Nature, Nurture, and Nonalcoholic Fatty Liver Disease

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Dr Cohen received his Ph.D. from the University of Cape Town then did postdoctoral studies at UT Southwestern where he has remained ever since. His research focuses on the genetic basis of metabolic disorders that contribute common diseases such as heart disease and diabetes. Together with his scientific partner Dr. Helen Hobbs he has identified genes that play major roles in the metabolism of fats cholesterol and triglycerides and elucidated the biological roles of their protein products.

Purpose & Overview

The overall goal of this Grand Rounds lecture is to update physicians on new developments in Nonalcoholic Fatty Liver Disease (NAFLD) and the role of genetics in elucidating the pathogenesis of this disorder.

Objectives

i) To describe the identification of genes associated with hepatic steatosis and their implications for the pathogenesis of NAFLD.

ii) To highlight the limitations of interpreting observational studies, whether cross-sectional or longitudinal.

iii) To illustrate the utility of genetics in interpreting epidemiological correlations.

Triglycerides and energy storage

Early in eukaryote evolution, triglycerides (TGs) emerged as the preferred storage nutrient to buffer against fluctuations in energy demand and availability. The ubiquitous selection of TG for this role is due to two physicochemical properties:

i) TGs provide greater caloric density (9 kCal/g) than do carbohydrates (4.5 kCal/g) or proteins (4 kCal/g)
ii) TGs are insoluble in water so they can accumulate to high levels with no adverse osmotic or colloidal effects on cells.

In higher organisms, TGs are stockpiled in adipocytes and accumulate in other cell types only under unusual circumstances. For example, migratory birds store large quantities of TGs in the liver as an energy source in preparation for prolonged seasonal flights, a propensity that has been exploited to produce the culinary delicacy, fois gras. Like migratory birds, some humans who consume excess calories deposit fat in the liver. Unlike birds, fatty liver in humans is maladaptive and can have severe clinical consequences.

The spectrum of nonalcoholic fatty liver disease (NAFLD)

Nonalcoholic fatty liver disease (NAFLD) comprises a spectrum of disorders extending from steatosis to cirrhosis and hepatocellular carcinoma. Hepatic TG content can be quantified by noninvasive imaging modalities, but liver biopsies are required to determine the stage of the disease. Steatosis has been defined as a hepatic TG level exceeding the 95th percentile for lean, healthy individuals (i.e., > 55 mg per g liver), and pathologically as the presence of cytoplasmic TG droplets in more than 5% of hepatocytes. Histological sections containing only increased liver fat and lobular inflammation are often referred to as simple steatosis. If the histology also includes evidence of hepatocyte injury (hepatocyte ballooning and cell death) and/or collagen deposition (fibrosis), the condition is referred to as nonalcoholic steatohepatitis (NASH). It is not known if steatosis always precedes NASH or if simple steatosis and NASH are distinct disorders. Between 10-29% of subjects with NAFLD progress to cirrhosis within 10 years (*1*) and 4-27% of individuals with NASH-induced cirrhosis develop hepatocellular carcinoma (*2*).

The pathogenesis of hepatic steatosis in humans

Hepatic steatosis arises from an imbalance between TG acquisition and removal. TGs are assembled by coupling three fatty acids to a glycerol backbone via ester bonds. The fatty acids used for hepatic TG formation are derived from three sources: diet, *de novo* synthesis, and circulatin FFAreleased by adipose tissue. Dietary fats taken up in the gut are packaged into TG-rich chylomicrons and delivered to the systemic circulation. In rats, ~80% of the TG in chylomicrons is hydrolyzed by lipoprotein lipase (LPL), releasing free fatty acids (FFA) for uptake by peripheral tissues. The remaining ~20% is delivered to the liver (*4*). Extrapolating from these experiments, a typical American diet (100 g of fat) furnishes the liver with ~20-30 g of TG each day, equivalent to one-half of the total TG content of an average liver.

Feeding also promotes *de novo* synthesis of FFA from acetyl-CoA by increasing levels of insulin and the availability of substrate. Insulin stimulates sterol regulatory element-binding protein-1c (SREBP-1c), a transcription factor that upregulates the enzymes that catalyze lipogenesis (*5*). Glucose activates another transcription factor, carbohydrate responsive element-binding protein (ChREBP), which promotes lipogenesis and activates the gene encoding liver-type pyruvate kinase, a key regulatory enzyme in the formation of acetyl-CoA from glucose, thus providing substrate for FFA and TG synthesis (*5*).

