Mechanisms of Lipid Droplet Accumulation in Steatotic Liver Diseases

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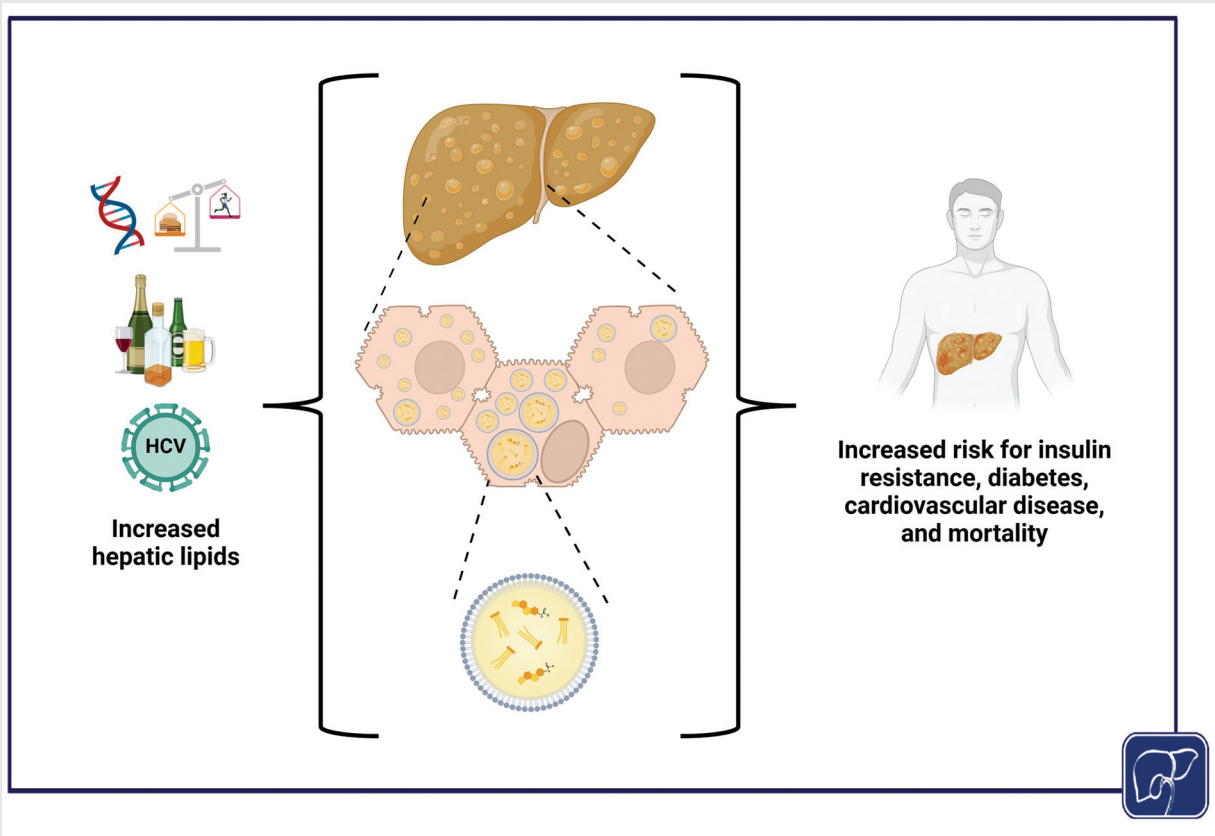
Graphical Abstract



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Abstract

The steatotic diseases of metabolic dysfunction-associated steatotic liver disease (MASLD), alcohol-associated liver disease (ALD), and chronic hepatitis C (HCV) account for the majority of liver disease prevalence, morbidity, and mortality worldwide. While these diseases have distinct pathogenic and clinical features, dysregulated lipid droplet (LD) organelle biology represents a convergence of pathogenesis in all three. With increasing understanding of hepatocyte LD biology, we now understand the roles of LD proteins involved in these diseases but also how genetics modulate LD biology to either exacerbate or protect against the phenotypes associated with steatotic liver diseases. Here, we review the history of the LD organelle and its biogenesis and catabolism. We also review how this organelle is critical not only for the steatotic phenotype of liver diseases but also for their advanced phenotypes. Finally, we summarize the latest attempts and challenges of leveraging LD biology for therapeutic gain in steatotic diseases. In conclusion, the study of dysregulated LD biology may lead to novel therapeutics for the prevention of disease progression in the highly prevalent steatotic liver diseases of MASLD, ALD, and HCV.

Keywords

- steatotic liver disease
- lipid droplet
- lipid droplet proteins
- perilipins
- MASLD
- alcohol-associated liver disease
- hepatitis C

Lay Summary

Lipid droplets (LDs) are a metabolically active component inside of cells. In the liver, LDs are made from fatty acids and are used to store other lipids, lipid toxic compounds, and signaling molecules. The fats stored inside an LD or at the surface can be used for the cell membrane or for energy. However, two diseases—metabolic dysfunction-associated steatotic liver disease (MASLD) and alcohol-associated liver disease (ALD)—are caused by the overaccumulation of LDs, and hepatitis C uses LDs for its viral life cycle. By understanding the development of LDs in three of the most common liver diseases, we may be able to develop novel therapeutics to prevent disease progression.

Lipid droplet (LD) organelles are highly conserved organelles that are at least 3.1 billion years old.[1] LDs are the intracellular hubs of lipid metabolism in all cells and are increasingly acknowledged as the disease-defining organelles of several hepatic steatotic diseases. Debated as a bona fide organelle as recently as 2006,[2] LDs have since emerged as the organelles that harbor most of the genetic risk for two common hepatic steatotic diseases, metabolic dysfunction-associated steatotic liver disease (MASLD, formerly called nonalcoholic fatty liver disease) and alcohol-associated liver disease (ALD). LDs are also required for the viral life cycle of the third major hepatic steatotic disease, hepatitis C virus (HCV). Thus, for three of the most common causes of liver disease worldwide, LD organelles are intimately involved in disease susceptibility and pathogenesis.

The advent of advanced imaging and molecular and genetic tools has both increased our understanding of the biology of LDs and prompted new, yet still unexplored questions about this organelle. For example, much is now known about the biogenesis of LDs and the possible fates of LDs. Less is known about the signals that regulate LD size and composition and how the potential fates are determined. In addition, while our understanding of LD biology and consequent pathogenesis in some diseases like MASLD appears to have garnered consensus,[3] our understanding of other diseases like ALD and HCV lags.

Here, we review the current understanding of hepatocellular LD biology, from their formation as nascent LDs to their involvement in the pathogenesis of three diseases, MASLD, ALD, and HCV. We also take this opportunity to outline gaps in our current understanding as they relate to these diseases and highlight recent efforts to leverage LD biology for therapeutics in steatotic diseases.

