Role of Cholesterol-Associated Steatohepatitis in the Development of NASH

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The rising prevalence of nonalcoholic fatty liver disease (NAFLD) and NAFLD-related cirrhosis in the United States and globally highlights the need to better understand the mechanisms causing progression of hepatic steatosis to fibrosing steatohepatitis and cirrhosis in a small proportion of patients with NAFLD. Accumulating evidence suggests that lipotoxicity mediated by hepatic free cholesterol (FC) overload is a mechanistic driver for necroinflammation and fibrosis, characteristic of nonalcoholic steatohepatitis (NASH), in many animal models and also in some patients with NASH. Diet, lifestyle, obesity, key genetic polymorphisms, and hyperinsulinemia secondary to insulin resistance are pivotal drivers leading to aberrant cholesterol signaling, which leads to accumulation of FC within hepatocytes. FC overload in hepatocytes can lead to ER stress, mitochondrial dysfunction, development of toxic oxysterols, and cholesterol crystallization in lipid droplets, which in turn lead to hepatocyte apoptosis, necrosis, or pyroptosis. Activation of Kupffer cells and hepatic stellate cells by hepatocyte signaling and cholesterol loading contributes to this inflammation and leads to hepatic fibrosis. Cholesterol accumulation in hepatocytes can be readily prevented or reversed by statins. Observational studies suggest that use of statins in NASH not only decreases the substantially increased cardiovascular risk, but may ameliorate liver pathology. *Conclusion:* Hepatic FC loading may result in cholesterol-associated steatohepatitis and play an important role in the development and progression of NASH. Statins appear to provide significant benefit in preventing progression to NASH and NASH-cirrhosis. Randomized controlled trials are needed to demonstrate whether statins or statin/ezetimibe combination can effectively reverse steatohepatitis and liver fibrosis in patients with NASH. (*Hepatology Communications* 2022;6:12-35).

Nonalcoholic fatty liver disease (NAFLD) encompasses a wide histological spectrum of disease ranging from simple steatosis and nonalcoholic steatohepatitis (NASH) to cirrhosis and hepatocellular carcinoma.[1] It is the most common cause of chronic liver disease worldwide, with a prevalence estimated to be 24%-26%.[2,3] The prevalence of NASH in North America is estimated to be about 24.1%, with the highest prevalence reported in the Middle East (31.7%) and South America (30.4%).[3] About 41% of patients with NASH experience progression of fibrosis with an incidence of stage 3 or 4 fibrosis of 68 per 1,000 person-years.[1,3,4] NAFLD/NASH is currently the second leading indication for liver transplantation and is expected to become the number-one indication in the next few years, with excellent long-term posttransplant survival. [1-3,5,6]

Abbreviations: 2-Oxo, 2-oxoglutarate; ABC, ATP-binding cassette transporter; ACAT2, acyl-CoA:cholesterol acyltransferase enzyme; ApoB, apolipoprotein B; BSEP, bile salt export pump; CASH, cholesterol-associated steatohepatitis; CE, cholesterol ester; CVD, cardiovascular disease; ER, endoplasmic reticulum; FC, free cholesterol; FDFT1, farnesyl diphosphate farnesyl transferase 1; FXR, farnesoid X receptor; HDL, high-density lipoprotein; HMGCoAR, 3-hydroxy-3-methylglutaryl coenzyme A reductase; HSC, hepatic stellate cell; Ihh, Indian hedgehog; IL, interleukin; KC, Kupffer cell; LD, lipid droplet; LDLR, low-density lipoprotein receptor; LXR, liver X receptor; MBOAT7, membrane-bound-O-acyltransferase domain-containing protein 7; MetS, metabolic syndrome; mRNA, messenger RNA; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; nCEH, neutral cholesterol ester hydrolase; NF-κB, nuclear factor kappa B; NLRP3, NOD-, LRR-, and pyrin domain-containing protein 3; NPC1L1, Niemann-Pick type C1 like 1 protein; oxLDL, oxidized low-density lipoprotein; PNPLA3, patatin-like phospholipase domain-containing protein 3; ROS, reactive oxygen species; Scap, SREBP cleavage activating protein; SERCA, sarco/endoplasmic reticulum Ca2+-ATPase; SR-B1, scavenger receptor class B type 1; SREBP, sterol regulatory element binding protein; T2DM, type 2 diabetes mellitus; TGF-β, transforming growth factor β; TNF-α, tumor necrosis factor α; UPR, unfolded protein response; VLDL, very low density lipoprotein.

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Many risk factors for developing hepatic steatosis have been identified including the metabolic syndrome (MetS), obesity, insulin resistance with hyperinsulinemia, personal or family history of type 2 diabetes mellitus (T2DM), and dyslipidemia.[1,3] Of these risk factors, T2DM and insulin resistance are very common in NAFLD and may play a pivotal role in NAFLD/NASH development.[3,7-10] Despite these known risk factors, we do not yet know the causative factor(s) without which development and progression of NASH cannot possibly occur in certain patients. In this review, we examine the data supporting the hypothesis that hepatic cholesterol is a key pathogenetic factor driving the development of NASH at least in a subset of patients, and propose the term cholesterol-associated steatohepatitis (CASH) to describe this mechanistic pathway by which hepatic cholesterol may result in the development of steatohepatitis. These considerations are critical, because unless a causative agent is uncovered, it is unlikely that a highly effective treatment of NASH will ever be identified. Although there is mounting evidence that cholesterol may also lead to hepatic carcinogenesis, we will not focus on the association between cholesterol and hepatocellular carcinoma in this review.

Pathogenesis of NASH: Conceptual Models and the Role of Cholesterol

Historically, the two-hit hypothesis proposed a stepwise progression from normal liver to hepatic steatosis and then to NASH.[11,12] This theory postulates that insulin resistance is the "first hit," which promotes accumulation of fatty acids in the liver, leading to steatosis.[13] Hyperinsulinemia results in increased lipolysis from peripheral adipose tissue and altered hepatic gene transcription, which promotes free fatty acid uptake and *de novo* lipogenesis.[13-15] Oxidative stress is the "second hit," resulting from increased oxidation of fatty acids, and causing reactive oxygen species (ROS) formation, lipid peroxidation, DNA damage, mitochondrial dysfunction, and release of proinflammatory cytokines.[16,17] These cellular mechanisms result in hepatocyte damage, inflammation, and fibrosis, characteristic of steatohepatitis.[16,17]

More recently, a "multiple parallel hits" hypothesis has been proposed, in which multiple cellular mechanisms, working simultaneously to cause a "perfect storm," result in hepatic inflammation and

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progression to NASH.[18] Cellular mechanisms that could be altered and lead to inflammation include abnormal lipid metabolism, mitochondrial oxidative injury, endoplasmic reticulum (ER) stress, genetic polymorphisms, altered immune responses, and gut microbiome dysbiosis.[18-20] It is postulated that the accumulation of lipotoxic lipids within the liver, which interact with pro-inflammatory signals, causes these cellular abnormalities, which leads to inflammation and fibrosis.[21] Although triglycerides are the most common lipids in the liver by far, it is likely that they represent a "safe" storage molecule for fatty acids.[22] Instead, it is the accumulation of other lipotoxic lipids, such as cholesterol (and potentially free fatty acids, diacylglycerol, ceramides, and others), which are postulated to result in cellular dysfunction.[21,23,24] Cholesterol has a relatively "safe" storage option (i.e., its esterification to cholesterol esters [CEs]); however, hepatic *free* (i.e., unesterified) cholesterol is highly toxic to multiple cellular processes and organelles even if only slightly increased.[25] Thus, we propose that in a subset of patients with NASH, hepatic cholesterol accumulation results in the development of cholesterol-associated steatohepatitis (CASH) and is the main driver of the necroinflammation and fibrosis causing NASH, while dietary, genetic, and lifestyle co-factors either lead to the accumulation of hepatic cholesterol or interact which hepatic cholesterol to promote NASH, as shown in Fig. 1.

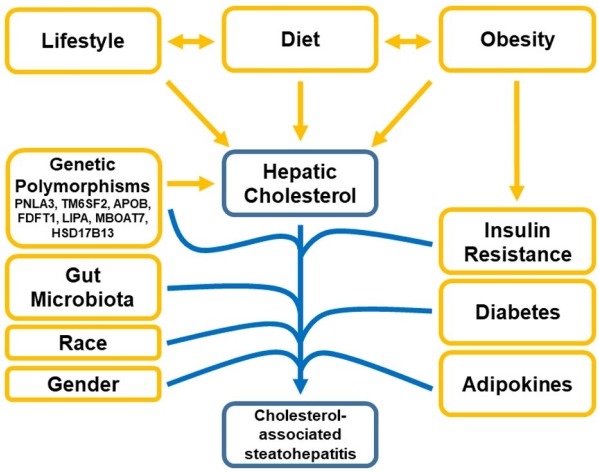


FIG. 1. Model of the CASH hypothesis. In the CASH model, hepatic cholesterol accumulation is the main driver of cellular derangement, causing NASH in a subset of patients, whereas dietary, genetic, and lifestyle co-factors either lead to the accumulation of hepatic cholesterol (yellow arrows) or interact which hepatic cholesterol to promote CASH (blue arrows). Abbreviations: FFA, free fatty acids; HSD17B13, 17β hydroxysteroid dehydrogenase 13; LIPA, lysosomal acid lipase; and TM6SF2, transmembrane 6 superfamily member 2.

