN* with the degree of liver steatosis as well as with signs of lobular inflammation and perivenular fibrosis, which are consistent with NASH.¹¹⁶ For *GCKR* rs780094 and *PPP1R3B* rs4240624, although not statistically significantly associated with NAFLD, the direction of effect and the effect size in our cohort were similar to the GOLD Consortium's results.¹¹⁶ It is likely that the inability to achieve statistical significance is due to a lack of power to discern the effect of variants of modest effect size. The absence of even a trend for association of *LYPLAL1* rs12137855 with NAFLD may be due to differences among populations, a false negative finding, or a false positive finding in the initial GOLD Consortium study. Assuming a 24% population prevalence of NAFLD, our sample had 80% power to detect an OR of 1.48 for presence of steatosis for a variant with allele frequency of at least 0.05 at $p < 0.05$. Using a higher population prevalence of 50%, which may be more suitable for a bariatric surgery population, our sample had 80% power to detect an OR of 1.29 for a variant with an allele frequency of at least 0.05 at $p < 0.05$.

Our data revealed that the *PPP1R3B* SNP rs4240624 was not significantly associated with liver steatosis, lobular inflammation, or hepatocyte ballooning.¹¹⁶ However, this variant was the only SNP in our study that showed a trend toward association with cirrhosis ($p = 0.07$). In the GOLD Consortium meta-analysis, *PPP1R3B* was associated with fatty liver identified on CT scan, but it was not associated with histological features of NAFLD. As hypothesized by Speliotes et al (2011)⁹², this may suggest that *PPP1R3B* is related to liver steatosis but not hepatocyte inflammation and fibrosis. Alternatively, the SNP may be associated with the intrahepatic accumulation of a substance that resembles fat accumulation on abdominal radiographic imaging and causes cirrhosis (i.e. glycogen, which accumulates in glycogen storage disease type IV that is characterized by glycogen precipitation in the liver causing cirrhosis).

Our data also showed distinct patterns of serum lipids for some NAFLD-associated variants. The GOLD Consortium meta-analysis found no association between lipid levels and *PNPLA3* rs738409[G]. Our data suggest a relationship between the NAFLD-associated G allele of this SNP and lower levels of total and HDL-cholesterol in the full cohort ($p = 0.03$).¹¹⁶ This may be a type I error or alternatively due

to a difference in ascertainment since all subjects in our study were selected for extreme obesity. With regard to serum TG, there was no association between this SNP and serum TG levels in the overall cohort, but there was a trend toward an association with lower TG levels in patients with NAFLD ($p = 0.08$).¹¹⁶ In support of this finding, the relationship between this SNP and serum TG levels has been reported by others.^{91,120} As in the GOLD Consortium meta-analysis, we found a relationship between NAFLD-associated *NCAN* rs2228603[T] and lower serum LDL-cholesterol, total cholesterol and TG levels, particularly in those with NAFLD.¹¹⁶ Interestingly, this association was not found in equally obese patients without NAFLD, suggesting that NAFLD and lower serum lipids may be mechanistically linked in rs2228603[T] carriers. Since NAFLD is clinically associated with higher serum lipids, this seemingly paradoxical finding suggests that more than one NAFLD subtype may exist. Elucidation of the causative gene/variant(s) at this locus may uncover a novel disease mechanism in which sequestration of TG in the liver, either as a result of increased TG uptake and/or decreased TG lipolysis, results in lower serum TG levels. By contrast, NAFLD-associated *GCKR* rs780094[T] was associated with higher serum TG¹¹⁶; this finding is consistent with the

findings of the GOLD Consortium meta-analysis as well as other studies.¹²¹⁻¹²³ Finally, we did not detect any association between the *LYPLAL1* or *PPP1R3B* SNPs and serum lipids.¹¹⁶ It is possible that the lack of association is due to an inadequate sample size and power, especially for *PPP1R3B*, in which the GOLD Consortium reported increased LDL- and HDL-cholesterol.

One of the limitations of our study is that our cohort was predominantly composed of Caucasian subjects. It would be important to use a more ethnically diverse population since studies have shown ethnic differences in hepatic steatosis.^{124,125}

Neurocan, the protein product of *NCAN*, is a chondroitin sulfate proteoglycan primarily expressed in the nervous system and is thought to be involved in cell adhesion and migration.¹²⁶⁻¹²⁹ SNP rs2228603 is located in exon 3 of *NCAN* and encodes a non-conservative non-synonymous mutation (Pro92Ser), which is predicted by the software tool PolyPhen-2 to alter protein structure and function.¹³⁰ While neurocan itself has not yet been shown to be expressed in the liver or to directly affect lipid metabolism or hepatic steatosis, it is becoming increasingly recognized that the central nervous system (CNS) is an important regulator of peripheral glucose and

triglyceride metabolism.¹³¹⁻¹³³ The liver is highly innervated by both sympathetic and parasympathetic nerves.¹³⁴ Bruinstroop et al. have shown that postprandial serum triglyceride levels were significantly elevated in parasympathetic or sympathetic denervated rats compared to sham-operated animals.¹³⁵ Robertson et al. demonstrated that vagal stimulation increases hepatic secretion of very low density lipoprotein (VLDL) triglyceride.¹³⁶ Since *NCAN rs2228603* has been associated with increased liver fat and decreased serum triglyceride^{92,137}, it is plausible that this variant is also associated with a brain-liver axis that, when deregulated, increases the risk for NAFLD. Furthermore, this axis may be modulated by increased dietary fat intake, as suggested by a 50% increase in hepatic VLDL synthesis after fat intake.¹³⁸ Further evidence for a central control of the liver lipid metabolism comes from studies of neuropeptide Y (NPY), a 36-amino acid peptide neurotransmitter secreted by the hypothalamus that has glucose and lipid regulatory effects in the liver. Fasting rats treated with intracerebroventricular injections of NPY had a 2.5-fold increased VLDL secretion into the bloodstream.¹³⁹ Intracerebroventricular administrations of an NPY-Y5 receptor agonist reproduced this effect, while an NPY-Y1

receptor antagonist decreased VLDL secretion. These findings demonstrate that the CNS can control VLDL secretion. However, the *NCAN* locus contains at least 20 genes in a 500-kb region on chromosome 19p13.^{137,140,141} Therefore, fine mapping of this locus and functional studies of the neighboring genes are needed to determine if they play a role in NAFLD.

In summary, in light of the increased risk for NAFLD conferred by obesity, we have extended the findings by the GOLD Consortium to include a large population of bariatric surgery patients in whom liver steatosis, inflammation, and fibrosis had been documented histologically. Specifically, we have shown that variants in *PNPLA3* and *NCAN* are associated not only with liver steatosis, but also NASH, a more progressive and clinically ominous manifestation of NAFLD. *NCAN* is a gene expressed in brain and thus may identify an untapped potential role for the CNS in the mechanism of fatty liver. Alternatively variants in other genes at this locus may be responsible for the NAFLD phenotype. Fine mapping and functional studies will be required to identify the causative genes/variants, which may provide insight into a novel pathway for NAFLD development, leading to new strategies for the prevention and treatment of this disease. In addition, follow-up

analyses of the effects of gastric bypass surgery on liver steatosis, inflammation and dyslipidemia may provide additional insight into the potential role of these loci as determinants of metabolic responses to weight loss.

CHAPTER 4: GENE EXPRESSION

Introduction

Although the *NCAN* SNP rs2228603 has been associated with NAFLD, *NCAN* is not highly expressed in liver tissue. Therefore, we sought to determine if liver mRNA levels of other genes at this locus are associated with liver steatosis and rs2228603 genotype. The premise that the *NCAN* locus is associated with hepatic lipogenesis is supported by a recent RNA interference (RNAi) study of genes from lipid GWA studies that implicated several genes from the *NCAN* locus in cholesterol regulatory function.¹⁴² RNAi and high-content screening microscopy were used to profile 133 genes at 56 loci associated with blood lipid traits, cardiovascular disease, and/or myocardial infarction for roles in regulating cellular free cholesterol levels and LDL uptake. A high number of trait-associated genes were found to have conserved cholesterol-regulatory function in cells, with several GWAS loci, including the *NCAN* locus, harboring multiple genes of likely functional significance.¹⁴² For *GATAD2A*, siRNA knockdown positively correlated with decreased cellular levels of LDL receptor, a major determinant of blood LDL levels. For *NCAN*, *HAPLN4*, *TM6SF2*, and *GATAD2A*, siRNA

knockdown positively correlated with increased levels of cellular total free cholesterol.¹⁴² Therefore, we sought to characterize hepatic gene expression of 3 genes from the *NCAN* locus - *HAPLN4*, *GATAD2A*, and *TM6SF2*. (Figure 8)

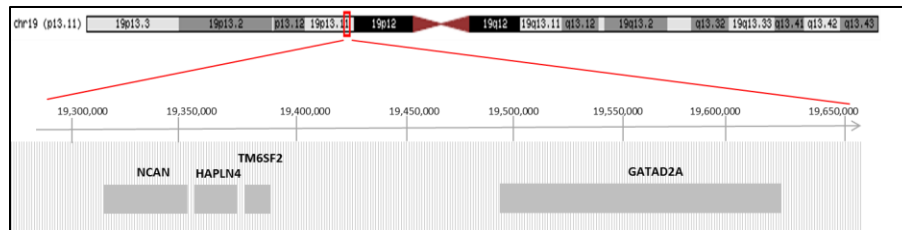


Figure 8: NCAN Locus Genes

HAPLN4

Hyaluronan and proteoglycan link protein 4 (HAPLN4) belongs to the four member HAPLN gene family. Collectively, the four HAPLN genes are expressed in most vertebrate tissues, suggesting that the hyaluronan-dependent extra-cellular matrix is essential to the structure and function of various tissue types. Each HAPLN gene is physically linked to one of the large aggregating CSPG genes and they are all organized similarly with the signal peptide, Ig domain and two proteoglycan tandem repeats encoded on separate exons.¹⁴³ The *HAPLN4* gene is 7156 bases long, comprised of 5 exons, and is physically linked to the *NCAN* gene.^{143,144} Based on the primary amino acid sequence, all four HAPLN proteins are predicted to

bind hyaluronic acid; HAPLN1 and HAPLN2 have been shown to bind to hyaluronan (HA).¹⁴⁵ Complexes of hyaluronan and chondroitin sulfate proteoglycan (HA-CSPG aggregates) can interact with other extracellular matrix and cell surface components through binding interactions of the large CSPG core protein, the chondroitin sulfate and/or the HA chain. Therefore, the HA-CSPG aggregate may provide an important nucleus around which an extensive matrix can be organized.