Hepatocyte LD Biology

The nascent LD emanates from the outer membrane of the endoplasmic reticulum (ER; ►**Fig. 1**). This process is mainly driven by the coordinated activity of enzymes such as diacylglycerol O-acyltransferase 1 (DGAT1) and DGAT2 and acetyl-CoA acetyltransferase 1 (ACAT1) and ACAT2, which esterify fatty acids (FAs) and cholesterol, respectively. DGAT1 is only present in the ER membrane and uses diacylglycerol (DAG) derived from cytosolic triglyceride lipolysis to re-synthesize triglycerides in the ER lumen. DGAT2 localizes to both the ER and the LD membrane and synthesizes de novo triglycerides from FAs.[4,5] ACAT1 converts cholesterol into cholesteryl esters in the cytosol which are then incorporated into LDs to prevent cytotoxicity from cholesterol crystals. However, the product formed by ACAT2 is translocated to the ER and released in lipoproteins.[6,7] The resulting neutral lipids, namely triglycerides and cholesterol esters, form the core of hepatocellular LDs. The budding of nascent LDs from the ER outer

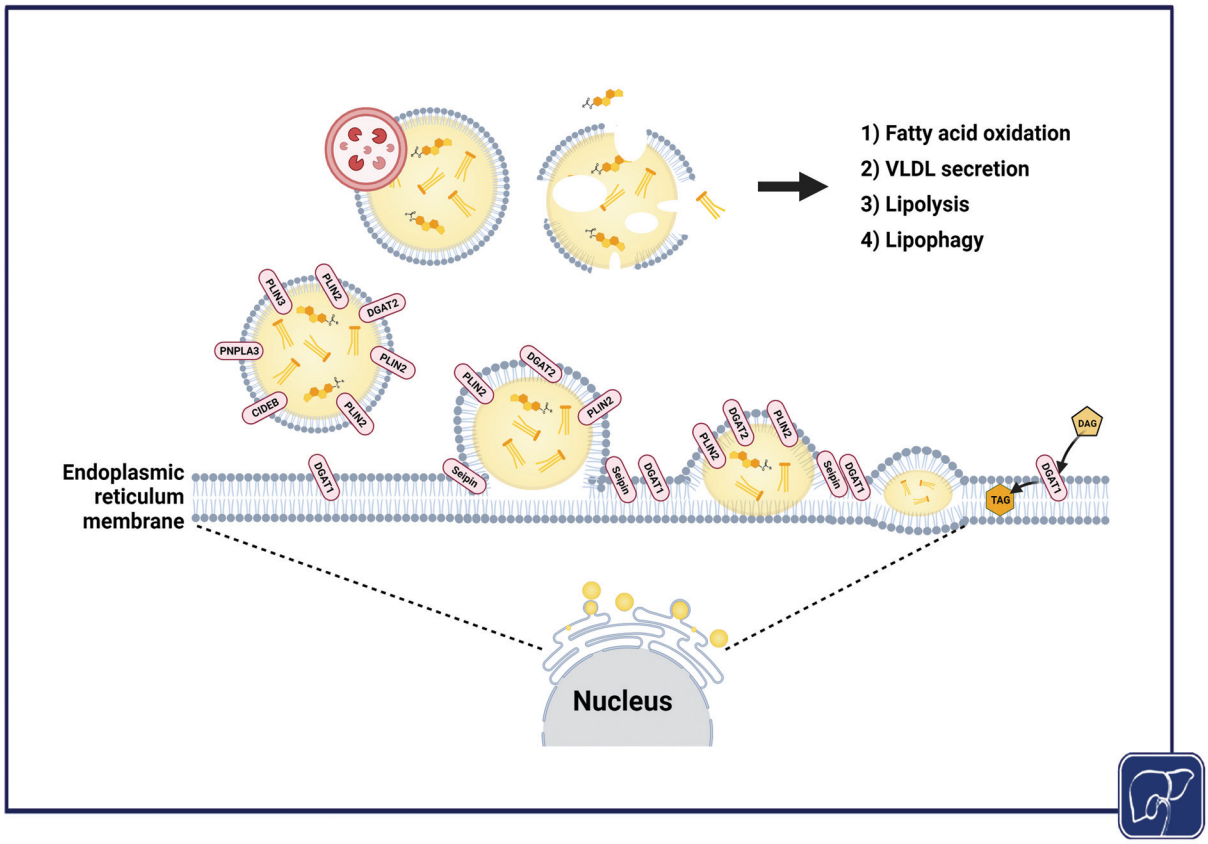

Fig. 1 Lipid droplet biogenesis in hepatocytes. A diagram describing the nascent development of an LD in the ER. Briefly, DAGs are converted by DGAT1 on the ER membrane to TAGs and stored in the ER intermembrane space. TAGs, cholesterol esters, and other neutral lipids accumulate in the ER intermembrane and causing the membrane to bleb outwards. The LD is released from the ER membrane coated with LD proteins, such as DGAT2, CIDEB, PLIN2, PLIN3, and PNPLA3. The LD can be maintained to store neutral lipids and can undergo lipolysis or lipophagy. The released fatty acids can be used for fatty acid oxidation or VLDL secretion or undergo lipolysis or lipophagy. This figure was created with BioRender.com. DAG, diacylglycerol; ER, endoplasmic reticulum; LD, lipid droplet; TAG, triacylglycerol.

membrane is facilitated by phase separation of neutral lipids within the ER bilayer, the direction of LD curvature (i.e., positive curvature toward the cytosol or negative curvature toward the ER lumen), and the engagement of several proteins that comprise the LD assembly protein complex.[8–11] Here, the protein seipin plays a critical role. Seipin is an evolutionarily conserved protein required for LD formation and budding. Recently, a model was proposed that seipin forms a cage-like structure whose conformation determines either triglyceride phase separation or LD growth and budding.[12] Seipin and other proteins involved in the LD assembly protein complex ultimately determine initial LD size and composition.[13–17] The mature hepatocyte LD is composed primarily of a neutral core of triglycerides and cholesterol esters. We have also demonstrated that the LD fraction includes bioactive lipids such as ceramides whose accumulation interferes with insulin signaling and other essential cellular processes.[18,19]

The neutral lipid core is enveloped by a phospholipid monolayer of multiple LD proteins. These proteins can either traffic from the ER bilayer to the LD membrane (Class I LD proteins) or remain associated with cytosolic LDs (Class II LD proteins). Patatin-like phospholipase domain-containing protein 3 (PNPLA3), a Class I protein, is particularly noteworthy, as it increases the risk of MASLD and ALD.[20–24] In hepatocyte LDs, perilipin (PLIN) proteins predominate as Class II proteins. There are five types of PLINs (PLINs 1–5), and others and we have demonstrated that in addition to regulating lipid metabolism, these proteins are involved in glucose metabolism.[25–27] Another Class II protein family is the CIDE family, a polymorphism of which has been associated with reduced risk of MASLD.[28,29]

Once formed, the mature LD has several potential fates largely dependent on the cell's nutrient state. The LD can remain a storage vesicle for neutral lipids; be catabolized through lipolysis or lipophagy whose release of FAs can be used for FA oxidation; or undergo repackaging into very low-density lipoprotein (VLDL) for secretion. An imbalance between LD formation and catabolism leads to hepatic steatosis. Steatosis, in turn, can cause the signature molecular, structural, and functional changes that characterize the initial stages of MASLD, ALD, and HCV.