Hepatic Cholesterol Metabolism

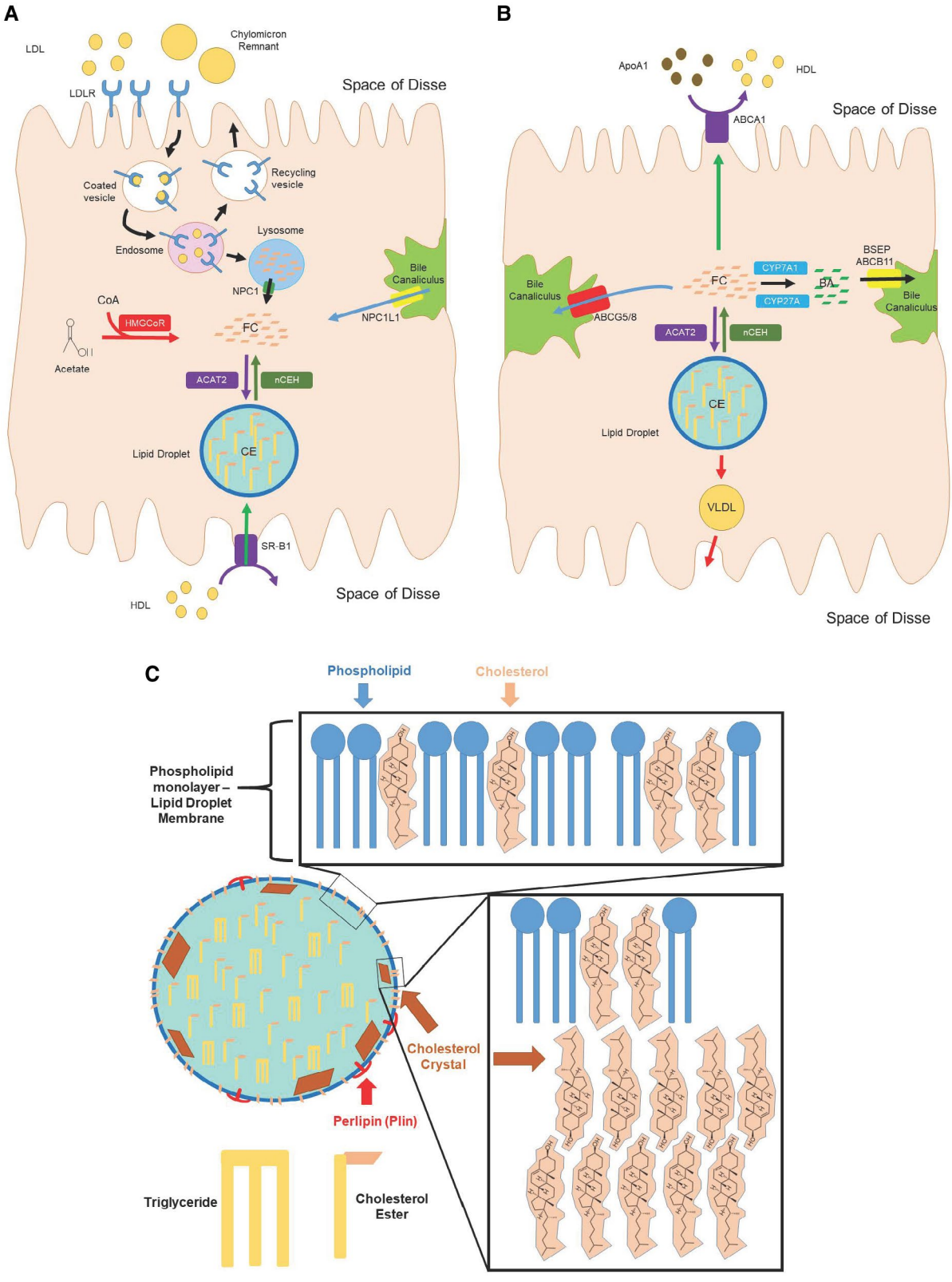
The liver is the most important organ that controls body cholesterol homeostasis. In the nonpathologic state, the mouse liver has a relatively low cholesterol concentration (132 mg/kg), but it has a high flow of sterols through the liver every day, consistent with its role in lipoprotein and bile acid synthesis and homeostasis (143 mg/kg/day).[26] When the sum total of the pathways involved in synthesis and uptake of cholesterol (FIG. 2A) exceeds the pathways that lead to removal of cholesterol (FIG. 2B), cholesterol accumulates in hepatocytes.[27]

A critical component of the CASH hypothesis is that the liver (not adipose tissue) is the body's storage site for excess cholesterol. Excess cholesterol is stored in the liver within hepatocyte lipid droplets (LDs) as CEs.[28] Once previously believed to be inert storage vessels, LDs have now been recognized as metabolically active organelles within cells that serve a wide variety of functions. LDs are derived from the ER and consist of a core of neutral lipids (CEs and triglyceride) that are surrounded by a phospholipid monolayer, studded with a diverse array of proteins.[29,30] The phospholipid monolayer contains FC, which affects LD membrane properties, including surface and line tension, size, and interaction with other LDs[29,30] (FIG. 2C).

REGULATION OF CHOLESTEROL HOMEOSTASIS

Cholesterol homeostasis is tightly regulated by a number of nuclear transcription factors, three of which have also been linked to NAFLD pathogenesis: sterol regulatory element binding protein-2 (SREBP-2), farnesoid X receptor (FXR), and liver X receptor (LXR) (FIG. 3).

SREBPs are a family of membrane-bound transcription factors that sense membrane cholesterol and fatty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Triglyceride
Cholesterol Ester

FIG. 2. Cholesterol trafficking through the hepatocyte. (A) Cholesterol uptake and synthesis. Dietary cholesterol is absorbed in the jejunal mucosa through NPC1L1, incorporated into chylomicrons (CMs), and reaches the liver in CM remnants. CM remnants are taken up by the liver through interaction of the apoE protein on the CM remnant and LDLR on hepatocytes, which also binds to circulating LDL particles through interaction with apoB-100 on the LDL surface. After binding to the LDLR, the complex undergoes receptor-mediated endocytosis, processing through the late endosome/lysosome compartment, and transport into the metabolically active pool of cholesterol in the cytosol through NPC1. CE taken from HDL particles are selectively transported into the cytosol through SR-B1, followed by hydrolysis through nCEH to join the metabolically active pool of cholesterol in the cytosol. Cholesterol can also be taken up from bile through NPC1L1 on the canalicular membrane of hepatocytes, when cells are deprived of cholesterol. Finally, cholesterol can also be synthesized *de novo* through the HMGCoAR, which is tightly regulated by SREBP-2, the principal transcriptional activator of HMGCoAR. (B) Cholesterol secretion and excretion. Transport of cholesterol out of the cell is performed primarily through members of a superfamily of ABC transporters that use ATP to transport lipids across membranes. ABCA1 is a transmembrane protein present on the basolateral plasma membrane of hepatocytes that removes lipids from the cell membrane to an extracellular acceptor apolipoprotein ApoA-I. ABCA1 interacts with lipid-free apoA-1 to generate nascent HDL particles, promoting cholesterol efflux from the cell. On the canalicular membrane of hepatocytes, ABCG5 and ABCG8 form a heterodimer that functions to excrete sterols into the bile. Cholesterol may also be secreted into the circulation in the form of VLDL particles. Finally, cholesterol may be converted to bile acids and excreted into bile through BSEP, an ABC transporter (ABCB11) located on the canalicular membrane of hepatocytes. In the classical pathway, the rate-limiting step for cholesterol conversion into bile acid is the microsomal cytochrome P450 CYP7A1, which results in 7-hydroxycholesterol; however, alternative pathways include the mitochondrial CYP27A enzyme and 25-hydroxylase enzyme, forming 27-hydroxycholesterol or 25-hydroxycholesterol, respectively. (C) Hepatocyte LD. The LD membrane consists of a monolayer of phospholipids, and FC and is covered with proteins, including perilipins. The interior of the LD consists of triglycerides and CEs. When the concentration of FC within the LD membrane exceeds the saturation threshold, FC can precipitate as cholesterol crystals in the periphery of the LD. Abbreviations: apoA-1, apolipoprotein A-1; apoB-100, apolipoprotein B-100; apoE, apolipoprotein E; BA, bile acid; CM, chylomicron; CoA, coenzyme A; NPC1, Niemann-Pick type C1.