Like *NCAN*, *HAPLN4* is expressed primarily in CNS tissue¹⁴³, but it is also found in the CD44 receptor and mediates adhesive interactions during inflammatory leukocyte homing¹⁴⁶. CD44 is the primary receptor for HA and has several functions, including attaching the extracellular matrix to cell surfaces and controlling the migration of activated lymphocytes to sites of inflammation¹⁴⁷. CD44 has a single Link module that forms part of its HA-binding domain.¹⁴⁸⁻¹⁵⁰ CD44 is expressed by most cells, including macrophages and hepatocytes, and has been implicated in many biological processes, including cell adhesion and inflammatory cell migration.¹⁵¹⁻¹⁵³ CD44 has also been identified on activated hepatic stellate cells and therefore may reflect extracellular matrix metabolism and hepatic inflammatory activity.¹⁵⁴

CD44 expression is elevated in liver and white adipose tissue (WAT) during obesity¹⁵⁵, suggesting a possible regulatory role for *CD44* in hepatic steatosis and subsequent inflammation. Kang et al (2013) showed that *Cd44*-deficient mice had a reduced susceptibility to the development of high fat-diet-induced hepatic steatosis.¹⁵⁶ This was associated with decreased expression of genes involved in FA synthesis and transport, de novo TG synthesis and TG accumulation as well as decreased expression of various inflammatory and cell matrix genes.¹⁵⁶ In addition, it has been demonstrated that mice fed a lipogenic diet have elevated *Cd44* expression on monocytes, T-cells and dendritic cells, while *Cd44*^{-/-} mice were protected from inflammation when fed a lipogenic diet feed.¹⁵⁷ HA binding of leukocytes was absent early during lipogenic diet feeding but significantly increased by week 4 in the susceptible mice.¹⁵⁷ Finally, it has been shown that hepatic expression of the *CD44* gene is upregulated in morbidly obese NASH patients.¹⁵⁸

GATAD2A

Because NAFLD alters gene expression, it may affect hepatic protein and enzyme levels. The regulation of such abnormal patterns can be determined by epigenetic

mechanisms, the most studied of which is DNA methylation on CpG sites that are commonly present in high concentrations in gene promoter regions. DNA methylation works with histone modification to regulate gene activity, and these regulatory mechanisms control the level of gene transcription.¹⁵⁹ *GATAD2A* (also known as *p66α*) is 123,103 bases long and encodes the GATA zinc finger domain containing 2A, a 68 kDa transcriptional repressor that interacts with the methyl-CpG-binding domain.^{144,160} The methyl-cytosine binding domain 2 (MBD2)-nucleosome remodeling and deacetylase (NuRD) complex comprises at least one homolog of six core proteins, including *p66α*.¹⁶⁰ The MBD2-NuRD complex recognizes methylated DNA and silences expression of associated genes via histone deacetylase and nucleosome remodeling functions. Structural work has shown that a coiled-coil interaction between the MBD2 and the *GATAD2A/p66α* proteins recruits the chromodomain helicase DNA-binding protein to the NuRD complex and is necessary for MBD2-mediated DNA methylation-dependent gene silencing in vivo.¹⁶¹

DNA methylation depends on the availability of methyl groups from S-adenosylmethionine (SAM).¹⁶² Dietary methyl groups come from foods containing folate, choline and betaine. Dietary depletion of these methyl donors

decreases hepatocellular SAM concentration while dietary supplementation increases SAM concentration.¹⁶² Animals fed a diet deficient in methyl donors have hypomethylated DNA; these changes occur not only in global methylation but also in the methylation of specific genes.¹⁶³⁻¹⁶⁵ DNA methylation changes modulate susceptibility to obesity as well as the effect of diet-induced liver injury.^{166,167}

The role of epigenetic changes in the development of hepatic steatosis was studied by feeding mice a lipogenic methyl-deficient diet that causes liver injury similar to that seen in human NASH. The development of hepatic steatosis was accompanied by prominent epigenetic abnormalities, including severe loss of genomic cytosine methylation.¹⁶⁸ In another study, rats fed an obesogenic high-fat-sucrose diet developed liver steatosis, while methyl donor supplementation with a cocktail containing betaine, choline, vitamin B12 and folic acid exerted a protective effect on liver fat accumulation.^{169,170} The methyl donor supplementation associated decrease in liver steatosis was associated with increased methylation of genes involved in obesity and lipid metabolism, including *Fasn*, *Lepr*, and *Srebf2*.¹⁷⁰ After showing that DNA methylation of the *MTTP* promoter was selectively increased in the livers of patients with NAFLD, Chang et al (2010)

demonstrated that berberine was able to reverse high fat diet-induced hypermethylation of the *MTTP* promoter, reverse abnormal expression of *MTTP*, and alleviate high-fat diet-induced hepatic steatosis by increasing secretion of TG-rich VLDL particles from the liver.⁸⁵

It has been shown that a methyl-depleted high fat diet can downregulate hepatic *MTTP*, the protein needed for assembly and secretion of apoB-containing lipoproteins. *MTTP* mRNA expression was negatively correlated with the DNA methylation of specific CpG sites.¹⁷¹ Alternatively, replenishing methyl stores with betaine supplementation decreased methylation of *MTTP* gene CpG sites and ameliorated high-fat diet-induced hepatic steatosis.¹⁷¹ Therefore, decreased *MTTP* mRNA due to gene methylation may play a role in the pathogenesis of hepatic steatosis.

Genome wide association studies have identified a single nucleotide polymorphism near *GATAD2A* that contributes to the risk for type 2 diabetes which, like NAFLD, is associated with the metabolic syndrome.¹⁷² By testing comprehensive liver DNA methylation profiles in a large group of patients with either mild or severe NAFLD and correlating differences in liver DNA methylation with differences in liver gene expression, Murphy et al (2013)

discovered many functionally relevant methylation differences that distinguish mild from severe NAFLD.¹⁷³

TM6SF2

At the start of this research project, less was known about *TM6SF2*. It is a 9,028 base gene that encodes a 42 kDa protein that is a member of the transmembrane 6 superfamily.¹⁴⁴ Two isoforms of *TM6SF2* are produced by alternative splicing; the gene product is a transmembrane helical protein whose function is unknown.¹⁷⁴ As part of the EUROIMAGE Consortium sequencing project, Carim-Todd et al (2000) isolated and characterized a novel gene on chromosome 15 called *TM6SF1* that encoded a 370 amino acid product with enhanced expression in spleen, testis and peripheral blood leukocytes. They also identified *TM6SF2* on chromosome 19q12 as the paralogue to *TM6SF1*, with an overall similarity of 68% and 52% identity at the protein level.¹⁷⁴ The corresponding orthologous genes in mouse of human *TM6SF1* and *TM6SF2* showed a high degree of amino acid sequence conservation.

To further examine the role of individual *NCAN* locus genes in NAFLD, we measured hepatic mRNA levels from a subset of Geisinger bariatric patients with and without hepatic steatosis. Our aim was to determine if gene

expression levels of these candidate genes are associated with hepatic steatosis and *NCAN* SNP rs2228603 genotype.

Methods

Samples

During bariatric surgery in 1,092 Geisinger Medical Center patients, a wedge biopsy (250–300 mg) was obtained from the right lobe of the liver (10 cm to the left of the falciform ligament). A portion was flash-frozen in liquid nitrogen for subsequent analysis while the remainder was used for routine histology; NAFLD was diagnosed with standard histological criteria.¹⁷⁵ For this phase of the project, we selected 69 patients with extremes of fatty liver disease (either grade 0 with <5% steatosis or grade 3 with >66% steatosis) and grouped them according to NCAN SNP rs2228603 genotype. (Table 4)

		<i>Liver Steatosis</i>	
		Grade 0	Grade 3
<i>NCAN rs2228603 genotype</i>	CC	n = 22	n = 16
	CT	n = 19	n = 12

Table 4: Patients Grouped by Liver Steatosis Grade
& NCAN SNP Genotype

Quantitative reverse transcriptase PCR (qRT-PCR)

RNA was isolated from each liver sample with TRIzol and purified using the RNeasy Micro Kit (Qiagen, Venlo,

Netherlands). A 2 µg aliquot of DNase treated hepatic RNA was reverse transcribed into cDNA using the Reverse Transcription System (Promega, Madison, WI). Using Taqman probes, qRT-PCR was performed to determine mRNA expression levels of the 3 candidate genes in the *NCAN* locus. β -*actin*, a housekeeping gene, served as a positive control. The primers were custom designed by the manufacturer (Roche, San Francisco, CA). (Table 5)

Gene	NCBI Gene Reference	Context Sequence	Assay ID
TM6SF2	NM_001001524	CTGGAGGCATCGGCCAGGCACAGTT	Hs00403495_m1
HAPLN4	NM_023002	TGGACCTGGAAGGCGTGGTCTTTCC	Hs00604587_m1
GATAD2A	NM_017660	CCCCTCAGCACAGGCGGGACCCTTG	Hs00214293_m1
ACTB	NM_001101	CCTTTGCCGATCCGCCGCCCGTCCA	Hs99999903_m1

Table 5: Candidate Gene Primers

Standard reactions (7 µL) were assembled as follows: 5.0µL of PCR buffer Master Mix, 0.5µL of primer, 3.0µL of cDNA template and 1.5µL of Diethylpyrocarbonate (DEPC) water. For the negative controls, cDNAs were replaced by DEPC water. Each assay was carried out in triplicate using a LightCycler® 480 System real-time PCR platform (Roche, San Francisco, CA). Forty amplification cycles were performed, with each cycle consisting of 94°C for 15 seconds followed by 59°C for 1 minute. Each sample was analyzed in

triplicate. Amplification and dissociation curves generated by the LightCycler® 480 Software application were used for gene expression analysis. The $2^{-\Delta\Delta CT}$ method¹⁷⁶ was used to calculate relative differences in gene expression.