Metabolic Dysfunction-Associated Steatotic Liver Disease (Formerly, Nonalcoholic Fatty Liver Disease)

MASLD is a chronic liver condition characterized by the accumulation of LDs in more than 5% of liver mass.[30,31] MASLD is a progressive condition that can advance to steatohepatitis (MASH [metabolic dysfunction-associated steatohepatitis])—defined as hepatocyte injury due to inflammation and hepatocellular ballooning. Steatosis in MASLD is categorized as macrosteatosis (hepatocytes with large LDs and a peripherally displaced nucleus) or microsteatosis (hepatocytes with small [typically less than 1 micron] LDs in a foamy appearing cytoplasm with a centrally positioned nucleus), the latter phenotype portending a worse prognosis (►**Fig. 2**).[32] The progression of steatosis to MASH can be followed by the development of fibrosis, liver cirrhosis, liver failure, and potentially hepatocellular carcinoma (HCC).[33,34] While the mechanisms behind the pathogenesis and progression of MASLD are not fully elucidated, LDs play a critical role.

In the context of MASLD, LDs serve major functions. First, LDs are sites of lipid synthesis and storage to protect the cell from cytotoxic FAs and other bioactive lipids. Second, through neutral lipid lipolysis or autophagy, LDs are also important sources of the FAs used to synthesize essential lipids needed for membrane biogenesis. Although dysregulation of this balance leads to an accumulation of LDs, the increased LD capacity remains insufficient to mitigate the ER-stress-inducing FAs. And in some individuals who harbor genetic polymorphisms of LD proteins, the inability to depend on counterregulatory mechanisms that protect against LD accumulation adds insult to injury.[34] Ultimately, this unmitigated LD accumulation has effects on both hepatocyte and microvasculature structure which spurs progression to MASH.

LDs and ER Stress in MASLD

A reduced capacity of LDs to successfully bud from the ER bilayer membrane can lead to lipotoxic ER stress, a widely acknowledged catalyst for MASLD progression.[35] LD budding from the ER is normally a highly regulated process that depends on the aforementioned phase separation of neutral triglycerides, the directionality of LD curvature, and the engagement of LD proteins. In MASLD, hepatocytes are exposed to excess FAs which are then directed to the ER for esterification. The accumulation of bioactive FAs such as DAGs favors negatively curved nascent LDs that fail to bud from the ER membrane and consequently have reduced access to the LD proteins that promote neutral lipid LD storage.[36] The disruption in ER homeostasis is called ER stress and has implications for other normal ER functions, including its regulation of the unfolded protein response (UPR). Dysregulation of the UPR leads to an accumulation of unfolded or misfolded proteins in the ER, and the resultant proteotoxic ER stress can lead to lipid dysregulation and the further accumulation of lipids in the ER.[37] An unmitigated UPR can also lead to hepatic inflammation, inflammasome activation, and cell death.[38]

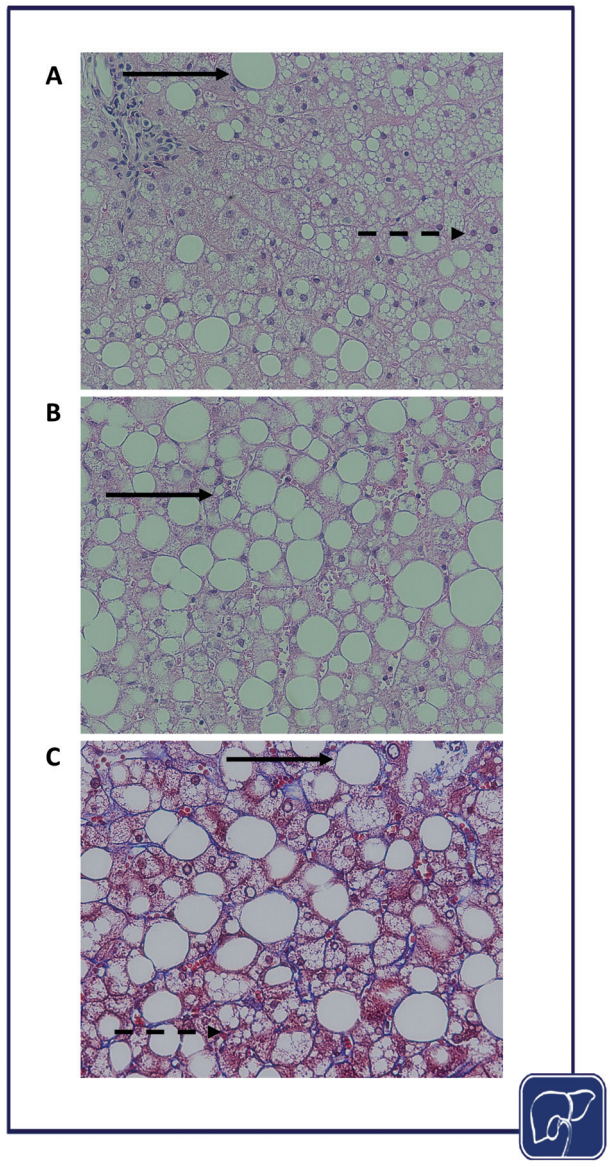

Fig. 2 Histology of steatosis in human livers. (**A**) H and E of MASH with a mixed macro- and microsteatosis pattern. (**B**) H and E of MASH with macrosteatosis. (**C**) Trichromstain of MASH showing pericellular fibrosis with mixed macro- and microsteatosis. *Solid arrow*: macrosteaotic hepatocyte with peripherally displaced nucleus. *Dashed arrow*: microsteatotic hepatocyte with centrally positioned nucleus. MASH, metabolic dysfunction-associated steatohepatitis.

Genetics of the LD Proteome in MASLD

The mature LD engages multiple LD proteins and enzymes involved in lipid metabolism, membrane trafficking, and protein degradation. The five PLIN LD proteins predominate in hepatocytes, and several PLINs have known roles in MASLD. In general, PLINs promote LD structure and maintain the integrity of the neutral lipid core. While PLIN1 is predominantly expressed in adipocyte LDs, it can be de novo expressed in both adult and pediatric nonalcoholic steatohepatitis (NASH).[39–41] The exact role of this upregulation in MASH is unknown but may relate to the established role of PLIN1 in inhibiting lipolysis—a function that would serve to reduce the burden of FAs.

PLIN2 is the most abundant perilipin LD protein and, others and we have demonstrated its upregulation in MASLD.[39,41] PLIN2 promotes storage of neutral lipids within *large* LDs by impeding access of the lipolytic enzyme adipose triglyceride lipase (ATGL); inhibiting FA oxidation; and protecting against lipophagy.[42] We have also demonstrated that PLIN2 promotes insulin resistance through the positive regulation of ceramides —another potential mechanism for lipotoxicity and oxidative stress in MASH.[26,43–45] Recently, the PLIN2 S251P polymorphism was discovered to promote the accumulation of *small* LDs[46] and increase insulin sensitivity.[47] While there may be an increased association of the polymorphism with MASLD,[46,48] we have not found an increased prevalence in patients with MASLD when using a large and diverse population dataset (published abstract).[49]

Less is known about PLINs 3–5 in MASLD. PLIN3 is a ubiquitous LD protein expressed on nascent and small LDs. In the liver, PLIN3 (like PLIN2) is found in both hepatocytes and hepatic stellate cells in humans with MASLD.[39] In mice, PLIN3 promotes steatosis and insulin resistance, and conversely, silencing of PLIN3 reduces steatosis and improves insulin sensitivity.[25] PLIN4 is highly expressed in adipose tissue,[50] but to date, there have been no reports of its role in MASLD. PLIN5 is expressed in oxidative tissues, hence its prior name of Oxpatperilipin (OXPAT). These tissues include brown adipose tissue, cardiac and skeletal muscle, and liver.[51,52] PLIN5 regulates hepatic triglyceride metabolism through promoting LD storage and inhibiting lipolysis.[52,53] PLIN5 expression is increased in MASLD[54]; however, unlike PLIN2, PLIN5 tends to protect against hepatic insulin resistance providing evidence that steatosis per se does not impair hepatic insulin signaling.[55]