acid content and modulate the transcription of genes involved in cholesterol and fatty acid synthesis and uptake.[31,32] SREBP-1 is primarily involved in fatty acid, triglyceride, and phospholipid pathways, whereas SREBP-2 is involved in cholesterol metabolism.[33,34] SREBP-2 is a resident of the ER, where it is bound to SREBP cleavage activating protein (Scap).[35] When Scap senses cholesterol depletion, SREBP-2 is transported to the Golgi complex, where it is cleaved to the active form and enters the nucleus to activate the transcription of genes for cholesterol synthesis and uptake, including 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGCoAR) and low-density lipoprotein receptor (LDLR).[35,36]

FXR is a nuclear receptor that senses bile acids and is extensively involved in bile acid, lipid, and glucose homeostasis.[37-39] In the liver, FXR up-regulates scavenger receptor class B type 1 (SR-B1), resulting in increased uptake of high-density lipoprotein (HDL) cholesterol from the circulation, increases ATP-binding cassette transporter (ABC) G5 and G8 (ABCG5/G8) specifically in mice, and bile salt export pump (BSEP) synthesis, resulting in biliary excretion of cholesterol and bile acids, but also inhibits CYP7A1 preventing cholesterol conversion to bile acids.[37,40,41] FXR also promotes removal of triglycerides from hepatocytes by increasing β-oxidation and decreasing lipogenesis.[42] In hepatic stellate cells (HSCs), FXR creates a quiescent and pro-apoptotic phenotype, which promotes liver fibrosis resolution.[43]

LXRs are nuclear cholesterol sensors that are activated by high intracellular oxysterols.[44,45] Following activation by oxysterols, LXRα forms a heterodimer with retinoid X receptor.[44] LXRα results in reverse cholesterol transport and hepatic cholesterol metabolism by increasing the expression of macrophage ABCA1/G1, resulting in increased HDL levels, and increasing hepatic, macrophage, and intestinal ABCG5/G8, causing net cholesterol excretion from the body, but also increases LDLR degradation on hepatocytes.[46-50] In rodent models, LXR also induces expression of CYP7A1, resulting in cholesterol conversion to bile acids; however, this phenomenon is not seen in humans.[51] Additionally, duodenal Niemann-Pick type C1 like 1 protein (NPC1L1) expression has been shown to be negatively correlated with LXR expression, inhibiting intestinal cholesterol absorption, resulting in fecal excretion of cholesterol.[52] LXRα agonists in mice demonstrated reduced hepatic inflammation and fibrosis by decreasing cholesterol-mediated activation of hepatic Kupffer cells (KCs) and HSCs.[53,54]

CHOLESTEROL ESTERIFICATION

To prevent the toxic effects of FC within hepatocytes, FC is esterified to CE and stored in hepatocyte LDs.[55] One of the enzymes responsible for cholesterol esterification in hepatocytes is acyl-CoA:cholesterol acyltransferase enzyme 2 (ACAT2).[56] ACAT2 is a

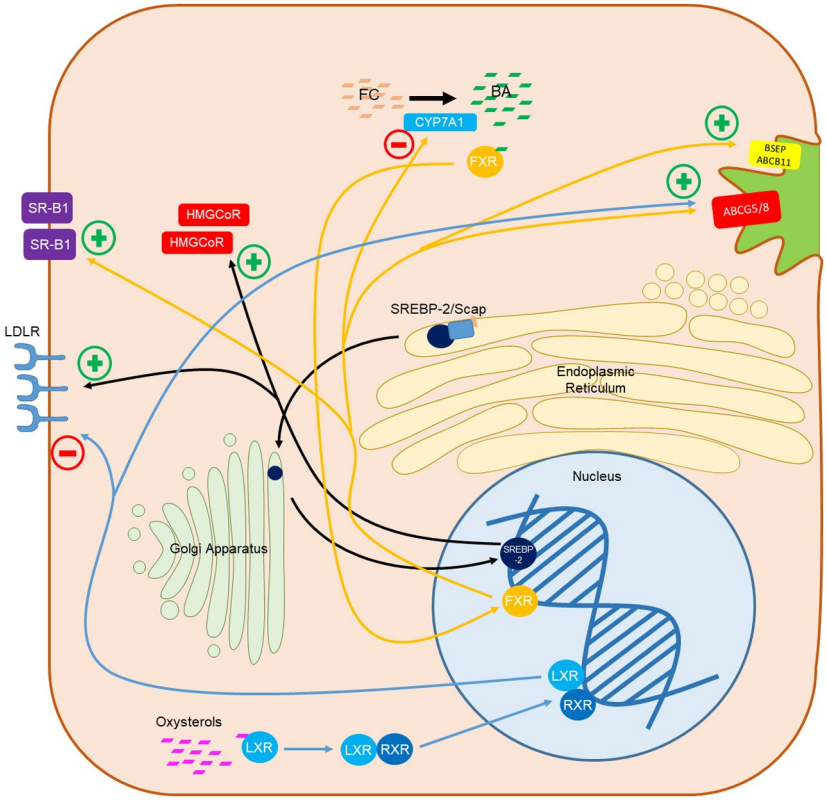

FIG. 3. Regulation of cholesterol homeostasis: The nuclear receptors SREBP-2 (black arrows), FXR (orange arrows), and LXR (blue arrows) are intimately involved in regulating cholesterol metabolism in a number of different mechanisms. The SREBP-2/Scap complex senses cholesterol content in the ER, and when cholesterol levels are low, SREPB-2 disassociates with Scap, travels to the Golgi apparatus where it is cleaved, and then promotes transcription of genes involved in cholesterol synthesis and uptake. FXR senses bile acids and triggers the transcription of SR-B1 and ABCG5/8, but inhibits the activity of CYP7A1, preventing further bile acid formation. LXR binds to oxysterols in the cell, and then, after combining with retinoid X receptor, up-regulates ABCA1, CYP7A1, and ABCG5/8 transcription, but down-regulates LDLR transcription. Abbreviations: BA, bile acid; and RXR, retinoid X receptor.

transmembrane protein found in the ER in the liver but not in adipose tissue; it integrates newly formed CEs into the ER membrane, which can either be incorporated into apolipoprotein B (ApoB) or bud off to form LDs.[29,30,57,58] When FC is needed by the hepatocyte, ACAT2 is down-regulated and neutral cholesterol ester hydrolase (nCEH) hydrolyzes CE to FC.[55,59]

Hepatic Cholesterol Accumulation in NASH

In the setting of NAFLD, numerous derangements to hepatic cholesterol homeostasis have been identified, which lead to the accumulation of hepatic cholesterol.[60] In mice, both hyperinsulinemia and inflammation lead to loss of the inhibitory effect of elevated plasma cholesterol on Scap/SREBP-2, resulting in hepatic cholesterol accumulation.[61-63] Increased levels of nuclear SREBP-2, HMGCoAR messenger RNA (mRNA), HMGCoAR protein, and HMGCoAR dephosphorylation, resulting in the active form of the enzyme, have been demonstrated in patients with NAFLD/NASH.[60,64] Despite elevated nuclear SREBP-2 levels, LDLR levels are actually down-regulated in patients with NAFLD/NASH, but an alternative hepatic scavenger receptor for oxidized low density lipoprotein (oxLDL) particles, CD36, is increased relative to the severity of steatosis.[15,60,63,64] Export of cholesterol out of the cell is also decreased,

with decreased mRNA levels of ABCA1, ABCG1, and ABCG5.[60,63]

Typical esterification/de-esterification activity in healthy individuals is determined by the relative concentration of ACAT2, while the concentration of nCEH remains relatively constant.[55] However, in patients with NAFLD, there is a 6-fold higher concentration of nCEH compared with healthy controls.[60,63,65,66] Increased expression of nCEH in animal models was also associated with reduced expression of CYP7A1 and CYP27A.[59] These cellular abnormalities, coupled with the decreased expression of ABC cholesterol exporters noted previously, result in the accumulation of toxic FC within hepatocytes.