Statistical Analysis

All data were provided as the mean \pm standard error of the mean (SEM) and analyses were performed using Microsoft Excel. Student t-tests were applied to calculate statistical significance for the data sets; a p-value <0.05 indicated a statistically significant difference between groups.

Results

All 3 candidate genes in the locus - *HAPLN4*, *TM6SF2*, and *GATAD2A* - were expressed in the hepatic tissue of both patients with NAFLD and patients without NAFLD.

GATAD2A mRNA levels were higher in rs2228603 genotype CC patients without NAFLD (Group A) than in CT patients with NAFLD (Group D) ($p=2.97E^{-3}$). (Figure 9) It was unclear if this difference in mRNA level was associated with NAFLD or with rs2228603 genotype. If the T allele were associated with NAFLD, then the T allele would be associated with lower *GATAD2A* gene expression, as well. However, among all patients with genotype CC (Groups A and C), *GATAD2A* mRNA levels were lower in those with NAFLD (Group C) compared to those without NAFLD (Group A) ($p=5.75E^{-7}$). (Figure 9) This difference among CC homozygotes suggested that there was not a straightforward causal effect of the *NCAN* SNP T allele on liver steatosis phenotype or *GATAD2A* mRNA levels. Furthermore, among patients with genotype CT (Groups B and D), *GATAD2A* mRNA levels are actually higher in NAFLD patients (Group D) compared to those without NAFLD (Group B) ($p=8.46E^{-4}$). (Figure 9)

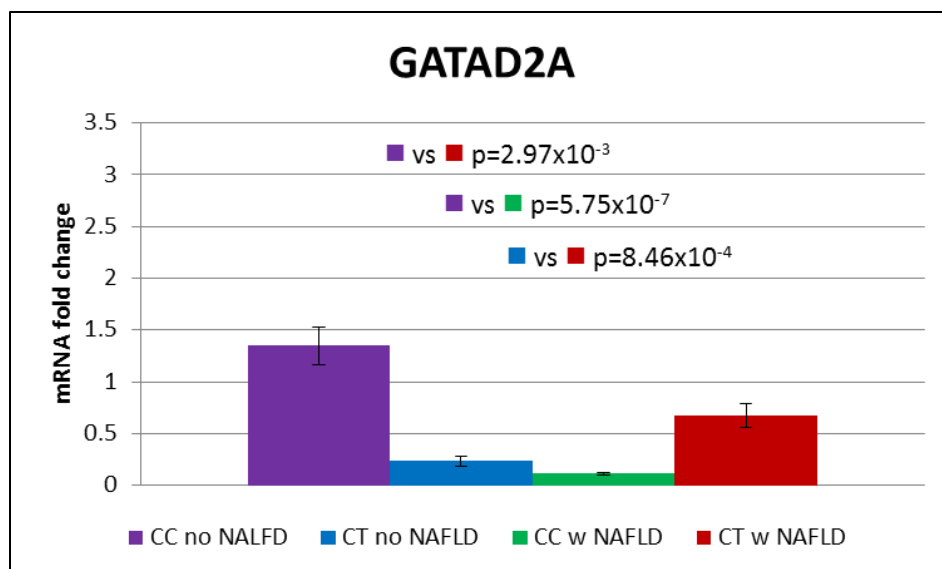


Figure 9: GATAD2A mRNA Hepatic Expression

There was no statistically significant difference in *TM6SF2* mRNA levels between genotype CC patient without NAFLD and genotype CT patients with NAFLD ($p=0.058$). (Figure 10) However, among patients with the CC genotype (Groups A and C), *TM6SF2* mRNA levels were lower in those with NAFLD (Group C) compared to those without NAFLD (Group A) ($p=3.05E^{-3}$). (Figure 10) This difference among CC homozygotes suggested that there was not a straightforward causal effect of the NCAN SNP T allele on liver steatosis phenotype or *TM6SF2* mRNA levels. These findings suggested an association between NAFLD and lower *TM6SF2* mRNA levels, however, among patients with genotype CT (Groups B and D), there was no difference in *TM6SF2* mRNA level between

patients with NAFLD (Group D) and patients without NAFLD (Group B) (p=0.10). (Figure 10)

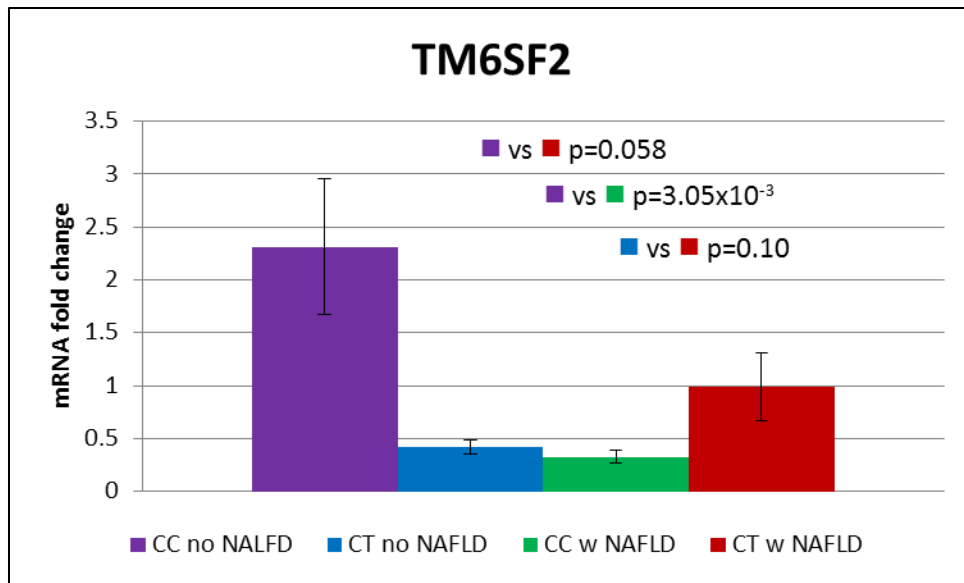


Figure 10: TM6SF2 mRNA Hepatic Expression

There were no statistically significant differences in *HAPLN4* mRNA levels between any of the groups. (Figure 11)

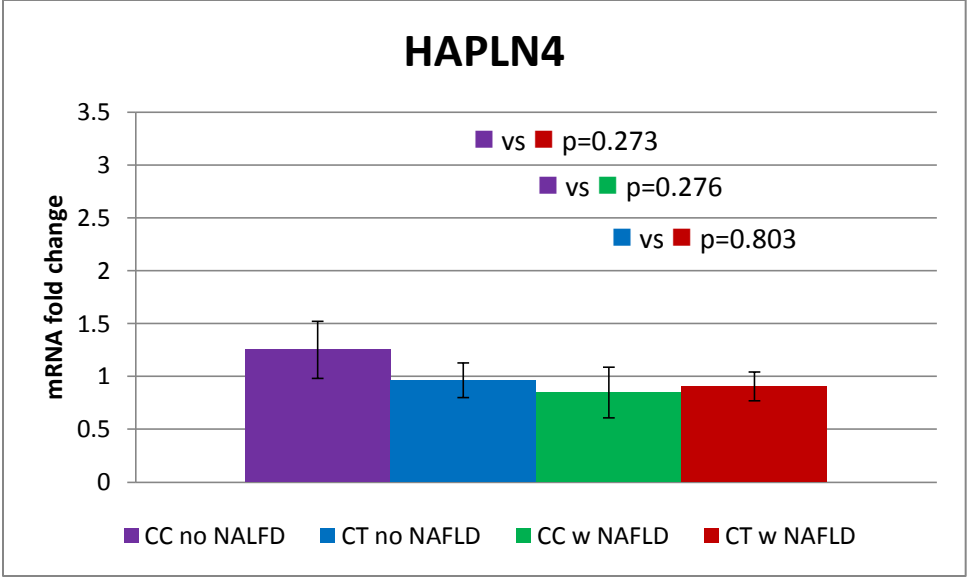


Figure 11: HAPLN4 mRNA Hepatic Expression

Discussion

Since *NCAN* is not expressed in the liver, the NAFLD-associated SNP rs2228603 is likely marking a neighboring gene that may be involved in the pathogenesis of liver steatosis. By comparing patients with extremes of hepatic steatosis that have been grouped based on *NCAN* SNP rs2228603 genotype, we showed that rs2228603 may affect the way in which levels of the *NCAN* locus genes *GATAD2A* and *TM6SF2* are associated with liver steatosis. For *GATAD2A*, lower levels were associated with steatosis among patients with the CC genotype, while higher gene expression levels were associated with steatosis among patients with the CT genotype. Similarly, lower *TM6SF2* expression levels were associated with steatosis among patients with the CC genotype; there was only a trend toward statistical significance for the relationship between higher expression levels and steatosis among patients with the CT genotype. *HAPLN4* mRNA levels did not differ among the four groups.

These gene expression studies identified *GATAD2A* and *TM6SF2* as potential contributors to liver steatosis, but they did not explain the underlying mechanism(s) whereby they promote or inhibit hepatic lipid accumulation. It is possible that the *NCAN* SNP associated with liver steatosis is an expression quantitative locus (eQTL), which is a

genomic locus that regulates mRNA expression levels and pattern. An example of this type of regulation is the lactase persistence phenotype that maps 5' distal to *LCT*, a gene coding for the small intestine lactase enzyme.¹⁷⁷ An intronic noncoding stretch of DNA without any known function contains a distal enhancer specific to enterocytes producing lactase.¹⁷⁸ However, one of the greatest difficulties in eQTL analysis is proving a causal relationship between genomic variation and phenotype. Many eQTL SNPs have been associated with disease phenotypes but the association does not indicate the role of individual variants.^{179,180} Nicolae et al (2010) determined that SNPs associated with complex human traits, like diabetes and obesity, are more likely to be eQTLs.¹⁸⁰ Therefore, genetic risk factors for complex traits often affect phenotype by altering the amount or timing of protein production rather than by changing the type of protein produced.