While there is still much to learn about the role of perilipin proteins in the context of MASLD, these studies do demonstrate the critical roles of the LD proteome. Indeed, we recently estimated that there are at least 77 genetic polymorphisms involved in MASLD pathogenesis, and many of these genetic variations are in LD protein genes.[56] The most validated of these proteins is the Class I ER bilayer LD protein PNPLA3. The substitution of isoleucine to methionine in position 148 (I148M) of PNPLA3 causes a missense variant PNPLA3 I148M that fails to disengage from LDs due to a defect in its ability to be degraded by the ubiquitin proteosome system or by autophagy.[20,24] Because this variant PNPLA3 also demonstrates reduced hydrolase activity, LDs accumulate in individuals who harbor this polymorphism. The consequent increased MASLD risk and severity associated with this variant[57] occurs in the absence of effects on insulin sensitivity.[58] Exactly how the polymorphism promotes liver disease progression is unknown but may result from the variant-induced shifts of the LD lipid composition toward higher polyunsaturated FAs and reduced phosphatidylcholine.[59] Reduced phosphatidylcholine impairs the hepatocyte's ability to synthesize VLDL particles[60] (which further compromises the cell's ability to reduce the burden of hepatocyte lipids) and increases membrane fluidity, the consequence of which can be loss of cell membrane integrity.[61]

While several genetic polymorphisms of LD proteins tend to increase MASLD risk and promote its pathogenesis, others are protective. For example, a polymorphism of the LD protein hydroxysteroid 17-β dehydrogenase 13 (*HSD17B13*) results in a splice variant (rs72613567:TA) that codes for a truncated protein. This variant is not associated with changes in LD neutral lipid accumulation but is associated with reduced aminotransferase levels, higher platelet counts, and protection against the progression of steatosis to NASH.[62] A loss-of-function variant (rs143404524) was subsequently reported in black and Hispanic individuals that had no effect on steatosis. These data suggest that the benefit of the *HSD17B13* polymorphism stems not from its global effects on LD concentration but rather from its effects on LD lipid composition. Indeed, the *HSD17B13* splice variant increases several hepatic phospholipids, including phosphatidylcholine.[63] In contrast to the expected effects of the PNPLA3 variant on membrane fluidity, this shift is likely to stabilize membrane fluidity and integrity, thus improving overall cell function. Notably, in individuals who have both the *HSD17B13* splice variant and *PNPLA3* I148M polymorphism, *HSD17B13* mitigates the effects of the *PNPLA3* polymorphism demonstrating that the interplay of LD proteins is as critical to understand as their individual functions.

The most recent LD protein genetic polymorphism to have gained attention in its ability to reduce MASLD risk is cell death-inducing DNA fragmentation factor α-like effector B (CIDEB).[28] CIDEB is a Class I LD protein that is highly expressed in hepatocellular LDs, the ER membrane, and Golgi apparatus.[64] It has several known functions including promoting LD–LD lipid transfer, fusion of small and large LDs, and lipidation and maturation of VLDL particles within the Golgi apparatus.[65,66] Loss of function and missense CIDEB variants are associated with lower liver fat and aminotransferase levels and protect against MASLD, cirrhosis, viral hepatitis, and ALD, thus demonstrating CIDEB's (and by extension, LD proteins') broad role in normal hepatic function.[28] The protection garnered by these CIDEB polymorphisms is most pronounced in obese individuals and in those with PNPLA3 polymorphisms, making CIDEB modulation an attractive therapeutic target for individuals with the highest MASLD risk.

LD Effects on Hepatic Morphology in MASH

The idea of steatosis as a benign or "simple" stage of disease has not been borne out by what we now understand about MASLD pathogenesis. Indeed, there are a myriad of molecular and genetic reasons why LD accumulation is pathologic to the liver.[34] Emerging data also demonstrate that LD accumulation has deleterious effects on both hepatocellular and hepatic structures that spur NASH development.

MASH is classically defined as the presence of ballooned hepatocytes, which can be found on routine hematoxylin and eosin (H and E) staining, but the clinical reliability and utility of this designation has come under scrutiny.[67] Ballooned hepatocytes are generally thought to be a step beyond steatosis and are characterized by substantial LD accumulation, dilated ER, and cytoskeletal injury with Mallory–Denk

body formation.[68–70] These large, deranged hepatocytes represent a lipotoxic phase of injury in which FAs and free cholesterol are released from the LD core, thus causing hepatic inflammation.[71] Lipotoxicity is exacerbated by the activation of c-Jun M-terminal kinase signaling pathways that cause cellular stress, inflammation, apoptosis, mitochondrial dysfunction, and inhibit B-oxidation.[72–74] While much attention has been paid to the MASH-defining ballooned hepatocyte, less well recognized are the ultrastructural changes that can occur because of hepatocyte LD accumulation during steatosis.

Steatosis itself impairs both hepatic blood flow and the hepatic parenchymal microcirculation. In humans with MASLD, sinusoidal portal hypertension is related to steatosis grade.[75] In vitro, large LDs can also distort hepatocyte nuclei and chromatin structure.[76] In preclinical rodent models, LD-laden hepatocytes compress the space of Disse, the subendothelial space that lies between the liver sinusoidal endothelial cells and hepatocytes. Under normal conditions, this region regulates blood flow (70% portal vein and 30% hepatic artery).[77] In severe MASLD, compression of the space of Disse by the steatotic hepatocytes causes hypoxia by increasing intrahepatic resistance and portal venous pressure, thereby reducing oxygen supply. Tissue hypoxia stimulates angiogenesis and type I collagen,[78] promotes inflammatory cytokines, and activates hepatic stellate cells that results in extracellular matrix deposition and fibrosis development.[57] In rabbits given high cholesterol diets, sinusoidal blood flow was reduced by half due to moderate steatosis and was decreased further in rabbits with severe steatosis.[79] In rats fed a methionine–choline-deficient diet for several weeks, significant portal hypertension was observed in severely steatotic rats despite the absence of significant inflammation or fibrosis on standard H and E.[80] In scanning electron microscopy, mouse steatotic hepatocytes are observed to directly constrict and distort sinusoids. Capillarization (i.e., defenestration) of sinusoids ensues which impairs hepatic perfusion, promotes space of Disse collagen deposition, and traps leukocytes, which then incite a NASH-inducing inflammatory cascade.[81] These ultrastructural changes that result from hepatocellular LD accumulation cannot be detected on routine H and E and do not initially lead to overt clinical manifestations. This may explain why steatosis has often been considered a benign, nonpathologic stage of liver disease. To the contrary, hepatocellular LD accumulation can lead to significant remodeling of the hepatic microenvironment which over time promotes the progression from steatosis to advanced stages of MASLD.[82]