Dietary Cholesterol and NASH

Human studies consistently support the association between cholesterol intake and the development of NASH or cirrhosis (Table 1). A nested case-control analysis of the multiethnic cohort, a large prospective study with over 215,000 older-adult participants in Hawaii and California, showed a positive association between dietary cholesterol intake and development of NAFLD with cirrhosis.[67] Another study, representative of the U.S. population, reported that dietary cholesterol consumption (but not total fat consumption) was significantly associated with the development of cirrhosis from all etiologies of liver disease combined.[68]

Experimental animal models (e.g., mice, rats, rabbits, gerbils, pigs) also consistently demonstrate that the addition of dietary cholesterol leads to progression of liver disease to fibrosing steatohepatitis and cirrhosis (Table 2). These studies generally show that while dietary fat intake alone causes the development of only simple steatosis without substantial necroinflammation or fibrosis, the addition of dietary cholesterol causes the progression from steatosis to NASH. Studies in some animal models, such as Ossabaw swine, showed that marked steatosis is not always necessary for the development of dietary cholesterol-induced ballooning degeneration, KC activation, and fibrosis.[69,70]

Genetic Polymorphisms Associated With NASH Are Related to Hepatic Cholesterol Metabolism

Many human genetic polymorphisms that have been strongly linked to NAFLD, NASH, and NASH-related cirrhosis appear to be related to hepatic cholesterol metabolism, although some clearly affect other lipids too. The most common and well-described is the patatin-like phospholipase domain-containing protein 3 (*PNPLA3*) I148M variant, which causes impairment of very low density lipoprotein (VLDL) secretion, LD remodeling, and hydrolase activity for triglycerides and retinyl esters.[71,72] Homozygous carries of the *PNPLA3* I148M variant have a greater risk of progressive steatohepatitis and fibrosis.[73] Carriers of the *TM6SF2* (transmembrane 6 superfamily member 2) E167K variant have impaired hepatic VLDL secretion, and are at higher risk for liver disease; however, they are at lower risk of cardiovascular events.[74] ApoB mutations, characteristic of familial hypobetalipoproteinemia, impair hepatic secretion of VLDL particles, which results in worsening steatosis, steatohepatitis, and cirrhosis.[75] Polymorphisms in farnesyl diphosphate farnesyl transferase 1 (FDFT1), encoding squalene synthase, the first enzyme in the sterol biosynthesis pathway, have been associated with NAFLD activity scores and fibrosis.[76] Patients with mutations in the *LIPA* (lysosomal acid lipase) gene, encoding lysosomal acid lipase, accumulate CEs and triglycerides in the liver, with progression to hepatic steatosis, fibrosis, and cirrhosis.[77] Nongenetic reductions in lysosomal acid lipase activity have been identified in patients with NAFLD, with higher reductions in lysosomal acid lipase activity, resulting in worsening disease.[78] Finally, a newly investigated protein, HSD17B13 (17β hydroxysteroid dehydrogenase 13), a LD enzyme with retinal dehydrogenase activity that also plays a key role in cholesterol and fatty acid metabolism, was found to have 5.9-fold higher hepatic expression in patients with NASH compared with controls.[79] Although it is intriguing that these polymorphisms appear to affect hepatic cholesterol homeostasis directly or indirectly, it is important to

TABLE 1. SUMMARY OF STUDIES INVESTIGATING THE ASSOCIATION BETWEEN DIETARY CHOLESTEROL INTAKE AND NAFLD/NASH IN HUMANS

Study	Population	Measurements	Results
Musso et al. 2003[149]	50 patients 25 NASH; 25 controls Mean age: 37	7-day alimentary record	Dietary intake richer in cholesterol in patients with NASH NASH: 506 ± 108 mg/dL Control: 405 ± 111 mg/dL *P* = 0.002
Allard et al. 2008[150]	73 patients referred for elevated liver enzymes and suspected NAFLD at a single center from Oct 2003 to Oct 2006 Mean age: Minimal findings: 46.8 ± 2.7 Simple steatosis: 44.7 ± 2.7 NASH: 47.7 ± 2.2	Self-reported dietary intake assessment	Increased dietary intake correlated with histologic disease severity Cholesterol consumption (mg/day): Minimal findings: 269.5 ± 27.5 Simple steatosis: 290.8 ± 28.1 NASH: 357.9 ± 37.5
Ioannou et al. 2009[68]	9,221 patients without evidence of cirrhosis followed for 13.3 years as part of the National Health and Nutrition Examination Survey Age: 25-74	24-hour dietary recall	Cholesterol consumption positively associated with cirrhosis and liver cancer Cholesterol consumption (mg/day): 0-156: 1 157-294: 1.52 295-510: 1.66 >511: 2.45 *P* = 0.007
Yu et al. 2013[151]	608 patients with hepatitis C enrolled in the Hepatitis C Antiviral Long-term Treatment Against Cirrhosis trial followed for 1.8 years Mean age: 51.0 ± 7.0	Responses to food frequency questionnaires at baseline and 1.8 years later	Each higher quartile of cholesterol intake was associated with a 46% increase in the risk of clinic or histologic liver disease progression Cholesterol consumption (mg/day): 32-152: 1 152-222: 1.51 224-310: 2.83 >310: 2.74 *P* = 0.004

TABLE 1. *Continued*

Study	Population	Measurements	Results
Mokhtari et al. 2017[152]	169 patients with NAFLD referred to two Hepatology clinics in Tehran, Iran in 2015 and 782 controls	Responses to a validated food frequency questionnaire	Dietary cholesterol intake was higher in cases compared with controls; greater egg consumption was associated with higher dietary cholesterol intake; greater egg consumption was associated with higher OR for NAFLD
	Mean age: Cases (NAFLD): 42.65 ± 12.21 Controls: 43.71 ± 14.52		Cholesterol consumption (mg/day): Cases: 263.41 ± 5.35 Controls: 315.31 ± 11.50 $P < 0.001$ Cholesterol consumption (mg/day) per egg consumption: <2/week: 226.40 ± 5.75 2-3/week: 291.95 ± 11.60 >4/week: 383.90 ± 9.53 $P < 0.001$
Noureddin et al. 2019[67]	>215,000 men and women living in Hawaii or California between 1993 and 1996 Age: 45-75 years	Responses to a validated quantitative food frequency questionnaire	Cholesterol intake positively associated with NAFLD with cirrhosis NAFLD: 1.16 ($P = 0.005$) NAFLD with cirrhosis: 1.52 ($P = 0.002$)
Yasutake et al. 2009[153]	56 patients with NAFLD diagnosed by ultrasound, CT, or liver biopsy at Kyushu Medical Center between Oct 2006 and Oct 2007 Mean age: Obese: 53.5 ± 12.3 Nonobese: 47.2 ± 14.8	Self-reported dietary intake	Cholesterol intake was significantly higher in nonobese patients with NAFLD compared to obese patients with NAFLD and healthy controls $P = 0.0378$

Abbreviations: CT, computerized tomography; OR, odds ratio.

TABLE 2. SUMMARY OF STUDIES INVESTIGATING THE EFFECTS OF DIETARY CHOLESTEROL IN INDUCING NAFLD/NASH IN DIFFERENT ANIMAL MODELS

Study	Animal Model	Diet	Age at Onset of Diet	Duration of Diet	Liver Histology Induced by Dietary Cholesterol	Mechanism
Cote et al. 2013[154]	Dawley female rats	40% fat and 1.25% cholesterol	8 weeks	7 weeks	Hepatic steatosis	Hepatic accumulation triglycerides and cholesterol Decreased FXRs Lower expression of HMGCoAR, FDFT1, and ABCG8
Ichimura et al. 2015[155]	Sprague-Dawley male rats	High-fat alone or in combination with 1.25% or 2.5% cholesterol	9 weeks	9 weeks	Fibrosing NASH and progression to cirrhosis	Diminished CPT activity and ABCG5
Ichimura et al. 2017[156]	Sprague-Dawley male rats	High-fat alone or in combination with 1.25% or 2.5% cholesterol	9 weeks	18 weeks	Fibrosing NASH and progression to cirrhosis	Diminished CPT activity, ABCG5, and BSEP
Moriya et al. 2012[157]	SHRSP5/Dmcr male rats	High-fat and high-cholesterol diet (25% palm oil, 5% cholesterol, 2% cholic acid)	10 weeks	2, 8, and 16 weeks	Fibrosing NASH	Altered TNF-α proinflammatory cytokine and NF-κB pathway
Yetti et al. 2013[158]	SHRSP5/Dmcr male rats	High-fat and high-cholesterol diet (25% palm oil, 5% cholesterol, 2% cholic acid)	10 weeks	2, 8, and 16 weeks	Fibrosing NASH Hepatic necrosis	Downregulation of caspase activity
Horai et al. 2016[159]	SHRSP5/Dmcr male rats	High-fat and high-cholesterol diet (25% palm oil, 5% cholesterol, 2% cholic acid)	6 weeks	2, 4, 6, 8, and 16 weeks	Fibrosing NASH	Eosinophilic inclusion bodies and mega-mitochondria
Csonka et al. 2017[160]	Wistar males rats	2% cholesterol, 0.25% cholate	6 weeks	12 weeks	Hepatic steatosis	Increased SCD1 and decreased FADS1 and FADS2
Matsuzawa et al. 2007[161]	C57BL/6J male mice	1.25% cholesterol and two different amounts fat (7.5% and 60%)	6 weeks	6, 12, or 24 weeks	Fibrosing NASH	Down-regulation of antioxidant enzymes
Savard et al. 2013[65]	C57BL/6J male mice	15% fat and/or 1% cholesterol	6 months	30 weeks	Fibrosing NASH	N/A
Vergnes et al. 2003[162]	C57BL/6J and C57BL/6ByJ male mice	7.5% fat, 0.5% cholate, and/or 1.25% cholesterol	3 months	3 weeks	Fibrosing NASH	Activation of HSCs, SAA family genes, histocompatibility antigens, Il-2rγ, Scyb9, and Samhd1
Desai et al. 2008[163]	C57BL/6J males mice	1.25% cholesterol, 0.5% cholic acid, and 16% fat	8-10 weeks	3 weeks	NASH	Mononuclear leukocyte infiltration in liver Enhanced MCP1, RANTES, and MIP2
Sumiyoshi et al. 2010[164]	C57BL/6J males mice	15% milk fat, 1.5% cholesterol, and 0.1% cholic acid	4 weeks	25 or 55 weeks	Hepatic steatosis Fibrosis Focal nodular hyperplasia	Elevated levels of MCP1 levels and PDGF-B protein
Ganz et al. 2015[165]	C57BL/6J male mice	High fat, 10% cholesterol, and high sugar supplement	8-10 weeks	8, 27, or 49 weeks	Fibrosing NASH	Enhanced levels of MCP1, TNF-α, and IL-1β Macrophage polarization toward an M1
Tu et al. 2017[166]	C57BL/6J male and female mice	15.8% fat, 1.25% cholesterol, and 0.5% cholate diet	8 weeks	3 weeks	Fibrosing NASH	Elevated FC, CEs, and cholic acid Changes to metabolism of sphingomyelins and phosphatidylcholines