During fasting, plasma levels of insulin fall, whereas levels of glucagon and epinephrine increase, stimulating TG hydrolysis in adipocytes. The first step in TG hydrolysis is catalyzed by adipocyte TG hydrolase (ATGL) (*6*). FFAs are released and transported to liver, mostly bound to albumin. FFAs in liver have three major fates: they can be re-esterified to TG and stored in lipid droplets, oxidized in mitochondria to produce energy and ketone bodies, or secreted as a constituent of very low density lipoproteins (VLDL). FFAs in liver are also incorporated into phospholipids and other lipids. The flux of FFA through the circulation amounts to ~100 g/day with 20% being extracted by the liver. Thus, the daily input of TG from the diet (20 g/day) and adipose tissue (20 g/day) approximates the entire TG content of the liver.

Observations in humans with rare monogenic disorders demonstrate that increased hepatic TG accretion by any of three pathways outlined above is sufficient to cause hepatic steatosis (*7*). Genetic disorders resulting in excessive food intake, such as leptin deficiency, or diseases that increase hepatic levels of glucose-6-phosphate, such as glycogen storage disease type 1a, cause massive hepatic steatosis. Patients with lipodystrophy have severe hepatic steatosis in part due to insufficient adipose tissue mass for TG storage. Since adipocytes are the sites of synthesis of both leptin and adiponectin, patients with lipodystrophy have low levels of both these proteins, which likely contribute to hepatic steatosis in this disorder (see below).

Genetic defects in proteins required to mobilize FFA from lipid droplets (ATGL or its cofactor CGI-58) or to oxidize FFA in mitochondria (the hydroxyacyl-CoA transferases) also cause hepatic steatosis (*7*). Mutations inactivating the mitochondrial aspartate-glutamate transporter (citrin) cause hepatic steatosis by disrupting the malate-aspartate shuttle.

The major export pathway for hepatic TGs is secretion into the blood as VLDL. Mutations in the structural protein of VLDL [apolipoprotein (apo)B], or in the protein that adds TG to the nascent lipoprotein particle in the ER (microsomal TG transfer protein, MTTP) cause fatty liver. Individuals heterozygous for inactivating mutations in *APOB* produce fewer VLDL particles and have a 3-fold increase in hepatic TG relative to normal individuals (*8*).

Whereas single-gene mutations are important in rare forms of severe steatosis, the increased prevalence of NAFLD in the general population is due to changes in the quantity and composition of food consumed. The cardinal role of obesity in the development of hepatic steatosis is evident from cross-sectional correlations. In the Dallas Heart Study, hepatic steatosis is uncommon among lean individuals (9% in individuals with BMI <25 kg/m^2), and highly prevalent among the severely obese (51% in individuals with BMI >35 kg/m^2) (Fig. 3). Food composition also influences hepatic fat deposition. Carbohydrates in general and fructose in particular play important roles. Fructose consumption routes dietary carbons directly to liver in a form that is primed to enter biosynthetic pathways, including *de novo* lipogenesis. Unlike glucose, circulating fructose is taken up almost entirely by liver (*9*). Since fructose is phosphorylated at carbon 1 rather than carbon 6, it cannot be used to synthesize glycogen but instead is quantitatively converted to glyceraldehyde-3-phosphate, providing substrate for *de novo* lipogenesis. Fructose intake has progressively increased and may contribute to the increasing prevalence of NAFLD.

The relationship between obesity, insulin resistance and hepatic steatosis.

In liver, insulin inhibits glucose production and promotes fatty acid synthesis. With the development of hepatic insulin resistance, the inhibitory effect of insulin on glucose production is diminished, whereas the stimulatory effect of insulin on lipogenesis is retained. As noted above, obesity and insulin resistance promote hepatic steatosis by increasing *de novo* lipogenesis and the delivery of FFA to liver from the diet and peripheral tissues. The relative contributions of the three pathways to hepatic steatosis in humans remains poorly defined: the only study that determined the source of

hepatic fat in humans found that 59% is derived from circulating FFA with lesser contributions from *de novo* lipogenesis (26%) and diet (15%)(*10*).