Clinically, steatosis in the context of MASLD is an independent predictor of patient outcomes and is associated with the onset of other metabolic diseases. Steatosis progressively increases the risk of type 2 diabetes. There is up to a four times risk of incident risk of diabetes development after steatosis develops and this risk increases up to 15-fold as steatosis severity worsens. Conversely, steatosis resolution can reduce the risk of incident diabetes on follow-up to a level comparable to individuals who have never had steatosis.[83] Steatosis also increases the risk of cardiovascular disease. Compared with patients who do not have steatosis, those with steatosis have a hazard ratio of 1.69 for cardiovascular events. Moreover, this risk is independent of other cardiovascular risk factors or amount of cardiac arterial stenosis.[84] There is also an association between hypertension and steatosis,[85] and steatosis is a predictor of mortality in acute myocardial infarction with hepatic fibrosis having only a slightly increased risk of mortality compared with steatosis.[86] Finally, steatosis more than 30% predicts graft failure when steatotic livers are transplanted.[87] While the specific mechanisms of this graft failure have not been fully established, it is likely that the aforementioned effects of LD accumulation on the hepatic microenvironment and tissue oxygenation impact graft suitability. These studies demonstrate that steatosis per se matters and that MASLD is not benign. To the contrary, steatosis is predictive of and associated with chronic metabolic diseases and death.

Alcohol-Associated Liver Disease

ALD is a major cause of liver disease worldwide and is responsible for over 50% of cirrhosis-related deaths.[88,89] Hepatic steatosis is the initial stage of ALD and results from alcohol's profound, deleterious effects on hepatic lipid metabolism and LD biology.[90,91]

Mechanisms of LD Accumulation in ALD

Although not as prominent as in patients with MASLD, increased de novo lipogenesis plays a role in ALD pathogenesis (►**Fig. 3**). Through the increased activation of several transcription factors, including sterol regulatory element binding transcription factor 1 (SREBF1/SREBP1c),[92–98] MLX interacting protein like (MLXIPL/CHREBP),[99,100] and peroxisome proliferator activated receptor gamma (PPARG),[101] alcohol increases hepatic FA synthesis. In addition to alcohol's promotion of FA synthesis, chronic alcohol increases hepatic uptake of nonesterified FAs. Through both exaggerated adipose tissue lipolysis[102,103] and increased hepatic clearance of intestinal chylomicrons,[104,105] alcohol causes an influx of nonesterified FAs. This influx can be abrogated by genetic deletions of key enzymes such as abhydrolase domain containing 5 and lysophosphatidic acid acyltransferase (ABHD5), an enzyme involved in adipose tissue lipolysis.[106] Alcohol also causes increased hepatic uptake of FAs through the upregulation of hepatic FA transporters. Namely, alcohol increases hepatic FA translocase/CD36, which normally has low hepatic expression.[107] As with ABHD5 inhibition, CD36 ablation prevents alcohol-induced hepatic lipid accumulation.[108,109] Finally, FAs can accumulate in the liver through impaired mitochondrial β-oxidation. Here, alcohol has two major effects on β-oxidation: excessive alcohol consumption damages mitochondria[110–113] and alcohol interferes with FA oxidation in favor of alcohol oxidation.[114,115]

Compounding the alcohol-induced increase in hepatic FAs is the inhibition of FA export and lipophagy. Normally, neutral lipids are exported from cells after re-incorporation into VLDL particles. VLDL is synthesized in the ER after lipidation by apoB100 and matures in the Golgi apparatus.

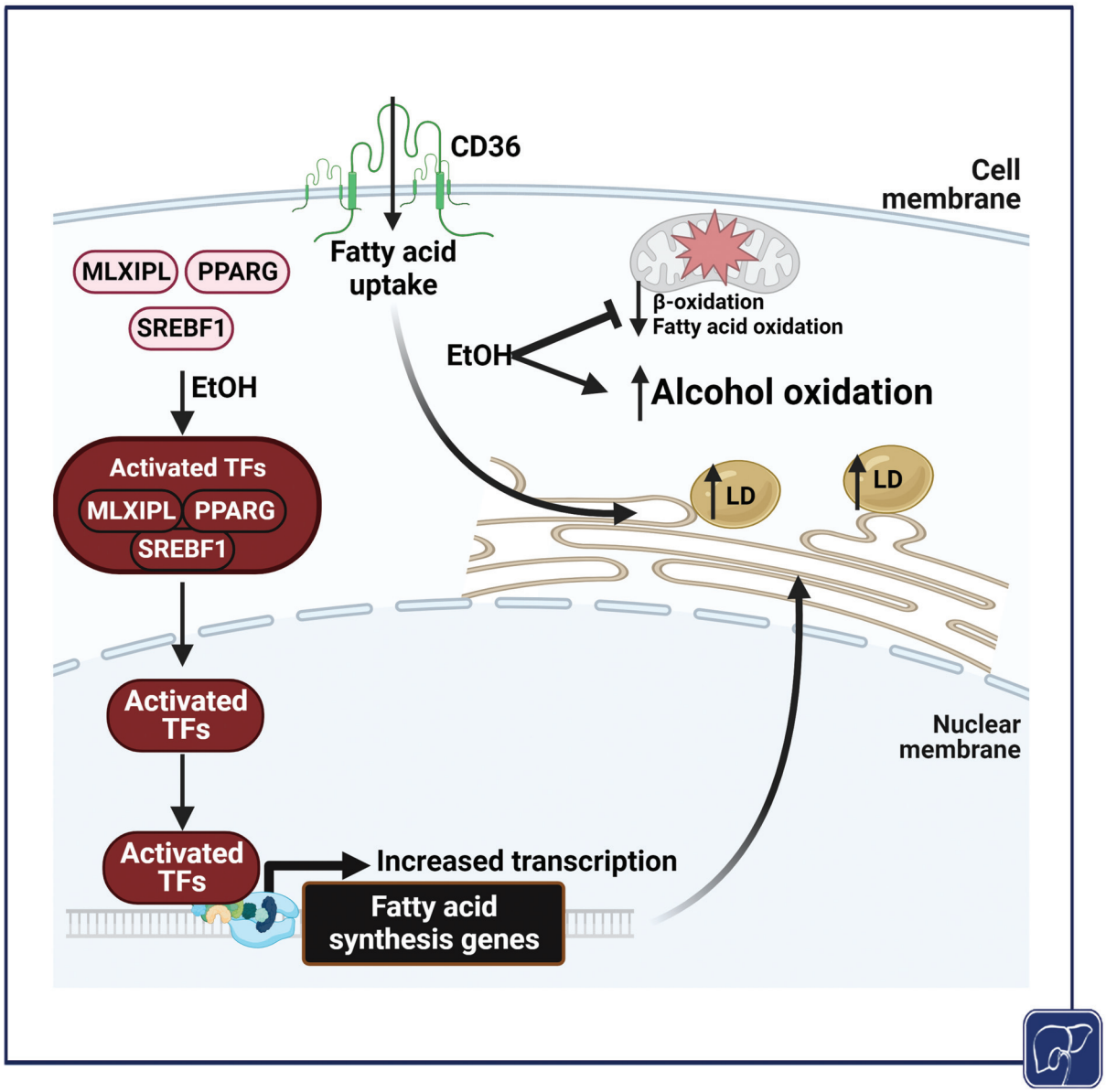

Fig. 3 Lipid droplet biogenesis in the presence of EtOH. A diagram describing the effects of EtOH on lipid droplet biogenesis. Briefly, in hepatocytes, exposure to EtOH causes increased activation of MLXIPL, PPARG, and SREBF1, which leads to increased transcription of fatty acid synthesis genes. Expression of the fatty acid uptake transporter CD36 is increased. EtOH exposure damages mitochondria and causes a pathway activity shift from β-oxidation and fatty acid oxidation to alcohol oxidation. The increased expression of genes and activities that produce lipids causes LD accumulation. This figure was created with BioRender.com. LD, lipid droplet.