TABLE 2. *Continued*

Study	Animal Model	Diet	Age at Onset of Diet	Duration of Diet	Liver Histology Induced by Dietary Cholesterol	Mechanism
Henkel et al. 2017[167]	C57BL/6J male mice	Soibean oil, 6-PUFA, and 0.75% cholesterol	8 weeks	20 weeks	Fibrosing NASH	Activation of KCs and enhanced expression of *Cdl2, Cxcl2, Tnf,* and *Osm*
McGettigan et al. 2019[168]	C57BL/6J male mice	One of six diets with variable amounts of fat (10% or 45% of total kilocals) and cholesterol (0.05%, 0.2%, and 2.0% of weight)	6-8 weeks	12, 20, or 24 weeks	Fibrosing NASH	Induction of tissue repair and regeneration phenotype in KCs and recruited infiltrating macrophages
Andres-Blasco et al. 2015[169]	HL-/- male mice	10.8% fat and 0.75% cholesterol	2 months	16 weeks	NASH	Dyslipidemia Increased NEFA Enhanced macrophages Circulating levels of MCP1 and Th17 T-cell subset
Chiu et al. 2010[170]	HL-/- female mice	21% fat and 0.15% cholesterol	21-23 weeks	12 weeks	Decreased hepatic steatosis	No dyslipidemia and IR
Wouters et al. 2008[171]	LDLR-deficient and ApoE2 knock-in male and/or female mice	21% fat and 0.2% cholesterol	13 weeks	2, 4, 7, and 21 days or for 7 days according to experiments	NASH	Macrophage accumulation in the liver, increase in lipid and inflammatory genes
Subramanian et al. 2011[172]	LDLR-deficient male mice	36.6% fat, 35.5% carbohydrate, and 0.15% cholesterol	10 week	24 weeks with diet	NASH	Macrovesicular steatosis, inflammatory cell foci, and fibrosis
Van Rooyen et al. 2011[63]	Alms1 mutant (foz/foz) and wild-type diabetes NOD B10 female mice	23% fat and 0.2% cholesterol	8 weeks	12 or 24 weeks	NASH	Increased macrophage, liver apoptosis, and fibrosis
Schierwagen et al. 2015[173]	apoE-/- mice	Western-type diet containing 1.25% of cholesterol	12 weeks	7 weeks	NASH	Hepatic fibrosis Up-regulation of TGF-β Increased hepatic collagen Activation of HSCs
Rodriguez-Sanabria et al. 2010[174]	apoE-/- vs. LDLR-/- male mice	20% fat and 0.25% cholesterol	10 weeks	6 weeks	NASH	Increased macrophages and inflammatory nodules (apoE, apoE-/-) vs. hepatic steatosis (LDLR-/-)
Kampschulte et al. 2014[175]	ApoE-/- LDLR-/- male mice	Western diet containing 5% cholesterol and 21% fat	4 weeks	35 weeks	Fibrosing NASH	Macrophage and T-cell infiltration, hepatic ROS accumulation, JNK activation Induction of PPAR-α
Kainuma et al. 2006[176]	Rabbits male	Standard diet containing 1% cholesterol	10 weeks	8-12 weeks	Fibrosing NASH	N/A
Ogawa et al. 2010[177]	Pathogen-free Japanese White male rabbits	Standard diet supplemented with 0.75% cholesterol and 12% corn oil	1 year	2 months	Fibrosing NASH (almost cirrhosis)	Induction of PPAR-γ and aP2, increased mRNA of TNF-β1 and collagen 1A1

TABLE 2. *Continued*

Study	Animal Model	Diet	Age at Onset of Diet	Duration of Diet	Liver Histology Induced by Dietary Cholesterol	Mechanism
Ipsen et al. 2016[178]	Guinea female pigs	15%-25% sucrose, 20% fat, and 0.35% cholesterol	10 weeks	16 or 25 weeks	Fibrosing NASH	Decreased microsomal triglyceride transfer protein mRNA and decreased hepatic VLDL secretion
Lee et al. 2009[70]	Ossabaw male and female swine	20% fructose, 46% fat, 2% cholesterol, and 0.7% cholate	5-10 months	24 weeks	Fibrosing NASH	N/A
Liang et al. 2015[69]	Ossabaw female swine	18% fructose, 43% fat, 3500 ppm methionine, and 700 ppm choline	6 months	24 weeks	Fibrosing inflammation without steatosis	Caspase 3/7–induced apoptosis

Abbreviations: ApoE, Apolipoprotein E; CPT, carnitine palmitoyltransferase; FADS, fatty acid desaturase; FDFT1, farnesyldiphosphate farnesyl-transferase 1; JNK, c-Jun N-terminal kinase; MCP1, monocyte chemotactic protein 1; MIP2, macrophage inflammatory protein 2; PDGF-B, platelet-derived growth factor B; PUFA, polyunsaturated fatty acids; RANTES, regulated on activation normal T cell expressed and secreted; SAA, serum amyloid A; Samhd1, SAM domain and HD domain 1; SCD1, stearoyl coenzyme A desaturase; Scyb9, small inducible cytokine B9.

emphasize that some also affect other lipids and that the specific mechanisms by which each polymorphism causes NASH are complex and not fully elucidated.

Mechanisms of CASH Development

Cholesterol accumulation results in dysfunction of many organelles within hepatocytes and activation of other liver cells critical to fibrosing steatohepatitis, such as KCs and HSCs. The fluidity of a cell's membranes, both the outer plasma membrane as well as membranes of internal organelles, is dependent on a precise ratio of FC to phospholipids, as well as the saturation status of the phospholipids.[80] FC accumulation within a cell membrane causes liquid-ordered rafts to become too rigid, which affects transmembrane proteins that require a degree of fluidity in order to function properly.[80] Figure 4 summarizes the processes by which hepatic FC accumulation leads to hepatocyte dysfunction (FIG. 4A).

CHOLESTEROL AND ER STRESS

The ER is responsible for a number of critical cellular functions, including folding and posttranslational modification of proteins, calcium storage, lipid-membrane biosynthesis, drug metabolism, regulating surviving and cell death signals, and signaling the production of cholesterol through Scap/SREBP-2.[33,34,81,82] Multiple cellular aberrations can lead to ER stress and impair the proper folding of proteins, including oxidative stress, calcium dysregulation, hyperglycemia, inflammation, and hypercholesterolemia.[83,84] Elevated FC/phospholipid ratio in the ER membrane impairs the action of sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) in mice, a pump that maintains high Ca^{2+} concentration in the ER lumen to facilitate protein folding (FIG. 4B).[83-86] Impaired functionality of SERCA results in decreased luminal calcium concentration, higher levels of unfolded proteins, and ER stress.[83-86] Activation of the unfolded protein response (UPR) leads to upregulation of key enzymes that alleviate ER stress by decreasing ER secretory load and enhancing protein folding. Conversely, in cases of chronic ER stress in mouse and human models, the UPR can actually

promote worsening steatosis, apoptosis, autophagy, or activation of the NOD-, LRR- and pyrin domain-containing protein 3 (NLRP3) inflammasome causing interleukin (IL) 1β production, pyroptosis, and hepatic inflammation.[83,87,88] In this way, ER stress leads to a positive feedback loop of worsening steatosis, ER stress, cell death, and inflammation characteristic of NASH.