In a recent GWAS, Mirkov et al (2012) studied the potential function of nine SNPs associated with serum metabolomic traits.¹⁸¹ They correlated the mRNA levels and hepatic lipid content with genotypes in normal liver tissue. They also compared their findings with the previously published eQTL data. Four SNPs were identified that may affect the transcription of nearby genes in liver

tissue and two genes were identified that may influence the accumulation of lipids in the liver.¹⁸¹ When compared with published eQTL data, SNPs in four of the genes were also in linkage disequilibrium with eQTLs significantly affecting expression of these genes.¹⁸¹ These findings suggest that genetic variants affecting serum metabolite levels may play a functional role in the liver.

Our gene expression studies favor further study of *GATAD2A* or *TM6SF2*. The next step based on our gene expression results is to perform functional studies that alter the amount of protein produced by one of these candidate genes in order to elucidate its role in NAFLD pathogenesis. *TM6SF2* is further supported as a candidate based on a recent exome-wide association study in a multi-ethnic population that tested 138,374 SNPs for association with hepatic TG content in 2,736 participants. Only three SNPs reached exome wide significance: rs738409 and rs2281135 in *PNPLA3* and rs58542926 in *TM6SF2*.¹⁸² The latter is an adenine-to-guanine substitution coding nucleotide 499 that replaces glutamate at residue 167 with lysine.¹⁸² It was not associated with other hepatic steatosis risk factors, including BMI, insulin resistance or alcohol intake.¹⁸² Carriers of the *TM6SF2* variant encoding the p.Glu167Lys variant had elevated hepatic TG content and the

association remained significant after adjusting for insulin resistance and alcohol intake.¹⁸² Furthermore, the effect of the *TM6SF2* variant on hepatic TG content was independent of the effect caused by the *PNPLA3* SNP rs738409, and there was no statistical interaction between the two risk alleles.¹⁸² Although the SNP at the locus most strongly associated with hepatic triglyceride content in GWAS was the *NCAN* SNP rs2228603, the *TM6SF2* variant rs58542926 remained associated with hepatic TG content after conditioning on rs2228603 and other SNPs from the region.¹⁸² Conversely, conditioning on the *TM6SF2* SNP removed the association between *NCAN* SNP rs2228603 and hepatic TG content,¹⁸² suggesting that the *TM6SF2* SNP is the variant responsible for associating the region with NAFLD.

One of the limitations of the gene expression phase of our project is that the samples have been obtained from patients who have been on a pre-bariatric surgery low-calorie diet which can result in reduced liver size.^{183,184} Therefore, changes in hepatic mRNA levels of the candidate genes due to the pre-bariatric diet cannot be ruled out. To remove this possibility, liver biopsy would have to be performed prior to the preoperative diet. Another limitation is that the study relies on detection of mRNA

level changes to identify candidate genes. Changes in mRNA level do not always correspond with changes in protein level. So even though *HAPLN4* did not show changes in mRNA level between the different groups, there may still be variations in expression or function at the protein level.

In summary, these gene expression results did not definitively establish *GATAD2A* as either promoting or inhibiting liver steatosis. Several genome wide association studies have identified a relationship between a *GATAD2A* SNP and serum triglyceride levels.⁵ Therefore, one possible explanation for the indeterminate results is that serum TG levels may mediate the relationship between liver steatosis and gene expression. In addition, the results do not establish *TM6SF2* as either promoting or preventing liver steatosis. Perhaps stratifying patients by serum TG levels or based on the presence or absence of diabetes may reveal a consistent association between *TM6SF2* levels and liver steatosis. It is notable that the pattern is the same for both *GATAD2A* and *TM6SF2*, with high mRNA levels associated with steatosis for rs2228603 genotype CC and low mRNA levels associated with steatosis for rs2228603 genotype CT. This suggests that *NCAN* SNP rs2228603 may determine whether increased or decreased levels of *GATAD2A* and *TM6SF2* are associated with liver steatosis.

CHAPTER 5: FUNCTIONAL GENE KNOCKDOWN

Introduction

Zebrafish Model

The zebrafish (*Danio rerio*) shares many cellular and physiological characteristics with other vertebrates and has been used as a model in vertebrate genetics since the 1960s. Zebrafish are freshwater fish that belong to the cyprinid family in the class of Actinopterygii (ray-finned fish).¹⁸⁵ Because it has the conventional vertebrate body plan, the zebrafish is well suited for studies modeling human energy metabolism.¹⁸⁶ The zebrafish digestive system is highly homologous to the mammalian digestive system, containing a liver, pancreas, gall bladder and a linear segmented intestinal track with both absorptive and secretory function.¹⁸⁷ Until the zebrafish starts to feed around 5 days post fertilization, the sole source of energy is the maternally derived yolk composed of neutral lipids (triacylglycerol, wax and sterol esters) and polar phospholipids.^{188,189} Compared to mammalian models, the strengths of zebrafish as a model organism for studying vertebrate physiology include external fertilization,

relatively rapid development, optical transparency, transgenic feasibility, and genetic tractability.¹⁹⁰

Several zebrafish mutants with hepatic steatosis have been characterized. *Foie gras (fgr)*, was identified in a re-screening of a library of lethal mutations for liver size. The *fgr* mutant is a null mutation and causes hepatomegaly with hepatocyte nuclear degeneration and steatosis.¹⁹¹ The Fgr protein is the zebrafish ortholog of human TRAPPC11, which is involved in ER-to-Golgi trafficking.¹⁹² The *fgr* mutation has been shown to cause hepatic ER stress that is linked to hepatic steatosis.¹⁹³

The homozygous mutant, *dpt*, was discovered in a chemical mutagen-induced screen for defects in exocrine pancreas.¹⁹⁴ This zebrafish mutant exhibits abnormal pancreatic ductal network and acinar marker expression in addition to hepatic steatosis and liver degeneration.¹⁹⁵ The gene affected by this mutant is *ahcy*, which encodes S-adenosylhomocysteine hydrolase (SAH), an enzyme needed to generate methyl donors for use in numerous biological processes. The *dpt* mutation increases expression of genes encoding enzymes of de novo lipogenesis and activates inflammation by increasing TNF- α production.¹⁹⁵ The *hi559* mutant contains an inactivating insertion in the *cdipt* gene and impairs the synthesis of phosphatidyl inositol

(PI).^{188,196} Absence of PI in the liver causes hepatocyte ER stress marked by activation of the unfolded protein response along with macrovesicular steatosis, hepatocyte ballooning, apoptosis and altered mitochondrial morphology.¹⁹⁶ The red moon (*rmn*) mutant encodes the plasma membrane protein Slc16a6a, a transporter for beta-hydroxybutyrate, the major fuel of fasting (as the fasted organism uses up its hepatic glycogen stores, fatty acids and amino acids are partially oxidized into acetoacetate and its reduction product β -hydroxybutyrate). Mutation of this hepatocyte exporter of β -hydroxybutyrate causes rerouting of liver-trapped ketogenic precursors into triacylglycerol, which results in hepatic steatosis.¹⁹⁷

The zebrafish is emerging as a powerful model system for investigating the pathways that contribute to hepatic lipid metabolism and NAFLD. Several new genomic techniques have been developed to perform more rapid targeted gene disruption in the zebrafish. Many have appreciated the strengths of unbiased forward genetics approaches in which one seeks to identify genes involved in a process through the screening of populations of fish that contain random modifications throughout the genome; carriers of interesting modified alleles are identified by the observation of specific displayed phenotypes and subsequent

mapping of the allele within the genome reveals genes that are associated with the observed biological process.¹⁹⁸ But when a candidate gene has been identified, there is a role for reverse genetics, in which a gene of interest is altered or disrupted and the phenotypic consequences are observed.¹⁹⁹ One powerful tool frequently used for gene knockdown in the zebrafish is the Morpholino oligonucleotide.

Morpholino Oligonucleotides

The morpholino-modified antisense oligonucleotide (MO) causes transient gene knockdown by binding to RNA and inhibiting protein synthesis.²⁰⁰ MOs are approximately 25 bases in length and are made up of nonionic phosphorodiamidate backbone with morpholine rings substituted for ribose.²⁰¹ Both types of MO applications - splice blocking and translation blocking - inhibit protein synthesis.²⁰² Splice blocking MOs bind and inhibit pre-mRNA processing by inhibiting the spliceosome components that splice the primary transcript to mature mRNA. Translational blocking MOs bind complementary mRNA sequences in the 5' untranslated region to interfere with ribosome assembly near the translation start site.

In the zebrafish, morpholinos are microinjected into the yolks of 1- to 8-cell staged embryos.²⁰³ MOs are water soluble and are very resistant to a wide range of proteases and nucleases; therefore, they are difficult to eliminate from the cell.²⁰⁴ They have excellent RNA-binding affinity and antisense efficacy. MO specificity is ensured by reversing the effect with an RNA "rescue" in which synthetic mRNA encoding the protein from the targeted locus is injected into the yolk of 1-2 cell embryos.²⁰⁵ Embryos injected with both the targeting MO and with the gene-specific mRNA, are compared to those injected with MO alone and those injected with mRNA constructs alone.²⁰²

MOs have been used to study liver steatosis in the zebrafish. Her et al (2013) used zebrafish to explore the role of CHOP-10, a protein induced by the transcription factor YY1, in liver steatosis. Transgenic zebrafish from lines expressing YY1 were injected with antisense MOs designed to block translation of *CHOP-10* mRNAs. Embryos injected with *CHOP-10* morpholino showed significant hepatic TG accumulation (as indicated by ORO staining) compared to uninjected embryos and embryos injected with control *CHPO-10* MO.²⁰⁶ To rescue the knockdown phenotype, the *CHOP-10* MO was co-injected with in vitro-transcribed zebrafish *CHOP-10* mRNA; the injection either completely or partially balanced

the morpholino effect on triglyceride accumulation of CHOP-10 morphants.²⁰⁶ Moreover, hepatic FA accumulation was associated with induction of FA-oxidizing systems that increased hepatic lipogenesis and oxidative stress.²⁰⁶ This study successfully used gene knockdown to reproduce the fatty liver phenotype.

We performed gene knockdown studies on *Tm6sf2* in the zebrafish to determine if we could reproduce hepatic lipid accumulation. We also determined if knockdown of *Tm6sf2* is associated with changes in the expression of genes involved in hepatic lipogenesis (*ACC*, *FASN*, *SCD1*), fatty acid oxidation (*CPT1*), and lipid transport (*CD36*, *FABP6*).