MTTP is a key enzyme that regulates VLDL synthesis, and alcohol impairs both MTTP and apoB100 syntheses. Alcohol also reduces the levels of S-adenosyl methionine (SAM) and the SAM-dependent enzyme phosphatidylethanolamine methyltransferase, which is responsible for the production of the phosphatidylcholine component of VLDL.[116–118]

The increase in intracellular lipids can be exacerbated by the inhibition of lipophagy by chronic ethanol exposure. Rab7 (member of the RAS oncogene family)—a small guanosine triphosphatase that regulates membrane trafficking—mediates hepatocellular lipophagy. Rats fed an alcohol diet were resistant to starvation-induced lipophagy likely through inhibition of Rab7; rat primary hepatocytes exposed to ethanol had similar characteristics.[119] Alcohol also has a direct effect on the biogenesis and recycling of lysosomes that actively remove LDs. Transcription factor EB (TFEB), which can promote microlipophagy and macrolipophagy, mediates lysosomal biogenesis for the removal of LDs. In mice, alcohol decreased TFEB-mediated lysosome biogenesis, whereas overexpression of TFEB protected mice from alcohol-induced steatosis.[120,121] In rats, activity of dynamin 2 (Dnm2/Dyn2), which is responsible for recycling lysosomes to maintain autophagy, was inhibited by alcohol and decreased lysosomes by 40%.[122] The mechanisms by which ethanol regulates Rab7, TFEB, and Dyn2 are unknown. Notably, we have demonstrated in an experimental model of ALD that restoration of lipophagy can be achieved by hepatic ceramide reduction,[18] demonstrating that the effects of alcohol on lipid pathways may be reversible.

Characterizing the mechanism of ALD abrogation by ceramides may provide targets for inducing lipophagy and alleviating alcohol-induced steatosis.

Alcohol and LD Proteins

Given the alcohol-induced increase in FA uptake and synthesis, it is not surprising that there is an attempt by hepatocytes to upregulate pathways that promote esterification and safe neutral lipid storage as a strategy to reduce lipotoxicity. For example, in mice, alcohol increases LD size through increased expression of the LD protein cell death inducing DFFA like effector C (Cidec/Fsp27),[123] which in humans is correlated with the degree of steatosis in patients with ALD.[124]

Alcohol also upregulates the LD protein PLIN2 whose role is to prioritize triglyceride storage at the cost of worsening LD catabolic processes such as lipolysis and lipophagy. Indeed, we have demonstrated experimentally that PLIN2 is required for the development of alcoholic steatosis and alcohol-induced insulin resistance.[26,43] However, we also observed that PLIN2-enveloped LDs harbor bioactive ceramides whose accumulation impairs insulin signaling. Thus, there appears to be an exquisite balance between healthy lipid storage within LDs and LD stagnation wherein the LD-associated lipids have no opportunity for catabolism. Catabolism of LDs is aided by LD mobility so that LDs can interact with other organelles. Here, PLIN3 attempts to exert a protective effect against intrahepatocellular accumulation of LDs. PLIN3 is an exchangeable and cytosolic perilipin protein whose expression is induced in mice chronically exposed to ethanol. In basal conditions, PLIN3 surrounds small, nascent LDs and spatially organizes LDs through the co-localization of the microtubule dynein subunit Dync1i1. While alcohol can upregulate PLIN3, alcohol disrupts the LD–microtubule interaction which results in small LDs being dispersed throughout the cytoplasm,[125] so-named microsteatosis. Alcohol also induces microtubule acetylation, which further impairs LD motility.[126] Thus, the overall effect of chronic alcohol exposure on the liver is unmitigated LD accumulation with little opportunity for LD catabolism. Akin to MASLD, this can lead to ER stress, inflammation, and hepatocyte cell death.

Genetics of the LD Proteome in ALD

Because of alcohol's myriad effects on lipid metabolism, it is not surprising that the majority of the known ALD genetic risk is attributable to LD proteins.[127,128] For example, the PNPLA3 missense I148M mutation is the highest risk allele for the development and severity of ALD.[20,129] The MBOAT7 rs641738 C > T variant is associated with an increased risk of steatosis and fibrosis, although the exact mechanisms are unknown.[130] In addition, as in MASLD, *HSD17B13* variants reduce the risk of cirrhosis and HCC in chronic alcohol users. Using four European cohorts, investigators examined 6,171 patients who either had alcohol-associated cirrhosis with or without HCC, were chronic alcohol misusers without liver disease, or were normal, healthy controls without a history of alcohol misuse or liver disease. The *HSD17B13* variant reduced the risk of cirrhosis by approximately 15% and, in men, reduced the risk of HCC by 10%.[131] Taken together, increased alcohol-induced hepatic FA uptake, inhibition of lipid export, reduction of FA oxidation, and promotion of de novo lipid synthesis and LD biogenesis are all pathways that converge with underlying genetic LD risk factors to determine one's individual susceptibility to ALD.

Hepatitis C

Hepatitis C is a chronic liver condition caused by the HCV that can cause hepatic steatosis, chronic hepatic inflammation, fibrosis, cirrhosis, and HCC. In the early 2000s, HCV was estimated to affect approximately 170 to 185 million people, but effective treatments have brought the global prevalence of this disease to approximately 58 million in 2019.[132–134] While the effects of HCV on liver inflammation are quite well understood, the effects on liver metabolism are increasingly being uncovered aided in part by the observation that HCV genotypes have differential effects on lipid metabolism. Namely, patients with HCV genotypes 1, 2, and 3 are prone to steatosis, but the etiology of steatosis in genotype 1 and 2 patients appears to stem from obesity, insulin resistance, and metabolic syndrome while steatosis in genotype 3 occurs in normal weight individuals.[135] While these differences may be confounded by differences in viral geographic distribution (and hence regional differences in body mass indices), there does appear to be true biologic differences in how these viruses negotiate hepatic lipid metabolism as well.

Lifecycle of HCV

HCV is a single-stranded, positive-sense RNA virus that relies on interactions between viral and host proteins for replication. First, the HCV virion circulates in the bloodstream as a low-density lipoprotein-viral particle (lipo-viral particle) or as a free particle (►**Fig. 4**).[136] The virion enters the cell via endocytosis through interactions with cell-surface receptors. The positive-strand RNA genome is released into the cytoplasm where it undergoes ER translation. Ten distinct proteins derive from cleavage of the polyprotein precursor by host and viral proteases.[137] Viral replication is accomplished through the engagement of a viral complex formed by both nonstructural viral and host proteases. Core is the first translated protein and forms the capsid which interacts directly with cytoplasmic host LDs.[138–140] The virion envelope is formed by the ER-interacting transmembrane E1 and E2 glycoproteins. Replicated viral RNA is trafficked to the virion by nonstructural protein 5A (NS5A), wherein the nucleocapsid is formed around the HCV genome.[141–143] Ultimately, infectious lipoviral particles are formed from the association of the mature virion with endogenous lipoproteins sourced from the core–LD interactions.[144] In addition to E1 and E2, HCV uses the host VLDL assembly mechanisms, such as apolipoprotein B (ApoB)[145] and ApoE,[146] to coat the nucleocapsid. The coating of the nucleocapsid by host proteins allows the virus to easily exit an infected cell and enter a new target cell.