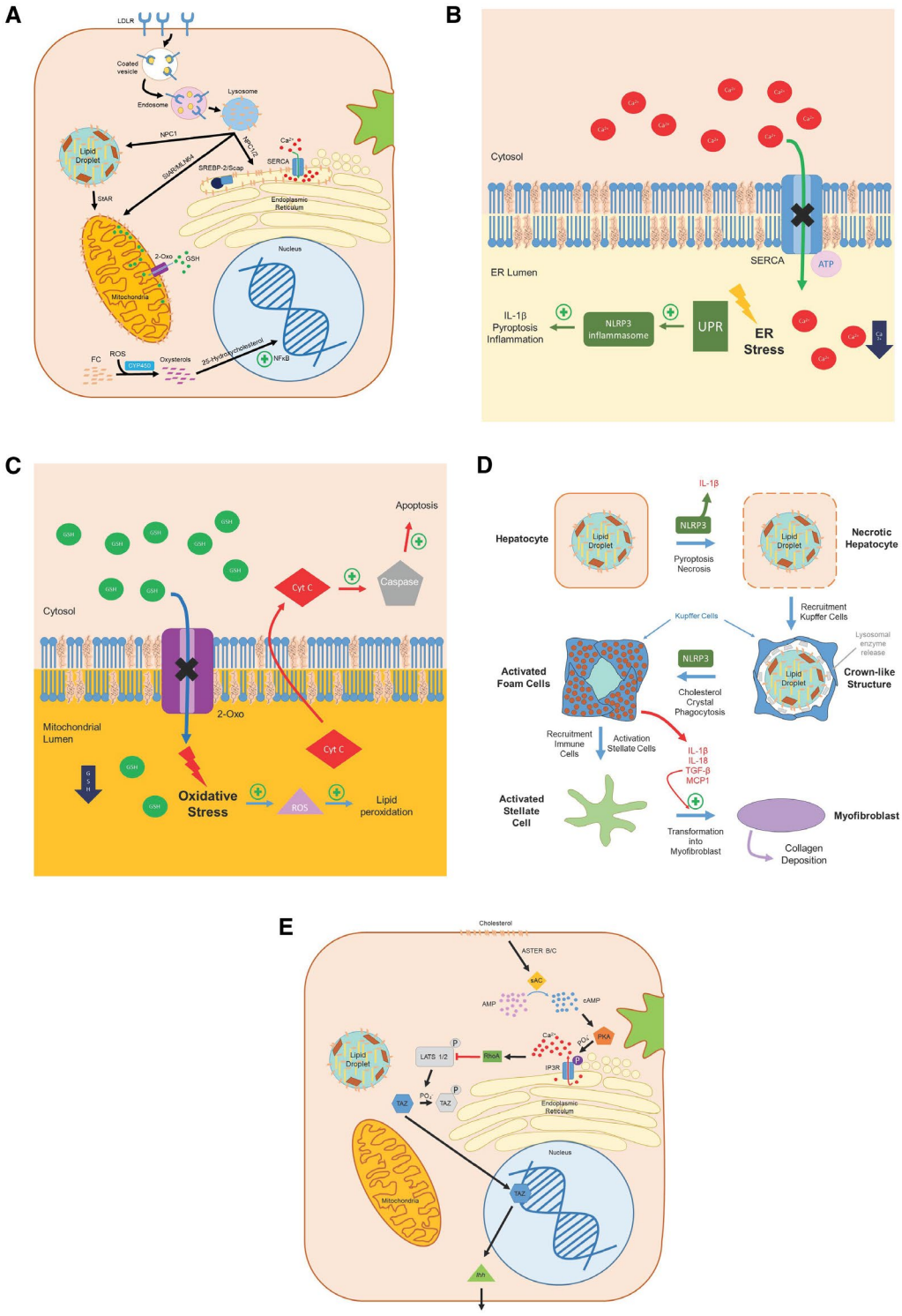

FIG. 4. Mechanisms of organelle dysfunction in cholesterol overload. (A) Overview of organelle cholesterol loading. Cholesterol entering the hepatocyte through LDL particles binds to the LDLR receptors and undergoes receptor-mediated endocytosis. That cholesterol is then trafficked through the late endosome and lysosome, and ultimately is transferred to different cellular organelles. NPC1 mediates transfer of cholesterol to lipid droplets, where it is stored; however, FC can form cholesterol crystals within the LDs. StAR/MLN64 transfers cholesterol from the lysosome to the mitochondria (or StAR can transfer cholesterol from the LD to the mitochondria), where it is typically used for synthesis of steroidogenic signaling molecules; however, it can also be deposited into the mitochondrial membrane and interfere with the function of 2-Oxo. NPC1/2 mediates transfer of cholesterol from the lysosome to the ER, where high cholesterol membrane content causes disruption of the calcium pump SERCA, decreasing the concentration of calcium in the endoplasmic reticulum lumen. FC in the cell can react with ROS through CYP450 enzymes and form oxysterols, which increases nuclear NF-κB signaling. (B) ER stress. Excess cholesterol in the ER leads to dysfunction of SERCA, lowers the luminal calcium concentration (stimulating the UPR), activation of NLRP3 inflammasome, and pyroptosis. (C) Mitochondrial dysfunction. Cholesterol loading in the mitochondrial interferes with 2-Oxo function, which depletes the mitochondrial glutathione pool, resulting in ROS generation, lipid peroxidation, release of cytochrome C, and trigger of apoptosis. Excessive ROS generation for cholesterol overload leads to the generation of toxic oxysterols, which triggers inflammatory signaling through NF-κB. (D) LD cholesterol crystallization and activation of inflammatory cells. Excessive FC in hepatocyte LDs leads to the formation of cholesterol crystal in the periphery of the LDs. LD cholesterol deposition results in activation of the NLRP3 inflammasome, which results in release of IL-1β, causing pyroptosis or necrosis. Processing of these cholesterol crystals by activated KC in crown-like structures causes release of proinflammatory signaling molecules, specifically IL-1B, IL-18, TGF-β, and MCP1, which recruits immune cells to the liver and transforms HSCs into myofibroblasts. Myofibroblasts elaborate collagen, which deposits in the liver and leads to fibrosis and cirrhosis. (E) The TAZ Pathway. FC accumulated on the plasma membrane gets internalized by ASTER B/C, which activates sAC. Elevations in cAMP levels results in phosphorylation of IP3R through PKA and causes release of Ca from the ER lumen. Elevated cytosolic Ca levels activates RhoA, which inhibits LATS1/2 through phosphorylation. LATS1/2 is unable to phosphorylate TAZ, and the dephosphorylated TAZ (active form) translocates to the nucleus to induce transcription of Ihh. Ihh is secreted out of the hepatocyte and is then able to induce profibrotic mRNA in HSCs, resulting in hepatic fibrosis. Abbreviations: AMP, adenosine monophosphate; ATP, adenosine triphosphate; Ca, calcium; cAMP, cyclic adenosine monophosphate; Cyt C, cytochrome C; IP3R, inositol 1,4,5-trisphosphate receptor; GSH, glutathione; LATS 1/2, large tumor suppressor 1/2; MCP1, monocyte chemoattractant protein-1; MLN64, metastatic lymph node 64 protein; NPC1, Niemann-Pick type C1; PKA, protein kinase A; PO^{4-}, phosphate; RhoA, ras homolog family member A; sAC, soluble adenylyl cyclase; and StAR, steroidogenic acute regulatory protein.

CHOLESTEROL IN MITOCHONDRIAL STRESS

The mitochondria membrane contains little cholesterol compared with other cellular membranes and is more susceptible to slight alterations in cholesterol membrane content.[89,90] Steroidogenic acute regulatory proteins transfer cholesterol from late endosome/lysosome (LE/LY) to the mitochondria for steroid synthesis in steroidogenic cells, and demonstrate a 7-15-fold increase in expression in patients with steatosis and NASH.[66,91] Increased delivery of cholesterol to the mitochondrial membrane results in dysfunction of membrane proteins such as 2-oxoglutarate (2-Oxo)[90] (FIG. 4C). When mitochondrial cholesterol content increases in mice and human HepG2 cells, the fluidity of the mitochondrial membrane is reduced, impairing the function of 2-Oxo and depleting the mitochondrial glutathione pool.[90,92] This sensitizes the hepatocyte to tumor necrosis factor α (TNF-α), promoting oxidative stress, lipid peroxidation, increased mitochondrial membrane permeability with cytochrome c release, and signaling for necrosis.[92] Indeed, studies evaluating elevated cholesterol content in mice and human HepG2 cells in mitochondria demonstrate experimental NASH.[90,92]

FORMATION OF TOXIC OXYSTEROLS

Formation of oxysterols within the cell occurs either through auto-oxidation of cholesterol in the setting of oxidative stress, or through hydroxylation by a number of cytochrome P450 monooxygenases, typically as an intermediary in the formation of CEs, bile acids, or steroid hormones.[93,94] Oxysterols are known to be potent signaling molecules, binding to LXRα in human hepatocytes and promoting reverse cholesterol transport, or binding to SREBP-2 and inhibiting *de novo* cholesterol synthesis.[36,44,95-98] Studies looking at both animal models and humans with biopsy-proven NASH show increased levels of oxysterols within the liver and subsequent liver damage, inflammation, and fibrosis.[99-101] One species of oxysterol, 25-hydroxycholesterol, has been demonstrated to enhance inflammatory signaling in rat hepatocytes through nuclear factor kappa B (NF-κB) activation, a key proinflammatory regulator; however, its sulfate derivative, 25-hydoxycholesterol-3-sulfate, actually

has anti-inflammatory properties.[102] In the rat mitochondria, oxysterols can promote signaling for cellular apoptosis.[101] These findings suggest a complex role of oxysterols in the pathogenesis of NASH with room for further experimentation.