A major unresolved question is whether NAFLD is a *cause* or *consequence* of insulin resistance. The association between hepatic steatosis and insulin resistance led to the hypothesis that excess TG in liver causes insulin resistance. Hepatic steatosis and insulin resistance occur together in several strains of genetically-modified mice. However, the notion that hepatic steatosis causes insulin resistance is contradicted by observations in mice with defects in diverse pathways that cause hepatic steatosis without insulin resistance: mice with reductions in fatty acid mobilization, fatty acid oxidation, cytokine signaling, and choline synthesis maintain normal, or improved insulin sensitivity, despite hepatic TG accumulation (*11, 12*). TG may act as a marker for another molecule, such as diacylglycerol (DAG), long-chain acyl-CoA, or ceramide, that interferes with insulin action. Increased cellular levels of DAGs are associated with activation of an atypical PKC that alters phosphorylation of IRS-1, resulting in lower insulin-stimulated glucose transport activity (*13*). Some mouse models that develop both hepatic steatosis and insulin resistance, such as those with long-chain hydroxyacyl acyl-CoA dehydrogenase deficiency, have elevated DAG levels (*14*); however other strains of mice with elevated levels of TG and DAG are not insulin resistant. The dissociation between hepatic lipid content and insulin resistance is exemplified by mice overexpressing acyl-CoA:DAG acyltransferase 2, the enzyme that adds the final FA to DAG to form TGs, in the liver (*15*). These mice have a 17-fold increase in hepatic TG with proportional increases in DAG, ceramides, and unsaturated long-chain fatty acyl-CoAs, yet their glucose and insulin tolerance is normal. Mice lacking CGI-58, the co-factor for ATGL (Fig. 2), have increased insulin sensitivity despite a 4-fold increase in hepatic TG content, and 2-fold increases in hepatic DAG and ceramide (*12*). Thus, hepatic accumulation of TG, DAG, ceramide or long chain fatty acyl-CoA does not invariably produce insulin resistance, at least in mice. It remains possible that the localization of these lipids in specific pools within hepatocytes contributes to insulin resistance.

In humans, naturally-occurring mutations provide a powerful tool to untangle mechanistic relationships between highly correlated metabolic traits. If increased hepatic TG content causes insulin resistance, then individuals with genetic variants that promote hepatic steatosis should be at increased risk of developing insulin resistance. An increasing number of genetic defects have uncoupled these two variables. Individuals with inactivating mutations in *APOB* have increased levels of hepatic TG yet maintain normal insulin sensitivity (*8*). Patients with deficiency of ATGL or CGI-58 have severe steatosis but are not insulin resistant (*7*). In population-based studies, a genetic variant in *PNPLA3* that is associated with hepatic steatosis is not associated with insulin resistance (see below) (*3*). Recently, variants of *APOC3* were reported to be associated with both hepatic steatosis and insulin resistance (*16*), but this association did not replicate in a large population-based cohort (*17*). Taken together, these findings are not compatible with the hypothesis that TG accumulation in hepatocytes causes insulin resistance in humans.

Inter-individual variation in susceptibility to hepatic steatosis

Although obesity and insulin resistance are the most prevalent risk factors for NAFLD, hepatic fat content varies significantly among individuals with equivalent adiposity, indicating that other factors contribute to this condition. One of these factors is gender. Prior to age 60, men are significantly more likely to develop steatosis than women (*18*), but at older ages, women predominate. Another factor is ethnicity. Asian-Indian men are particularly susceptible to the development of hepatic steatosis (*19*). In Dallas County, hepatic steatosis is more common in Hispanics (45%) and less common among African-Americans (24%) than in individuals of European ancestry (33%) (*18*). The higher prevalence of hepatic steatosis in Hispanics is due in part to a higher prevalence of obesity and insulin resistance in this

population, but the lower prevalence in African-Americans cannot be explained by ethnic differences in body mass index (BMI), insulin resistance, ethanol ingestion, or medication use.

Genetic susceptibility to NAFLD

Hepatic steatosis, NASH, and cirrhosis cluster in families (*20*) with the heritability of NAFLD being estimated to be ~39% (*21*). The first genetic variant consistently associated with NAFLD is a missense mutation (I148M) in PNPLA3 (also called adiponutrin) (*3*). This variant was initially identified through a genome-wide association (GWA) study of 9,299 nonsynonymous sequence variations and the association with hepatic steatosis has been confirmed in many independent studies [for review, (*22*)]. The frequency of the susceptibility variant (PNPLA3-148M) in ethnic groups mirrors the prevalence of NAFLD and accounts for ~70% of the differences in frequency of hepatic steatosis between Hispanics, African-Americans and individuals of European descent (*3*).