Methods

Morpholino Sequences and Microinjection

An antisense MO was used for translational knockdown of *Tm6sf2* in zebrafish embryos. The MO was designed to target the 5' UTR of the zebrafish *Tm6sf2* gene and was purchased from Gene Tools (Philomath, OR): ACCACTGGCCTGAAAATACAAAACA. The MO was diluted with Danieau buffer (58 mM NaCl, 0.7 mM KCl, 0.4 mM MgSO₄, 0.6 mM Ca(NO₃)₂, 5 mM HEPES, pH 7.6) to a concentration of 0.3 mM. Zebrafish eggs were fixed onto a 1.5% agarose plate and the MOs were microinjected into the embryos at the 1- or 2-cell stage using a PV820 Pneumatic Pico Pump microinjector (World Precision Instruments, Sarasota, FL). Uninjected embryos as well as embryos injected with five-nucleotide mismatch MOs served as negative controls. To demonstrate reversal of the MO effects, embryos were coinjected with the *Tm6sf2* MO and *Tm6sf2* mRNA (*Tm6sf2* from 6 days post fertilization (dpf) zebrafish-embryo total mRNA were cloned into the pGEM-T Easy vector [Promega, Milan, Italy]; the linearized plasmids were used as templates to transcribe synthetic mRNAs with the SP6 MEGAscript transcription kit [Ambion, Austin, TX]).

The efficacy of the MO was confirmed with qualitative PCR of *Tm6sf2* in samples from uninjected control fish and fish injected with the *Tm6sf2* MO. Standard reactions were formulated with optimal amounts of PCR buffer Master Mix, primer, cDNA template, and DEPC water. For the negative controls, cDNAs was replaced by DEPC water. Forty amplification cycles were performed with the following cycling conditions: 2 min at 50 °C and 10 min at 95 °C, followed by 45 cycles of 10s at 95°C, 5s at 60°C and 7s at 72°C. Amplification was confirmed by agarose electrophoresis stained with ethidium bromide.

Morphology

For whole-mount analysis of Oil Red O (ORO) staining, 5-10 larvae from each treatment group were fixed in 4% paraformaldehyde, washed with phosphate buffered saline (PBS), infiltrated with propylene glycol, and stained with 0.5% ORO in 100% propylene glycol. Stained larvae were washed with propylene glycol followed by several rinses with PBS and transferred to 80% glycerol. The stained larvae were viewed on a stereomicroscope and images were captured using a Zeiss AxioCam camera. Using image analysis software (ImageJ 1.45s, National Institutes of

Health, Bethesda, MD, USA), the liver was outlined and the percentage of liver area stained by ORO was quantified.

qRT-PCR of lipogenesis genes

For each lipogenesis-related gene, 10-20 zebrafish embryos were pooled from each treatment group: uninjected embryos, control MO injected embryos, *Tm6sf2* MO-injected embryos, and MO rescued embryos (co-injected with the *Tm6sf2* MO and *Tm6sf2* mRNA). RNA was isolated from pooled hepatic tissue using TRIzol and then purified using the RNeasy Micro Kit (Qiagen, Venlo, Netherlands). A 2 µg aliquot of DNase treated hepatic RNA was reverse transcribed into cDNA using the Reverse Transcriptase System (Promega, Madison, WI). Using Taqman probes, qRT-PCR was performed to determine mRNA expression levels of 5 genes involved in lipid homeostasis and cell death/stress²⁰⁷; *β-actin*, a housekeeping gene, served as a positive control. (Table 6)

Gene	NCBI Gene Reference	Forward primer	Reverse primer
Cd36	NM_001002363	5'-TGCGGCTCGCCATCATGACC-3'	5'-TCATGTCGCCACCGGGATG-3'
Fabp6	NM_001002076	5'-CCCGCTCTTCTTCTCCGCTCA-3'	5'-GTCACGGCCCTTTGCGATGACA-3'
Acc1	XM_001343907	5'-GCGTGCGCCGAACAATGGCAG-3'	5'-GCAGGTCCAGCTTCCCTGCG-3'
Fasn	XM_682295	5'-GGAGCAGGCTGCTCTGTGC-3'	5'-TTGCGGCCTGTCCACTCCT-3'
Scd1	NM_198815	5'-TTGCACTGCGTCCCGATGCC-3'	5'-GGCTCGTCGTCGGCAACCTC-3'
b-act	AF057040	5'-CCTGTATGCCAACACAGTGC-3'	5'-GAAGCACTTCTGTGAACGA-3'

Table 6: Hepatic Lipid Metabolism Gene Primers

Standard reactions were formulated with optimal amounts of PCR buffer Master Mix, primer, cDNA template, and DEPC water. For the negative controls, cDNAs was replaced by

DEPC water. Each assay was carried out in triplicate using a LightCycler® 480 System real-time PCR platform (Roche, San Francisco, CA). Forty amplification cycles were performed with the following cycling conditions: 2 min at 50 °C and 10 min at 95 °C, followed by 45 cycles of 10s at 95°C, 5s at 60°C and 7s at 72°C. Each sample was analyzed in triplicate. Amplification and dissociation curves generated by the LightCycler® 480 Software application were used for gene expression analysis. The $2^{-\Delta\Delta CT}$ method¹⁷⁶ was used to calculate relative changes in gene expression.

Statistical Analysis

All data were provided as the mean \pm standard error of the mean (SEM) and analyses were performed using Microsoft Excel. Student t-tests were applied to calculate statistical significance for the data sets; given the small sample size of each treatment group, the more stringent p-value <0.01 was used to indicate a statistically significant difference between groups.

Results

Morphology

Whole mount staining showed that larvae had differing percentages of hepatic lipid accumulation depending on the treatment group. Values that differed by more than 1.5 standard deviations from the group median percentage were considered outliers; one outlier was removed from the control MO group and one outlier was removed from the *Tm6sf2* MO group. The mean percentage of liver with ORO staining was 16.8% for the 5 uninjected larvae, 7.6% for the 4 control MO injected larvae, 41.8% for the 4 *Tm6sf2* MO injected larvae, and 9.8% for the 5 rescue injected (*Tm6sf2* MO + *Tmsf2* mRNA) larvae. (Table 7)

% Liver Stained with ORO				
	Uninjected	Control MO	MO	Rescue
fish 1	5.67	8.29	31.79	6.84
fish 2	26.51	6.03	57.62	17.84
fish 3	10.57	7.02	39.71	10.55
fish 4	14.50	9.26	38.03	8.08
fish 5	26.94			5.47
mean	16.84	7.65	41.79	9.76
s.d	9.55	1.42	11.09	4.89

Table 7: Percentage of Zebrafish Liver with ORO Staining

Uninjected fish had similar amounts of hepatic lipid staining as fish injected with the control MO ($p=0.098$). (Figures 12 & 13) However fish injected with the *Tm6sf2* MO had more hepatic lipid staining than uninjected fish ($P=0.012$) and more hepatic lipid staining than fish injected with the control MO ($p=0.008$). (Figures 12 & 13) Rescue fish injected with both the *Tm6sf2* MO and *Tm6sf2* mRNA had similar hepatic lipid staining as uninjected fish ($p=0.191$) and similar hepatic lipid staining as fish injected with control MO ($p=0.403$). (Figures 12 & 13) Compared to rescue fish, fish injected with the *Tm6sf2* MO had more hepatic lipid staining ($p=0.006$). (Figures 12 & 13) These findings suggest that the *Tm6sf2* MO causes hepatic lipid accumulation in the zebrafish embryo.

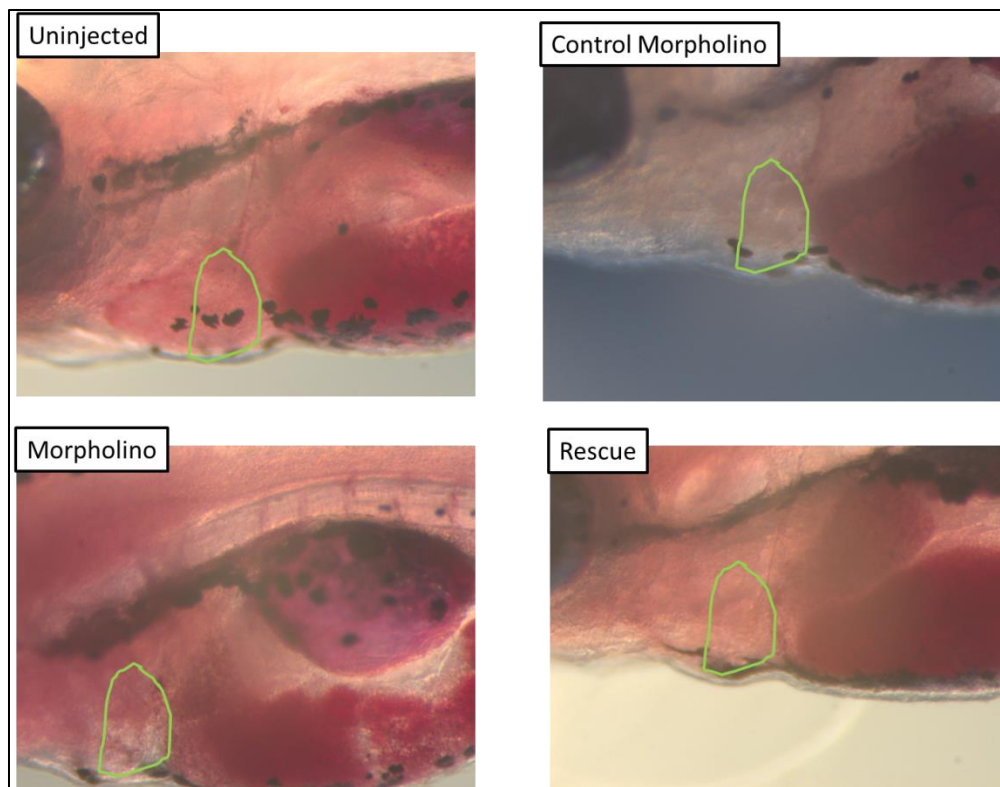


Figure 12: Zebrafish Hepatic Whole Mount ORO Staining (120X magnification)

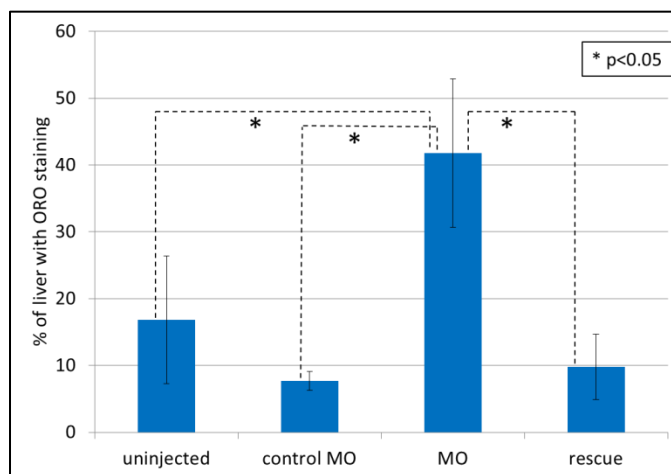


Figure 13: Comparison of Percentage of ORO Staining in Zebrafish Liver

The efficacy of the morpholino was confirmed by RT-PCR (data not shown).