CHOLESTEROL ACTIVATES KCs AND HSCs

In atherosclerotic plaques, cholesterol accumulation within macrophages results in the formation of foamy cells, and has been implicated as a prominent component of the inflammatory response found in these plaques.[103-105] In much the same way, mouse models demonstrate cholesterol accumulation within KCs, the resident macrophages in the liver, and appear to contribute to the inflammation that is characteristic of NASH.[106] As KCs in both mice and humans are not able to synthesize cholesterol *de novo*, they acquire cholesterol through uptake from the circulation, through LDLR-mediated endocytosis or scavenger receptors that bind oxLDL particles, or from processing remnant LDs of dead steatotic hepatocytes.[107-109] Uptake of oxLDL through the scavenger receptors, CD36 or SR-A, results in trafficking of oxLDL to the lysosome, where it gets trapped and cannot be exported out of the lysosome.[107,108,110] Unlike the LDLR pathway for cholesterol accumulation, the scavenger receptor pathway does not possess a negative feedback loop, leading to rapid accumulation of oxLDL in KC lysosomes and triggering hepatic inflammation.[107,108,110,111] Experiments in mouse models with scavenger receptor knockout/inhibition, or alleviation of lysosomal cholesterol accumulation, have shown improvement in the hepatic inflammation characteristic of NASH.[112-114]

HSCs, a type of nonparenchymal hepatic cell located in the space of Disse, are activated by fibrogenic cytokines elaborated by KCs, specifically transforming growth factor β (TGF-β) and TNF-α, resulting in transformation into myofibroblasts, which cause hepatic fibrosis.[115] Similar to KCs, experiments in mice show FC accumulates in HSCs by uptake from scavenger receptors, specifically lectin-like oxidized LDL receptor-1 (LOX-1), which directly activates HSCs via signaling through toll-like receptor 4 (TLR-4).[116,117] The LOX-1 IVS4-14 AG polymorphism, encoding a nontruncated splice isoform that was previously shown to confer higher cardiovascular disease (CVD) risk in homozygotes, was associated with increased severity of NASH in a study of 40 patients with biopsy-proven NASH and 40 matched controls.[118] Increased accumulation of FC leads to decreased lysosomal degradation of TLR-4, and sensitizes the cell to TGF-β signaling.[116]

CHOLESTEROL CRYSTALLIZATION

The hepatocyte LD represents one of the body's main storage sites for excess cholesterol, which is transferred to the LD membrane most likely through direct membrane contact sites with other organelles, including the ER, mitochondria, peroxisomes, and LE/LY. FC transferred to the LD membrane can be esterified to CE for "safe" storage. However, a high FC concentration can be reached in the LD membrane during this process. As the cholesterol concentration in the membrane increases, it eventually exceeds the ability of phospholipid head groups to cover all the cholesterol molecules, and excess molecules precipitate adjacent to the membrane, forming cholesterol monohydrate crystals (FIG. 4D). LD cholesterol crystals have been observed in steatotic hepatocytes in both patients with NASH and animal models of NASH.[28,105,109] In patients with biopsy-proven NAFLD, hepatocyte LD cholesterol crystals were observed almost exclusively in patients with NASH and not in patients with simple steatosis, suggesting that these cholesterol crystals are important in pathogenesis rather than innocent bystanders.[28]

Cholesterol crystals in subintimal atherosclerotic plaque macrophages are known to activate the NLRP3 inflammasome in humans and mice, mediating IL-1β and IL-18 release through the caspase 1 pathway.[119,120] It is plausible that cholesterol crystallization within hepatocyte LD also activates the NLRP3 inflammasome.[121] In mouse hepatocytes, NLRP3 activation causes pyroptosis, a form of programmed cell death marked by NLRP3 activation of caspase 1, DNA damage, and cell membrane pore formation, causing cell swelling and death.[122]

KCs that process dead hepatocytes with cholesterol crystals become exposed to these crystals and their proinflammatory effects. Following pyroptosis or necrosis of steatotic hepatocytes, their remnant LDs are encircled by KCs and form characteristic "crown-like structures" (CLSs), which secrete lysosomal

enzymes involved in the extracellular processing of LDs.[123-125] Processing of the LDs by lysosomal acid lipase results in the hydrolysis of CE to FC and further production of cholesterol crystals.[123,124] KCs that are exposed to cholesterol crystals are transformed into activated lipid-laden foam cells[123,124] through pathways that likely include activation of the NLRP3 inflammasome.[119,120,122-124] Given the central role of the NLRP3 inflammasome, it is no surprise that inhibition of this inflammasome in genetic and diet-induced mouse models of NASH resulted in decreased levels of inflammation and fibrosis.[126]

TAZ PATHWAY

A pathway has been identified recently that directly connects hepatocyte FC loading with hepatic fibrosis through HSC activation.[127] In 2016, Wang et al. showed that the transcription regulator TAZ was higher in both mouse models and patients with NASH; silencing of TAZ prevented or reversed features of steatohepatitis, but not steatosis; and expression of TAZ in models of steatosis induced steatohepatitis.[127] In most hepatocytes, TAZ is phosphorylated and in the inactive cytoplasmic state. However, models of NASH show increased dephosphorylation of TAZ to the active form, translocation to the nucleus, and transcription of target genes.[127] One of the target gene induced by TAZ is Indian hedgehog (*Ihh*), which can be secreted from hepatocytes and induces profibrotic genes in HSCs.[127] Wang et al. showed that silencing of TAZ in NASH models decreased gene expression of hepatocyte *Ihh* and subsequent profibrotic HSC mRNA.[127] A follow-up study published in 2020 showed that the process of TAZ activation is initiated by hepatocyte FC, which blocks proteosomal TAZ degradation through induction of soluble adenylyl cyclase and resulting Ca release from the ER.[128] This pathway (FIG. 4E**)** provides a direct link between increased hepatocyte FC levels and features of NASH.

Ezetimibe and Statins in NASH

Cholesterol-lowering medications (such as statins and ezetimibe) are very common in patients with NAFLD/NASH due to the high prevalence of hypercholesterolemia, diabetes, and CVD. In addition to their proven cardiovascular benefits, statins and ezetimibe also appear to have beneficial effects on NAFLD/NASH.[129-131] Table 3 summarizes studies that evaluated the effects of cholesterol-lowering medications on NAFLD/NASH, identified through a comprehensive review of the literature. Multiple small prospective studies in patients with either NAFLD or NASH assessed the effect of ezetimibe on steatosis, inflammation, and fibrosis. Although these studies have shown benefit in biochemical, metabolic, and histologic outcomes from ezetimibe therapy, the small size and relatively short follow-up of these studies limit their interpretation.[132-136] A meta-analysis performed in 2017 encompassing six studies and 273 patients with NAFLD or NASH suggested that ezetimibe improved serum liver enzymes, hepatocyte steatosis and ballooning, but had no effect on inflammation or fibrosis.[137]

Despite concerns about statin-induced hepatotoxicity, studies reported very rare incidence of statin-related adverse events in patients with liver disease.[138,139] *Post hoc* analysis of three large randomized, controlled, trials designed to evaluate the effect of statins on CVD, consisting of 11,587 patients, including 1,844 with elevated aminotransferases, demonstrated that statins resulted in improvement in serum aminotransferase levels and ultrasonographic steatosis.[129,140,141] In 2015, a multicenter cohort study consisting of 1,201 European patients who underwent liver biopsy for suspected NASH showed that the 107 patients who were taking statins had a protective effect from steatosis, inflammation, and NASH in a dose-dependent manner.[142] A multicenter, Italian cross-sectional study of 346 patients with diabetes with biopsy-proven NAFLD, confirmed that statins were independently associated with reduced odds of NASH and significant fibrosis.[143] Multiple small prospective trials using atorvastatin and rosuvastatin demonstrated improvement in biochemical, radiological, and histological features of NAFLD and NASH.[130,144,145] A systemic review of 121,058 patients with chronic liver disease showed that statins reduced the risk of portal hypertension, progression to cirrhosis or decompensated cirrhosis, and mortality.[131]

Meta-analyses of randomized controlled trials, including very large numbers of participants,