Homozygotes for the risk allele in *PNPLA3* (MM) have a ~2-fold higher hepatic TG content. The magnitude of the effect is strongly influenced by adiposity. Among lean individuals [body mass index (BMI) <25 kg/m^2], the effect of the M isoform is modest, whereas in the very obese (BMI>35 kg/m^2) homozygosity for the M isoform is associated with a 3-fold higher level of hepatic TG. Thus, the combined effects of genetic variation in *PNPLA3* and obesity constitute a true gene-environment interaction. A similar interaction is apparent when the population is stratified by homeostasis model of assessment-insulin resistance (HOMA-IR), an index of insulin sensitivity (Fig. 3B). It is not known which of these two factors, obesity or insulin resistance, is the primary driver of hepatic TG accumulation.

PNPLA3 is most highly expressed in adipose tissue and liver and is transcriptionally regulated by insulin through a signaling cascade that includes LXR and SREBP-1c; hepatic PNPLA3 mRNA levels are reduced to nearly undetectable levels during fasting and increase 80-fold with refeeding in mice (*23*). Over 90% of the PNPLA3 in hepatocytes is located in lipid droplets, which are specialized organelles that participate in protein partitioning, trafficking and degradation (for review, see *24*) as well as lipid storage.

What is the normal physiological role of PNPLA3 in the lipid droplet and how does the I148M isoform perturb TG homeostasis? PNPLA3 is a member of the PNPLA family, most closely resembling ATGL (PNPLA2) (*6*), and has both TG hydrolase and transacylase activity (*25,26*). PNPLA3 is predicted to be a monotopic membrane protein with the catalytic residues buried in a hydrophobic pocket. A ring of positively-charged residues surrounding the hydrophobic pocket likely interact with polar head-groups of membrane phospholipids. The isoleucine at position 148 forms part of a hydrophobic cleft that is predicted to be the site of substrate binding (*26*). In the protein encoded by the risk allele, the side chain of the substituted methionine at residue 148 extends further into the cleft and may obstruct accessibility of bulky lipids to the catalytic dyad. Consistent with this model, the I148M substitution eliminates TG hydrolase activity *in vitro* (*26*).

A parsimonious interpretation of these results is that the I148M substitution causes a loss-of-function and that hepatic steatosis results from a failure to hydrolyze TG; however, several lines of evidence from mouse studies are not consistent with this interpretation. First, inactivation of *Pnpla3* in mice fails to increase hepatic TG content, even in animals challenged with a high-fat, high-sucrose diet (*27,28*). Second, overexpression of PNPLA3-I148M in the liver causes an *increase* in hepatic TG content (*26*), suggesting the I148M substitution confers a gain-of-function. Thus, the mutation may act as a dominant-negative and interfere with TG hydrolysis by another lipase (*26*). Alternatively, the substitution may inhibit the TG hydrolytic activity of PNPLA3, yet preserve acyl-transferase activity. It is also possible that the effects of PNPLA3 on hepatic TG are an indirect consequence of its primary

catalytic role. PNPLA3-I148M may alter the phospholipid composition of lipid droplets and adversely affect the droplet-cytoplasm interface.

Additional genes associated with NAFLD

A GWA study of hepatic steatosis performed in 7,176 subjects (*29*) revealed multiple loci associated with NAFLD. None of the newly identified genomic intervals contained genes associated with rare Mendelian syndromes of hepatic steatosis, such as *APOB*, *ATGL*, *CGI-58*, or genes associated with lipodystrophy. The genomic regions associated with hepatic steatosis in this study included *PNPLA3*, *NCAN and PPP1R3B* (Table 1). Analysis of an independent cohort with histologically-defined NAFLD by the same group found an association with *NCAN, GCKR*, and *LYPLAL1,* but not with *PPP1R3B.* Loss-of-function alleles at two of the new genes, *GCKR* and *PPP1R3B*, are predicted to increase levels of glucose-6-phosphate. The biological role of *LYPLAL1* is poorly defined. *LYPLAL1* encodes a lysophospholipase-like protein that is expressed in adipose tissue and is associated with BMI and fat distribution in humans (*29*). Elucidation of the roles of these newly-identified genes may provide new insights into the metabolic pathways that contribute to common forms of NAFLD in the population.

More recently, our laboratory performed an exome-wide association study of ~150,000 coding sequence variants. Three of these variants were associated with increased liver fat at the exome-wide significance level: two in PNPLA3, the gene we previously associated with NAFLD, and one (encoding p.Glu167Lys) in TM6SF2, a gene of unknown function located near *NCAN*. The Glu167Lys TM6SF2 variant was also associated with higher circulating levels of alanine transaminase, a marker of liver injury, and lower levels of LDL-cholesterol, triglycerides and alkaline phosphatase in three independent populations (n > 80,000). Recombinant Glu167Lys TM6SF2 produced 50% less protein than wild-type TM6SF2 when expressed in cultured hepatocytes. Knockdown of Tm6sf2 in mice (by adeno-associated virus-delivered shRNA) increased liver triglyceride content 3-fold and decreased VLDL secretion by 50%. Taken together, these data indicate that TM6SF2 activity is required for normal VLDL secretion and that impaired TM6SF2 function causally contributes to NAFLD.