HCV-Induced Steatosis

Steatosis is a hallmark of HCV infection and results from HCV protein interactions with the LD or lipid metabolic

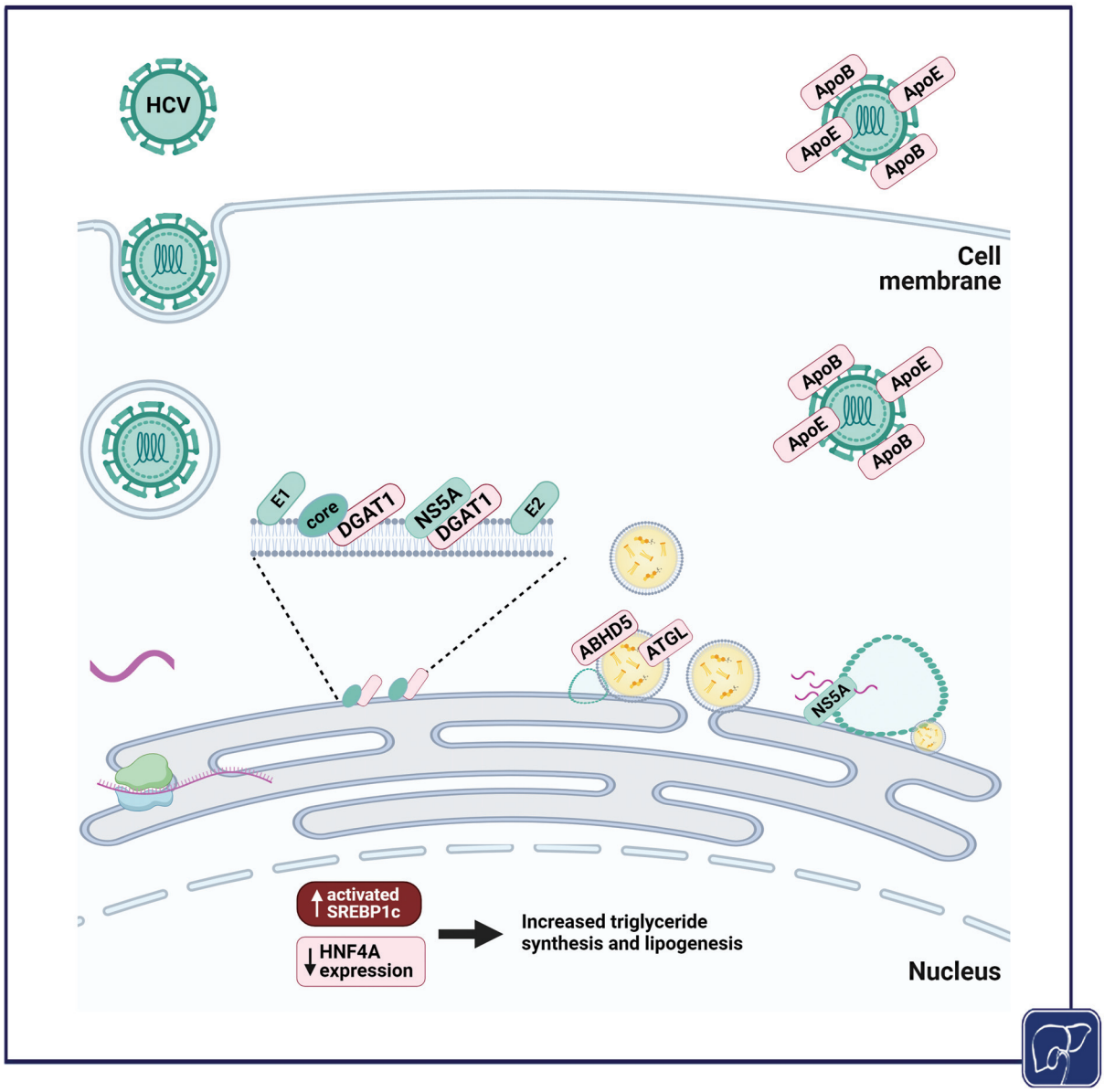

Fig. 4 The interaction of HCV and lipid droplets. A diagram describing the interaction of lipid droplets and lipid droplet proteins in the HCV cycle. Briefly, HCV is endocytosed into hepatocytes followed by release of the positive RNA. Host ribosomes translate the RNA and host proteases cleave the produce into the distinct HCV proteins. The HCV protein core co-localizes on the ER with host DGAT1 to form the capsid. The ER transmembrane HCV proteins E1 and E2 interact and form the virion envelope. NS5A, which also interacts with DGAT1, facilitates the translocation of other nonstructural HCV proteins and traffics the replicated viral RNA. HCV uses host ABDH5 and ATGL to initiate LD lipolysis to use the lipids for the virus cycle. ApoE and ApoB coat the nucleocapsid to allow the virus to leave the cell. HCV also causes increased SREBP1c activation and decreased expression of HNFA to increase triglyceride synthesis and lipogenesis. ER, endoplasmic reticulum; HCV, hepatitis C virus; LD, lipid droplet.

pathways.[147] The structural core protein increases the number of LDs and shifts the size distribution of LDs toward smaller droplets.[148] LD lipid composition appears to depend on HCV genotype, as core protein in HCV genotype 3a increases LD cholesteryl ester content and liver ceramides,[149] bioactive lipids that we have shown impair insulin signaling.[19] Core co-localizes with the ER membrane-associated enzyme DGAT1, which is required for efficient HCV assembly. With increased expression of the core and the overexpression of DGAT1, these two proteins gradually co-localize to the LD.[150] DGAT1 is also responsible for LD localization of NS5A from the ER. NS5A facilitates the translocation of other HCV nonstructural proteins, and inhibition of NS5A prevents the translocation of other nonstructural proteins, indicating a stepwise transition for HCV interaction from the ER to the LD.[151] Additionally, DGAT2, which is localized to the LD and is responsible for LD expansion,[152] does not associate with core or NS5A, reinforcing that DGAT1 must be functionally present for the HCV replication cycle.[150,153]

HCV also has an impact on lipogenesis. In mice, HCV activates SREBP1c to induce de novo triglyceride synthesis

while also inhibiting VLDL secretion of triglycerides and promoting LD formation.[154] Also, HCV induces lipogenesis by the downregulation of HNF4A. HNF4A regulates the expression of the microRNA mir-122, thereby inhibiting NF-κB-inducing kinase (NIK), an upstream lipogenic regulator of IκB kinase α (IKK-α). Decreased expression of HNF4A decreases mir-122 expression and leads to activation of NIK and IKK-α, promoting lipogenesis.[155] Conversely, HCV infection in liver cells can inhibit de novo lipogenesis by decreasing the expression of the long noncoding RNA Linc-Pint. Linc-Pint binds to serine/arginine-rich protein-specific kinase 2, which induces efficient splicing of genes in the lipogenesis pathway such as FA synthase.[156] Because HCV is known to induce steatosis, the decreased expression of Linc-Pint and subsequent inhibition of lipogenesis suggests that modulation of lipid metabolism by HCV is stage- and pathway-dependent. Additionally, HCV assembly uses α/β hydrolase domain-containing protein 5 (ABHD5/CGI-58) and ATGL to mediate LD lipolysis and lipoprotein morphogenesis.[157] In summary, HCV utilizes the host cell's capacity for lipid metabolism and induces steatosis to complete its life cycle. Hence, in addition to the direct cytopathic effects that result from the virus, the virus leaves in its wake a steatotic program that makes cells susceptible to the aforementioned ER stress.