TABLE 3. SUMMARY OF HUMAN STUDIES INVESTIGATING THE EFFECTS OF CHOLESTEROL-LOWERING MEDICATIONS ON NAFLD/NASH

Study	Study type	Medication	Study Population	Duration of Treatment	Results
Chan et al. 2010[132]	Randomized, single-blind placebo controlled trial	Ezetimibe vs. placebo	25 obese patients (ezetimibe, n = 15; hypocaloric diet alone, n = 10)	16 weeks	Improved hepatic steatosis, inflammation, and LDL-apoB-100 metabolism
Park et al. 2011[135]	Prospective long-term study	Ezetimibe	45 patients with newly diagnosed biopsy-proven NAFLD	24 months	Improved biochemical parameters (AST, ALT, hsCRP, TC, LDL, ox-LDL, and TG), visceral fat, and histologic features (steatosis, necroinlammation, ballooning, and NAS)
Takeshita et al. 2014[133]	Open-label randomized controlled clinical trial	Ezetimibe vs. placebo	32 patients with NAFLD (ezetimibe, n = 17; placebo, n = 15)	6 months	Improved hepatic fibrosis, increased long-chain fatty acids, and Hgb A1c
Loomba et al. 2015[136]	Randomized, double-blind, placebo-controlled trial	Ezetimibe vs. placebo	50 patients with biopsy-proven NASH (ezetimibe: n = 25; placebo: n = 25)	24 weeks	No significant difference in liver fat as measured by MRI-PDFF; no significant difference in biochemical parameters or histologic response
Nakade et al. 2017[137]	Meta-analysis	Ezetimibe	Six studies (two randomized-controlled; four single-arm trials) including 273 patients with NAFLD or NASH	24 weeks, four studies 48 weeks, one study 96 weeks, one study	Improved serum liver enzymes (AST, ALT, and GGT), hepatic steatosis, and ballooning
Athyros et al. 2006[130]	Prospective, open-label randomized study	Atorvastatin vs. fenofibrate vs. combination	186 nondiabetic patients with MetS and biochemical and ultrasonographic evidence of NAFLD	54 weeks	Significantly higher percentage of patients who no longer had evidence of NAFLD in the atorvastatin and combination groups, including reduction in hs-CRP, TG, LDL-C, TC, and glucose
Nelson et al. 2009[179]	Double-blind, randomized, placebo-controlled trial	Simvastatin vs. placebo	16 patients with biopsy-proven NASH, 14 completed the study, 10 underwent repeat biopsy at 1 year	12 months	No statistically significant improvement in serum aminotransferases, hepatic steatosis, necroinflammatory activity, or stage of fibrosis
Athyros et al. 2010[129]	*Post hoc* analysis of prospective, randomized intention-to-treat study (GREACE)	Atorvastatin vs. placebo	1,600 GREACE patients with coronary heart disease, 437 patients with moderately abnormal liver enzymes possibly associated with NAFLD (227 treated with statin)	3 years	Statin-treated patients had significant improvement in liver enzymes and reduction in cardiovascular events
Athyros et al. 2011[141]	*Post hoc* analysis of prospective randomized controlled trial comparing two LDL-C targets, <100 mg/dL (A2) or <130 mg/dL (B2)	Atorvastatin	1,123 ATTEMPT patients with MetS without diabetes or CVD, 326 with modestly elevated liver enzymes and ultrasonographic evidence of NAFLD	42 months	86% in the A2 group and 74% in the B2 group had resolution of NAFLD ($P < 0.001$), mean LDL-C and TG targets were higher in the B2 group compared with the A2 group

TABLE 3. *Continued*

Study	Study type	Medication	Study Population	Duration of Treatment	Results
Foster et al. 2011[144]	Prospective, randomized, placebo-controlled trial as part of the St. Francis Heart Study	Atorvastatin vs. placebo	1,005 patients, 80 with NAFLD at baseline	3.6 years	Treatment with atorvastatin plus vitamins C and E, significantly reduced the odds of NAFLD at the end of follow-up (70% vs. 34%, OR 0.29, $P < 0.001$)
Tikkan et al. 2013[140]	*Post hoc* analysis of a prospective randomized controlled trial (IDEAL)	Atorvastatin 80 mg/day vs. simvastatin 20-40 mg/day	8,863 IDEAL patients, 1,081 with ALT ≥ ULN	4.8 years	Major CVD event rates were 11.5% for simvastatin and 6.5% for atorvastatin; in patients with baseline elevated ALT, greater improvement in ALT was noted in atorvastatin group (–13.4 ± 27.5 vs. –8.8 ± 28.8; $P = 0.0073$)
Dongiovanni et al. 2015[142]	Multicenter cohort study	Statins (simvastatin 49%; rosuvastatin 27%; atorvastatin 17%; pravastatin 4%; fluvastatin 2%) vs. no statins	1,201 European patients who underwent liver biopsy for suspected NASH, 107 on statin therapy for at least 6 months	6 months	Statin use was associated with lower risk of steatosis (OR 0.09, $P = 0.004$), steatohepatitis (OR 0.25, $P < 0.001$), and fibrosis stage F2-F4 (OR 0.42, $P = 0.017$)
Kargiotis et al. 2015[145]	Prospective study	Rosuvastatin	20 patients with biopsy proven NASH, MetS, and dyslipidemia	12 months	Postintervention liver biopsy showed complete resolution of NASH in 19 of 20 patients, normalization of AST/ALT and GGT by the third treatment month, and normalization of ALP by the sixth treatment month
Nascimbeni et al. 2016[143]	Cross-sectional study	Statins (45%) (simvastatin 15%; pravastatin 6%; fluvastatin 2%; atorvastatin 53%; rosuvastatin 15%) vs. no statins (55%)	346 patients with diabetes with biopsy-proven NAFLD	N/A	Statins use was associated with a lower risk of NASH (OR 0.57, $P = 0.055$) and F2-F4 fibrosis (OR 0.47, $P = 0.011$)
Kim et al. 2017[131]	Systemic review and meta-analysis	Statins vs. no statins	13 studies (10 cohort studies, 3 clinical trials) in 121,058 patients with chronic liver disease, 46% exposed to statins	N/A	In patients with cirrhosis, statin use was associated with a 46% lower risk of decompensation (RR 0.54) and 46% lower morality (RR 0.54). In patients with chronic liver disease without cirrhosis, statin use was associated with a 58% lower risk of development of cirrhosis or fibrosis progression (RR 0.42). Statin use was also associated with a 27% lower risk of variceal bleeding or progression to portal hypertension (HR 0.73)

Abbreviations: ALP, alkaline phosphatase; ALT, alanine aminotransferase; apoB-100, apolipoprotein B-100; AST, aspartate aminotransferase; GGT, gamma-glutamyltransferase; GREACE, Greek Atorvastatin and Coronary Heart Disease Evaluation; Hgb A1c, hemoglobin A1c; HR, hazard ratio; hsCRP, high sensitivity C-reactive protein; MRI-PDFF, magnetic resonance imaging proton density fat fraction; NAS, NAFLD activity score; OR, odds ratio; ox-LDL, oxidized LDL; RR, risk ratio; TC, total cholesterol; TG, triglyceride.

demonstrated that statins resulted in a slightly increased risk of development of diabetes,[146,147] but the risk was low both in absolute terms and when compared with the reduction in coronary events. Specifically, treatment of 255 patients with statins for 4 years resulted in one extra case of diabetes (or approximately one case of diabetes per 1,000 patient-years). In observational studies, statin-treated patients had increased hepatic *de novo* lipogenesis through activation of SREBP-1c and up-regulation of genes involved in fatty acid and triglyceride metabolism, suggesting that activation of these genes contributes to insulin resistance and diabetes.[148] Because insulin resistance and diabetes are important risk factors for NASH, these findings raise some concern about the role of statins as potential NASH pharmacotherapies.

In summary, this evidence suggests beneficial effect of statins on steatosis, inflammation, fibrosis, portal hypertension and cirrhosis, and confirms the safety of statins for the treatment of dyslipidemia in patients with NAFLD and NASH as recommended by the American Association for the Study of Liver Diseases.[1] However, large, randomized, placebo-controlled trials of statins in NASH adequately powered for histological outcomes are lacking. Such studies are desperately needed but very difficult to design, as it may be considered unethical to randomize patients with NASH to placebo, given that most would fulfill criteria for being on a statin for cardiovascular reasons.

Conclusions

NASH is rapidly rising in prevalence worldwide and currently has no approved pharmacological treatments. In the near future, the number of liver transplantations for NASH will surpass all other indications for liver transplantation. The evidence presented in this review strongly supports the role of cholesterol in causing "cholesterol-associated steatohepatitis" (CASH) and should serve to focus efforts on targeting cholesterol lowering as a therapeutic option. This strategy has multiple advantages. First, statins are widely available, inexpensive medications with a proven track record of safety. Second, statins are proven to reduce cardiovascular mortality, which is the number-one cause of death in patients with NASH, and may have even greater cardiovascular benefits in patients with NASH.[129] Therefore, treatment of patients with NASH with statins would potentially simultaneously ameliorate both cardiovascular mortality as well as liver-related complications (e.g., cirrhosis and portal hypertension) and mortality. Randomized controlled trials of statins in patients with NASH or cirrhosis that are under way are eagerly awaited, while clearly more such studies are urgently needed.

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