Expression of Hepatic Lipid Metabolism Genes

We next examined the effect of *Tm6sf2* knockdown on hepatic lipid metabolism gene expression. For *Cd36* mRNA (Figure 14), levels were higher in fish injected with control MO compared to uninjected fish ($p=0.009$); this suggests non-specific MO effects. Furthermore, there was no difference in mRNA levels between fish injected with control MO and fish injected with *Tm6sf2* MO, ($P>0.01$); this also suggests nonspecific MO effects on *Cd36* mRNA level. Because of the nonspecific MO effects, the effects of *Tm6sf2* knockdown cannot be determined.

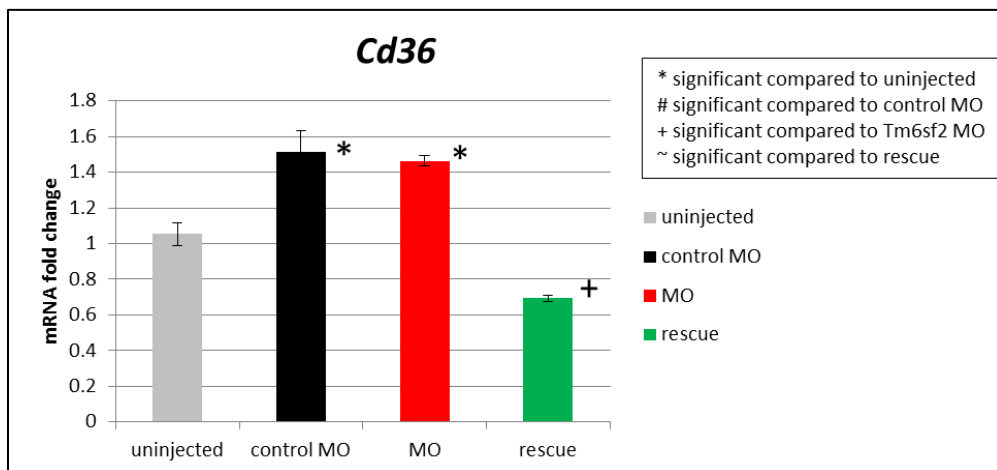


Figure 14: Zebrafish Whole Body *Cd36* mRNA Expression

For *Fabp6* mRNA (Figure 15), there was no difference between uninjected fish and fish injected with control MO ($p>0.01$), indicating that there were no non-specific MO effects. Levels were higher in fish injected with *Tm6sf2* MO than in uninjected fish ($p=0.002$). This indicates that *Tm6sf2* knockdown increased *Fabp6* expression. There was no difference between uninjected fish and rescue fish ($p>0.01$), indicating that reversal of the *Tm6sf2* MO effect with *Tm6sf2* mRNA returned *Fabp6* mRNA levels back to baseline. *Fabp6* mRNA levels were higher in fish injected with *Tm6sf2* MO than in fish injected with control MO ($p=0.003$), confirming that specific knockdown of *Tm6sf2* increased *Fabp6* expression. There was no difference in *Fabp6* mRNA level between fish injected with control MO and rescue fish ($p>0.01$), and levels were higher in fish injected with *Tm6sf2* MO than in rescue fish ($p=0.002$). Overall, specific knockdown of *Tm6sf2* increased *Fabp6* mRNA level.

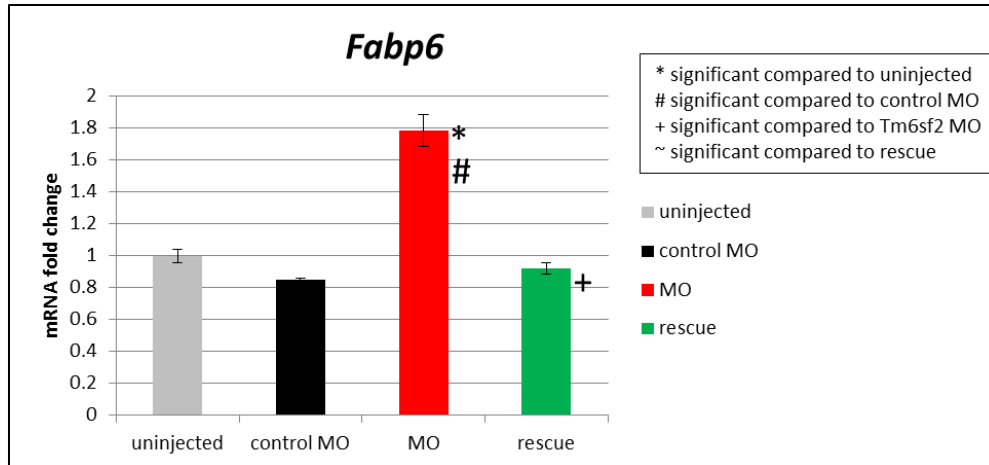


Figure 15: Zebrafish Whole Body *Fabp6* mRNA Expression

For *Cpt1* mRNA (Figure 16), there was no difference between uninjected fish and fish injected with control MO ($p=0.01$). This suggests that there were no non-specific MO effects. *Cpt1* levels were higher in fish injected with *Tm6sf2* MO than in uninjected fish ($p=0.0001$). This suggests that *Tm6sf2* knockdown increased *Cpt1* expression. There was no difference between uninjected fish and rescue fish ($p>0.01$), suggesting that reversal of the *Tm6sf2* MO effect with *Tm6sf2* mRNA returned *Cpt1* mRNA levels back to baseline. *Cpt1* mRNA levels were higher in fish injected with *Tm6sf2* MO than in fish injected with control MO ($p=0.002$), suggesting that specific knockdown of *Tm6sf2* increased *Cpt1* expression. Furthermore, *Cpt1* mRNA levels were higher in fish injected with *Tm6sf2* MO than in rescue fish ($p=0.001$). However, *Cpt1* mRNA levels were higher in

fish injected with control MO than in rescue fish (p=0.002), suggesting the presence of non-specific effects of the MO. Because of the nonspecific MO effects, the effect of *Tm6sf2* knockdown on *Cpt1* expression cannot be established.

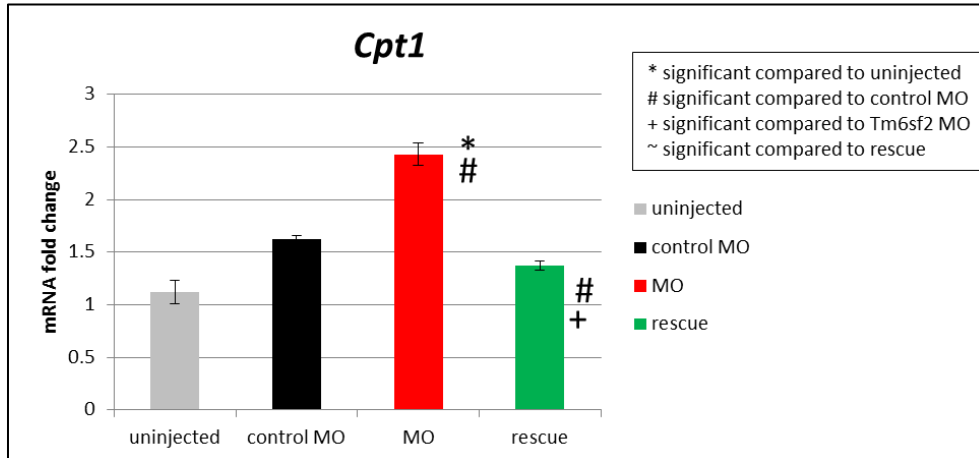


Figure 16: Zebrafish Whole Body *Cpt1* mRNA Expression

For *Acc1* mRNA (Figure 17), levels were higher in fish injected with *Tm6sf2* MO than uninjected fish (p=0.001), suggesting that *Tm6sf2* knockdown increased *Acc1* expression. In addition, levels were higher in fish injected with *Tm6sf2* MO than fish injected with control MO (p=0.001). This suggests that specific knockdown of *Tm6sf2* increased *Acc1* expression. However, there were higher *Acc1* mRNA levels in fish injected with control MO than uninjected fish. (p=0.001), suggesting that there were non-specific MO effects. There was no difference between fish injected

with control MO and rescue fish ($p > 0.01$), and levels were higher in fish injected with *Tm6sf2* MO than in rescue fish ($p = 0.002$). However, levels were higher in rescue fish than in uninjected fish ($p = 0.001$), suggesting that the mRNA did not completely rescue *Acc1* levels. Overall, because of the nonspecific MO effects, the effect of *Tm6sf2* knockdown on *Acc1* expression cannot be verified.