LD Proteins and Genetics of the LD Proteome in HCV

Several perilipin proteins are expressed in the livers of patients with HCV.[39,158] Among the perilipins, PLIN3 and PLIN5 are upregulated and have divergent roles regarding the protection of LDs during HCV infection. In mice with liver-specific NS5A expression, PLIN5 was found to co-localize with NS5A and protect against NS5A-induced lipolysis and lipotoxic injury.[159] Conversely, in a PLIN3 knockdown mouse study, the core required the presence of PLIN3 to interact with LDs and induce steatosis.[133] In human genetic studies, both PNPLA3 and HSD17B have associations with HCV. In a recent meta-analysis, the PNPLA3 I148M polymorphism increased the risk of fibrosis and cirrhosis by approximately 60% in HCV patients.[160] Finally, in a small case–control study, the *HSD17B13* splice variant protects against severe fibrosis in patients infected with HCV[161] and can be used to predict risk of HCC in HCV patients previously treated with direct-acting antivirals.[162]

Survey of Techniques to Study LDs in Steatosis

Many standard research techniques have been adapted to characterize LDs. Among the major methods, most frequently used by LD researchers are (1) histology and imaging and (2) LD isolation followed by characterization of LD structure and LD composition.

Histology, Imaging, and Quantifying LDs

A primary method to evaluate LDs in hepatocytes and other cells is to fix and stain samples. H and E staining provides a general pathology of LDs in cells such as differences between microsteatosis and macrosteatosis. More specific stains for LDs include BODIPY or LipidSpot610 by fluorescence microscopy[163–167] and Oil Red O for bright field and fluorescence microscopy.[168,169] Immunofluorescence assays have been multiplexed to quantify LDs and LD proteins.[163,164,168,170] Co-localization and immunostaining studies were used to initially determine the association and functions of HCV protein's core and NS5A with LDs.[171] To quantify LDs, stained LDs can be counted manually using tools such as Photoshop but this method is quite time-intensive. Several groups have developed more automated software to help quantify light and fluorescent quantification including CellProfiler,[172] Octave,[168] and ImageJ.[169] For example, CellProfiler uses the pixels form a scanned sample to quantify several parameters such as LD number, area, perimeter, and compactness; for a step-by-step guide to analyze and quantify LDs using CellProfiler, we recommend referring to Adomshick et al.[173] These techniques have been applied to LDs in a variety of organisms, cell types, and tissues including liver tissue, hepatocytes, muscle cells, bird oocytes, and yeast. Other visualization techniques used to evaluate LDs include Raman spectroscopy[174] and electron microscopy.[175]

LD Isolation and Characterization

The consensus that LDs are metabolically active organelles has led to a surge in LD-specific methods for isolation and characterization. LDs have a low density due to their neutral lipid core allowing researchers to initially separate cell debris and nuclei from less dense cellular components using high-speed centrifugation followed by ultracentrifugation to isolate the floating, white lipid layer on top of the solution. The first method developed was to use density gradient centrifugation with sucrose[176–181] or Ficoll.[178] Recently, we demonstrated that an organelle isolation kit could be used to separate LDs from other organelles, such as the ER and lysosomes in the mouse liver.[181] This allows direct analysis of LD-associated cellular components within the same samples. After isolation, LDs are washed and fraction purity is checked by western blotting using antibodies that are specific to different subcellular compartments,[182] such as PLIN2 for LDs.

Following isolation and purification of LDs, several methods have been employed to characterize LDs. First, -omics methods have been used to characterize LDs, primarily focused on the LD proteome and lipidome. This includes quantitative mass spectrometry[180,183] and H^1 NMR[184–186] for targeted and untargeted lipidomics. Mass spectrometry is also used for quantifying the LD proteome[187,188]; proximity labeling proteomics has also been used to biotinylate LD proteins for purification followed by mass spectrometry.[189] Additionally, fluorescence assays have been used to assess LD dynamic activities in live cells for interactions with the ER and other organelles[190] and lipolysis.[191,192] Binding affinities and inter-organelle interactions have been studied in isolated LDs and ERs,[182] and in artificial LDs.[193,194] Overall, visualization of LDs and characterization of their composition and function have relied on modifications to existing techniques, such as using LD- or LD-protein-specific fluorescent probes and -omics methods.

Leveraging LD Biology for Therapeutics

Now that more is understood about the role of LDs in the pathogenesis of hepatic steatotic diseases, investigators have leveraged this new knowledge to design therapeutic strategies. The majority of these attempts have been in NASH trials. To date, there have been over 800 clinical trials for NASH and the anticipated market for NASH therapeutics is estimated to be $35 billion by 2025.[195] The emerging role of LD proteins in MASLD pathogenesis provides another opportunity for novel, targeted strategies. For example, for patients who are homozygous for the PNPLA3 risk allele, the drug AZD2693 was developed to specifically lower mRNA expression of PNPLA3 by antisense oligonucleotide technology. A phase I, double-blind, randomized, placebo-controlled, multicenter study was launched in 2020 to administer subcutaneous injections of AZD2693 to NASH patients with F0–F3 fibrosis who are carriers of the PNPLA3 148M risk alleles.[196] While this strategy may be beneficial for steatosis reduction as seen in the antecedent preclinical study,[197] the impact of increased PUFA in the LD fraction that results from ASO administration will need to be better understood given the importance of these lipids on membrane fluidity. There may also be unintended effects as the PNPLA3 polymorphism is expressed in multiple tissues and this strategy would not only target the polymorphism but also wild-type PNPLA3, a protein that is critical for LD homeostasis. Another target launched by the same company, AZD7503, aims to phenocopy the protective effects of the *HSD17B* splice variant. A single-center, phase I study of this compound in MASLD patients was opened in 2022.[198] Regardless of the eventual results of these particular studies, the integration of genetic diagnostic and therapeutic strategies is likely going to be an integral part of liver disease management for steatotic diseases in the future.

Conclusion

LDs and LD proteins are key regulators of cellular lipid homeostasis, and their roles are being elucidated in the pathogenesis of steatotic chronic liver diseases. LDs and their associated proteins limit cellular toxicity associated with bioactive lipids and participate in the regulation of cellular energy. An imbalance in the formation and catabolism of LDs can result from overnutrition and alcohol and can also result from the attempt of viruses to support viral replication. Understanding both the individual functions of LDs and their proteins and the intersection of these proteins with the cellular environment will enable us to fine-tune LD biology in a way that provides better diagnostic and therapeutic solutions for patients at risk for developing or progressing from steatotic liver diseases.

Conflicts of Interest

R.M.C. receives research support from Merck, Inc, and has consulted for Intercept Pharmaceuticals and Astrazeneca.

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