What factors contribute to NAFLD progression?

The *PNPLA3* allele associated with steatosis is also associated with elevated serum levels of alanine transaminase (ALT) (*3, 30*), an enzyme released from injured hepatocytes, and with pathological features of NASH in biopsy samples (*31*). PNPLA3-I148M may also contribute more generally in the response of the liver to injury. In support of this notion, PNPLA3-148M greatly increases the odds of developing cirrhosis in alcoholics (*32*). The finding that PNPLA3 is associated with hepatic steatosis, NASH and cirrhosis provides compelling molecular evidence that NAFLD is indeed a disease continuum as shown in Fig. 1.

Longitudinal studies of NAFLD show that a significant number of individuals (up to 20-30%) improve between biopsies (*1*). The most compelling data suggesting that NAFLD can improve with intervention comes from the bariatric surgery literature. A recent meta-analysis of 15 studies, which included a total of 766 paired liver biopsies revealed that bariatric surgery improved steatosis in 92% of patients, improved steatohepatitis in 81%, and led to complete resolution in 70% (*39*). These data further support the theory that NAFLD is a disease continuum. To date, weight loss remains the most effective treatment.

Outlook

NAFLD is a major health problem arising from changes in diet. With recent advances in the treatment of hepatitis C, NAFLD is poised to become the primary indication for liver transplantation. Of

greatest concern is the increased prevalence of hepatic steatosis in children, where the disease may be more virulent (*41*).

Major questions remain regarding the epidemiology, pathogenesis, natural history, and treatment of this disorder. For example, why is the frequency of NAFLD reduced in individuals of African descent? Africans are particularly susceptible to disorders of glucose homeostasis associated with obesity, whereas they are relatively resistant to the effects on lipid metabolism. After controlling for body weight, Africans have lower plasma levels of TG and the men have higher levels of HDL-cholesterol than either Europeans or Hispanics. Elucidating the molecular basis for these ethnic differences may provide new therapeutic targets for the treatment of NAFLD and other components of the metabolic syndrome.

Improved methods for the early detection of NASH are also required. Hepatic steatosis can be diagnosed noninvasively but determining the presence of hepatic inflammation and fibrosis requires a liver biopsy, which is usually reserved for individuals with elevated levels of circulating liver enzymes. While having an elevated ALT increases the likelihood of having NASH, up to 59% of individuals with hepatic steatosis and normal ALTs have NASH on biopsy (*42*). Some patients with NAFLD present for the first time with cirrhosis. Development of noninvasive methods that detect inflammation and fibrosis in the liver are needed to more fully capture the natural history of this disorder and to test therapeutic approaches designed to halt or reverse disease progression. A further impediment towards the development of new therapeutic interventions is the lack of an animal model that recapitulates all the features of NAFLD (*11*). In the absence of an animal model that fully recapitulates the human disease, genetic studies in humans provide the best opportunity for delineating the molecular pathways that lead to steatosis, steatohepatitis and cirrhosis.

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Table 1. Loci associated with hepatic steatosis and NAFLD in a genome-wide association study (*29*).

		Steatosis n=7,176		**NAFLD** n=592 cases/1,405 controls	
Gene	**Protein**	**MAF**	**P-Value**	**Odds Ratio**	**P-Value**
PNPLA3	Patatin-like phospholipase domain-containing protein 3	0.23	4.3×10^{-34}	3.26	3.6×10^{-43}
PPP1R3B	Glycogen binding subunit of protein phosphatase 1	0.08	3.7×10^{-18}	0.93	NS
NCAN	Neurocan	0.07	1.2×10^{-11}	1.65	5.3×10^{-5}
GCKR	Glukokinase regulatory protein	0.39	NS	1.45	2.6×10^{-8}
LYPLAL1	Lysophospholipase-like 1	0.21	NS	1.37	4.1×10^{-5}

Hepatic steatosis was measured using multidetector computed tomography of the abdomen. Odds ratios for NAFLD were calculated using cases with biopsy-proven NAFLD and in ancestry-matched controls. MAF, minor allele frequency