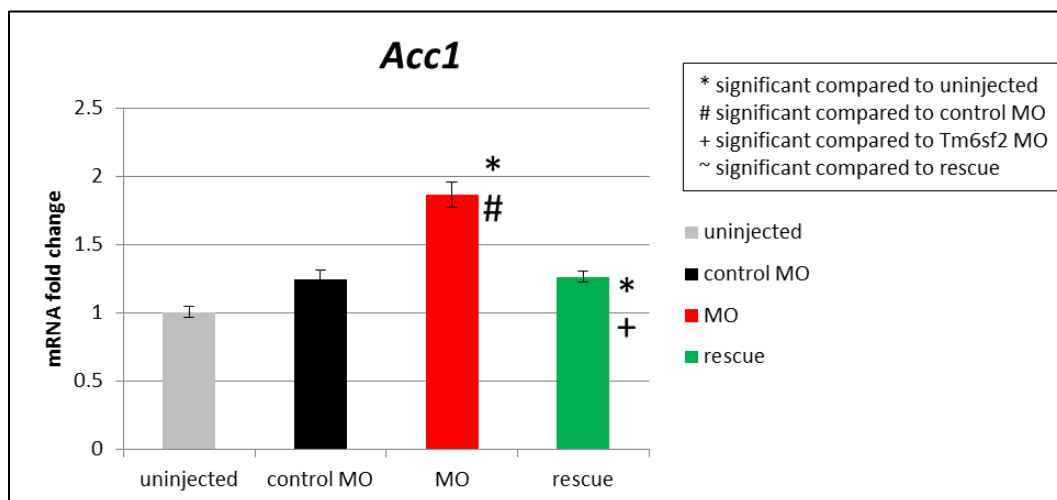


Figure 17: Zebrafish Whole Body *Acc1* mRNA Expression

For *Fasn* mRNA level (Figure 18), there was no difference between uninjected fish and fish injected with control MO ($p > 0.01$). This indicates that there were no non-specific MO effects. There was no difference in *Fasn* mRNA level between uninjected fish and fish injected with *Tm6sf2* MO ($p > 0.01$). This indicates that *Tm6sf2* knockdown does not increase *Fasn* expression. Furthermore, there was no difference between fish injected with control MO and

fish injected with *Tm6sf2* MO ($p>0.01$). This suggests that *Tm6sf2* knockdown does not increase *Fasn* expression. In addition, there was no difference between fish injected with *Tm6sf2* MO and rescue fish ($p>0.01$), which confirms a lack of effect of the *Tm6sf2* MO on *Fasn* mRNA level.

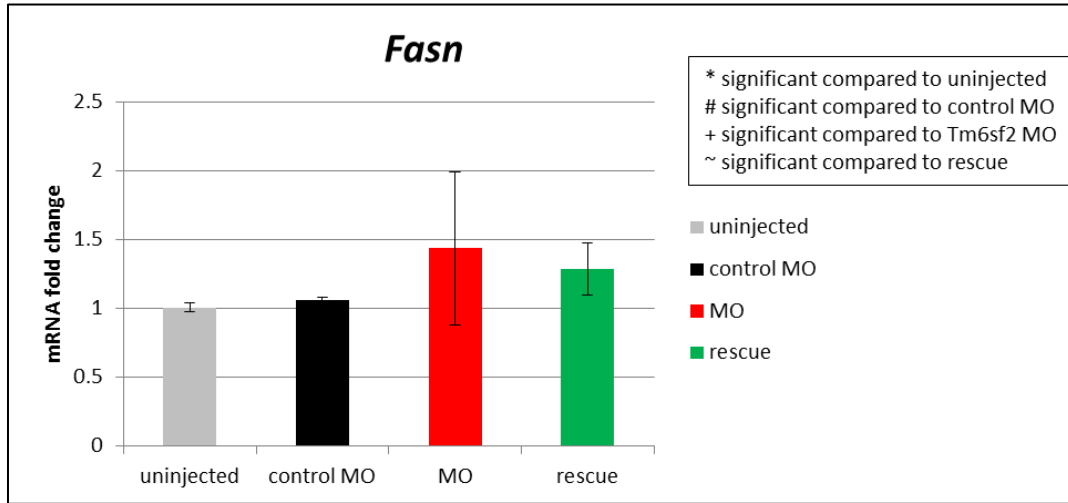


Figure 18: Zebrafish Whole Body *Fasn* mRNA Expression

For *Scd1* (Figure 19), mRNA levels were higher in fish injected with control MO than in uninjected fish ($p=0.004$). This suggests non-specific MO effects. Therefore, conclusions cannot be drawn regarding the effect of *Tm6sf2* knockdown on *Scd1* expression.

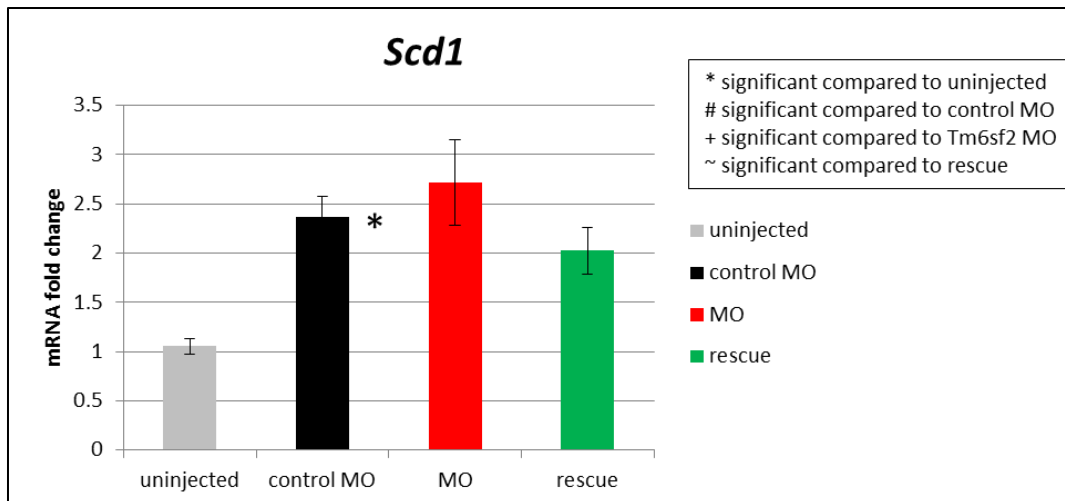


Figure 19: Zebrafish Whole Body *Scd1* mRNA Expression

Discussion

To further analyze the functional role of *TM6SF2* in hepatic steatosis, we used morpholino oligonucleotides to perform gene knockdown studies in the zebrafish model. We found that knockdown of the *TM6SF2* gene reproduced the liver steatosis phenotype. Along with the hepatic lipid accumulation, there was increased expression of *Fabp6*, a gene involved in fatty acid uptake and transport.

Kozlitina et al (2014) recently showed that the *TM6SF2* p.Glu167Lys substitution (rs58542926) was associated with NAFLD.¹⁸² Although *TM6SF2* protein is predicted to have seven transmembrane domains²⁰⁸, the specific biological function is not known. Human wild-type and mutant *TM6SF2* proteins were expressed in HuH-7 cells to determine the effect of the variation on *TM6SF2* expression and localization. While levels of *TM6SF2* mRNA were similar in cells expressing wild-type and mutant *TM6SF2*, levels of the mutant protein were reduced by 46%.¹⁸² To assess the effect of loss of *TM6SF2* function on hepatic TG content, recombinant adeno-associated viral vectors expressing short hairpin RNAs (shRNAs) were used to selectively decrease *Tm6sf2* transcript levels in the livers of mice. Hepatic inhibition of *Tm6sf2* increased hepatic TG content and decreased plasma total cholesterol and TG levels, while

mean serum ALT levels were unchanged.¹⁸² Because the combination of higher hepatic TG content and lower serum TG levels suggests a defect in VLDL secretion, the effect of *Tm6sf2* knockdown on VLDL secretion was examined by inhibiting intravascular lipoprotein lipase, which hydrolyzes the TG content of VLDL. Hepatic TG accumulation was markedly reduced in the knockdown mice compared to controls.¹⁸² These results suggest that wildtype *TM6SF2* may promote VLDL secretion and that the increased hepatic TG content associated with the *TM6SF2* variant results from a decrease in protein function.

To further assess whether *TM6SF2* may play a role in VLDL secretion from the liver, Holmen et al looked specifically at the functional effects of the *TM6SF2* p.Glu167Lys variant on serum lipid levels.²⁰⁹ After using an exome array to genotype 80,137 coding variants in over 5,000 subjects, 18 variants were followed up in 4,666 patients. They identified ten gene loci, including *TM6SF2*, with coding variants associated with a lipid trait.²⁰⁹ Most of these genes had well established functional roles in lipid metabolism, but *TM6SF2* had not been previously associated with blood lipid levels. The *TM6SF2* p.Glu167Lys variant reached genome wide significant association with serum total cholesterol.²⁰⁹ This SNP was in strong linkage

disequilibrium with the GWAS index SNP rs10401969 at the *NCAN* locus on chromosome 19p13.²⁰⁹ In this cohort, the *TM6SF2* rs58542926[T] variant showed stronger evidence for association with serum cholesterol than *NCAN* SNP rs2228603, which previously had been associated with both NAFLD and serum lipid levels. In this sample, the total cholesterol associations for the p.Glu167Lys *TM6SF2* coding variant and the GWAS index SNP rs10401969 were considered indistinguishable after conditioning on the *TM6SF2* variant since the total cholesterol association at the GWAS index SNP lost significance.²⁰⁹ This suggests that the *TM6SF2* variant is responsible for the association signal detected in the *NCAN* locus by the GWAS. To further determine whether *TM6SF2* might be the functional gene, they used northern blotting and western blotting to determine the distribution pattern of *Tm6sf2* in mice. Endogenous *Tm6sf2* mRNA and protein were both highly expressed in the liver, suggesting a potential role for *TM6SF2* in hepatic lipid metabolism.²⁰⁹ In subsequent functional studies, they transiently overexpressed human *TM6SF2* in mice using a liver-targeted adenovirus. In *TM6SF2*-overexpressing mice compared to controls, total cholesterol, LDL, and TG levels were increased while HDL levels were decreased.²⁰⁹ They also tested the impact of knockdown of *Tm6sf2* by transient

transduction of short hairpin RNA (shRNA) against *Tm6sf2* in mice using an adenovirus. The protein levels of TM6SF2 were decreased by 49% in the liver of *Tm6sf2* knockdown mice and fasting total cholesterol levels were decreased by 18.2% compared to controls.²⁰⁹ Since overexpression of *TM6SF2* increased total cholesterol and knockdown of *Tm6sf2* decreased total cholesterol, and the minor human allele is associated with reduced cholesterol, the results suggest that the variant results in decreased function of TM6SF2. However, neither *TM6SF2* overexpression nor *Tm6sf2* knockdown altered hepatic TG accumulation or serum ALT levels.²⁰⁹

Our *Tm6sf2* knockdown analysis of lipogenesis-related genes in zebrafish suggests a role for fatty acid binding proteins (FABPs) in NAFLD via TM6SF2. Because FAs are insoluble, they require chaperones like FABPs to transfer them throughout various cellular compartments.²¹⁰ There are several FABP isoforms and they are expressed throughout tissues that are involved in FA metabolism. Nine FABP protein-coding genes have been identified in the human genome, including liver (FABP1 or L-FABP), intestine (FABP2 or I-FABP) and ileum (FABP6 or Il-FABP).²¹⁰ These different isoforms were first named for the organ in which they were first identified or predominated, but their expression profiles are not exclusive to that specific organ. For

example, FABP1 is not only expressed in the liver, but also in the intestine, pancreas, kidney, lung and stomach. *FABP1-3*, 6 and 7 all exist on separate chromosomes in the human, whereas *FABP4*, 5, 8 and 9 all co-localize at chromosome 8q21.²¹⁰

Our functional results specifically suggest a role for *FABP6* in hepatic steatosis. *FABP6* is highly expressed in the ileum, and to a lesser extent in ovaries, placenta and the adrenal glands.²¹¹ *FABP6* binds both bile acids and FAs and interacts with the ileal bile acid transporter protein to aid in bile acid uptake and trafficking.²¹² Because the ileum plays a critical role in the enterohepatic circulation of bile acids, ileal FABP may affect hepatic lipid handling and other metabolic syndrome-related processes. For instance, in a fat assimilation study a variant of *FABP6* (Thr79Met) was the most significant marker associated with type 2 diabetes²¹³, with the Met allele of the Thr79Met substitution showing a decreased risk for diabetes.²¹⁴

Venkatachalam et al (2012) showed that feeding FAs to zebrafish induced the transcriptional upregulation of the steady-state level of *Fabp2* transcripts in the intestine and *Fabp3* transcripts in the liver.^{215,216} The modulation of *Fabp6* transcription by FAs in zebrafish implicates control

of these genes by PPAR interaction with peroxisome proliferator response elements (PPRE), most likely in *Fabp* promoters. Many putative PPREs were identified in the 5' upstream region and introns of the zebrafish *Fabp2*, *Fabp3* and *Fabp6* genes by silico analysis.²¹⁵

Therefore, it would be worthwhile to further analyze FABPs that are more highly expressed in the liver, particularly FABP1 (L-FABP). The promoter region of the *FABP1* gene contains several response elements involved in fat metabolism. The most understood of these is the PPRE, which contains a repeat sequence that binds and is activated by PPAR- α .²¹⁷ *FABP1* is upregulated by PPAR- α ligands²¹⁸ and FABP1 has been shown function in hepatic LCFA uptake, oxidation, esterification and secretion.²¹⁹ Mammalian studies have shown that gene transcripts of *FABP1* and *FABP2* in the liver and small intestine, respectively, are up-regulated by dietary FAs and peroxisome proliferators.²²⁰⁻²²⁴ A putative PPAR-binding site is located upstream of the *FABP2* transcription initiation site, where gene induction by benzafibrate is PPAR- α dependent.²²⁵ In addition, a sterol response element (SRE) has been found in the *FABP2* gene that is highly homologous with an SREBP bind site found in the promoters of the LDL receptor, HMG-CoA synthase and glycerol 3-phosphate acyltransferase.²²⁶

High fat feeding is a potent stimulator of hepatic FABP expression.²²⁷ *FABP1*^{-/-} mice were resistant to high fat diet induced liver TG infiltration compared to wildtype mice.²²⁸ Studies suggest that the *FABP1* and hepatic *MTTP* genes share the same transcriptional regulatory sequence in their promoter and their transcription is coordinately induced by PPAR- α and RXR- α agonists²²⁹; coordinated upregulation of both proteins would delivery sufficient FAs to the VLDL assembly and secretion pathway while their inhibition would decrease VLDL secretion without causing hepatic FA accumulation.^{230,231} Spann et al (2006) showed that simultaneous transcriptional repression of the two lipid transfer proteins, L-FABP and MTTP, which cooperatively shunt fatty acids into de novo lipogenesis and the transfer of lipids into VLDL respectively, act together to maintain hepatic lipid homeostasis and prevent TG accumulation.²²⁹

One limitation of our study is the small number of fish used for whole mount quantification of hepatic steatosis. In addition, ORO staining of histological sections of zebrafish liver would provide a more precise quantification of lipid accumulation. Another limitation of the study is that CD36 is the only other FA transport gene that we measured for mRNA expression. It would be

beneficial to check for changes in mRNA levels of other FA transport genes such as *slc25a10*, ApoA-IV, and *slc35b4*.

In summary, our results suggest a role for *TM6SF2* in NAFLD pathogenesis. Since *Tm6sf2*-knockdown-induced liver steatosis was associated with increased *Fabp6*, a FA transporter, the mechanism by which TM6SF2 may cause hepatic lipid accumulation may involve increased FA uptake that overwhelms the VLDL pathway of exporting TG from the liver.

CHAPTER 6: CONCLUSION

Because obesity is considered a risk factor for NAFLD, we extended the findings of the GOLD Consortium GWAS meta-analysis by studying a large population of bariatric surgery patients with intra-operatively obtained liver biopsies. We showed that genetic variation in *NCAN* is associated not only with liver steatosis but also with features of steatohepatitis. Since *NCAN* is not highly expressed in liver, it is probable that the genetic variant in *NCAN* is marking the involvement of a nearby gene. To determine if expression of any of the genes in the *NCAN* locus varies based on liver steatosis and *NCAN* SNP rs2228603 genotype, we compared patients with extremes of hepatic steatosis who had been grouped based on *NCAN* SNP rs2228603 genotype. For two of the genes in the *NCAN* locus, *GATAD2A* and *TM6SF2*, lower mRNA levels were associated with liver steatosis in patients with the *NCAN* rs2228603 CC genotype while higher levels were associated with liver steatosis in patients with the CT genotype. These findings suggest that *NCAN* SNP rs2228603 may affect the way in which levels of the nearby genes *GATAD2A* and *TM6SF2* are associated with liver steatosis. To further analyze the role of *TM6SF2*, we performed functional studies

in the zebrafish model and found that knockdown of *Tm6sf2* reproduced the liver steatosis phenotype and simultaneously increased hepatic expression of *Fabp6*.

None of the human FABP proteins are located on chromosome 19 with *TM6SF2* but our findings could be built upon by considering FABP SNPs associated with liver steatosis or serum cholesterol levels. A highly conserved missense mutation in exon 3 of the human *FABP1* gene results in a Thr → Ala substitution at position 94; carriers for this SNP have been shown to have higher baseline plasma free FA levels, lower BMI and smaller waist circumference than T94 homozygotes.²³² The T94 mutant is also associated with elevated fasting serum TG and LDL-cholesterol levels.²³³ A SNP in *FABP2* located at codon 54 (Ala → Thr) is a missense variant associated with dyslipidemia, IR and obesity.²³⁴⁻²³⁶ Finally, in a population study, carriers of the T-87C variant in the *FABP4* gene had significantly lower fasting TGs.²³⁷ Although the variant of *FABP6* (Thr79Met) showed decreased risk for T2DM, it did not show associations with traits related to lipid metabolism.²¹³

FABP1 in particular may be involved in the progression from simple liver steatosis to steatohepatitis. Chen et al (2013) explored a role for *Fabp1* in lipid metabolism in both hepatocytes and stellate cells. They found that it

plays a cell-specific role in regulating lipid metabolism in murine hepatocytes and stellate cells, with implication for hepatic stellate cell activation and for the development and progression of diet-induced NAFLD.²³⁸ FABP1^{-/-} mice have been shown to be protected against Western diet-induced obesity and hepatic steatosis through a series of adaptations in both hepatic and extrahepatic energy substrate use.²²⁸

It also would be worthwhile to return to the Geisinger bariatric cohort and determine if there are differences in hepatic expression of FABP mRNA and protein. Specifically, there may be a more clear relationship between liver steatosis and FABPs. These data may be further enhanced by evaluating patients with intermediate grades of steatosis. In patients with grade 1 or grade 2 steatosis, a change in FABP expression that corresponds with liver steatosis grade would support a functional role in NAFLD. For example, in a microarray analysis of hepatic gene expression, Guillen et al (2009) studied mRNA expression of 15 genes involved in lipid metabolism and found that ten of them showed significant associations among their expressions and the degree of hepatic steatosis.²³⁹

However, the mechanism by which TM6SF2 affects FABPs to cause hepatic steatosis remains to be determined. The

recent findings of Kozlitina et al (2014) suggest a role for *TM6SF2* in decreasing VLDL secretion. The role of hepatic-derived VLDL is to deliver energy in the form of TG to peripheral tissues and this redistribution of TG to the periphery also protects the liver from the accumulation of excess TG that may be involved in lipotoxicity. Since *FABP1* causes increased hepatic FA uptake, it may not decrease VLDL secretion but instead may overwhelm the ability of the VLDL secretion pathway to export hepatic lipids and thus lead to hepatic lipid overload and subsequent lipotoxicity. It has already been demonstrated that FA administration can increase apoB100 secretion by decreasing its intracellular degradation,^{240,241} but at higher doses for longer periods, FAs induce dose- and duration-related induction of ER stress, which in turn inhibits apoB100 secretion.^{73,242} Therefore, it is possible that increased fatty acid uptake decreases VLDL secretion by inhibiting apoB100.

In summary, our fine mapping of the *NCAN* locus has identified a role for this locus in NAFLD that is associated with lower serum TG. Our gene expression studies of *NCAN* locus candidate genes have identified *TM6SF2* as a possible contributor to NAFLD pathogenesis and our functional studies are consistent with a role for

TM6SF2 in hepatic lipid accumulation, possibly via a FABP.
Further study of FABPs may help to identify a mechanism for
NAFLD in which TG is sequestered in the liver.

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