HHS Public Access

Author manuscript

Nat Rev Gastroenterol Hepatol. Author manuscript; available in PMC 2019 January 04.

Published in final edited form as:

Nat Rev Gastroenterol Hepatol. 2017 June ; 14(6): 343–355. doi:10.1038/nrgastro.2017.32.

Lipid droplets and liver disease: from basic biology to clinical implications

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Abstract

Lipid droplets are dynamic organelles that store neutral lipids during times of energy excess and serve as an energy reservoir during deprivation. Many prevalent metabolic diseases, such as the metabolic syndrome or obesity, often result in abnormal lipid accumulation in lipid droplets in the liver, also called hepatic steatosis. Obesity-related steatosis, or NAFLD in particular, is a major public health concern worldwide and is frequently associated with insulin resistance and type 2 diabetes mellitus. Here, we review the latest insights into the biology of lipid droplets and their role in maintaining lipid homeostasis in the liver. We also offer a perspective of liver diseases that feature lipid accumulation in these lipid storage organelles, which include NAFLD and viral hepatitis. Although clinical applications of this knowledge are just beginning, we highlight new opportunities for identifying molecular targets for treating hepatic steatosis and steatohepatitis.

The liver is a central and major integrator of metabolism, and a properly functioning liver is essential to health. A variety of conditions result in abnormal lipid accumulation in the liver, socalled hepatic steatosis. Obesityrelated steatosis or NAFLD is the hepatic manifestation of the metabolic syndrome and represents a huge public health problem. The estimated

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Author contributions

All authors contributed to the research, discussion, writing and reviewing/editing of the manuscript before submission.

Competing interests statement

The authors declare no competing interests.

prevalence of NAFLD in the general population in North America alone is 20–30%, whereas the prevalence in adults with morbid obesity is 75–92%, and 60–70% in patients with type 2 diabetes mellitus[1]. China is poised for a major epidemic of hepatic steatosis and its ensuing complications, given the increasing incidence of obesity and the metabolic syndrome in that country[2]. More than 28% of Chinese men and 27% of Chinese women are overweight or obese[3]. Patients with hepatic steatosis are at risk of serious complications, including progression to steato hepatitis, fibrosis, cirrhosis, liver failure and hepato cellular carcinoma[4,5]. Currently, the only effective treatment option for NAFLD is weight loss; there are no pharmaceutical treatments approved[5], although a number are in development[6]. The net result is that NAFLD is the second leading liver disease among adults awaiting liver transplantation in the USA alone and is predicted to become the leading cause[7]. One could expect this trend to be repeated in other countries.

The excess lipids in hepatic steatosis are primarily neutral lipids, such as triglycerides and cholesterol esters. In hepatocytes and other liver cells (for example, hepatic stellate cells (HSCs) and Kupffer cells), neutral lipids are stored in dynamic organelles called lipid droplets (LDs). During the past decade, new insights have emerged with respect to LD biology and, in particular, their protein composition, lifecycle in cells, and how abnormalities in LD biology contribute to disease. Here, we review the basic biology of LDs that underlies hepatic steatosis and diseases that result in lipid accumulation in the liver. We do not comprehensively review hepatic steatosis or its progression (for this aspect, see other reviews elsewhere[4,8,9]), but instead offer a view of hepatic steatosis from the perspective of the basic biology of lipid storage in cells. For the purposes of this Review, we focus mainly on LD biology in hepatocytes.

Hepatic lipid metabolism

The liver is a central hub for lipid metabolism, with uptake, esterification, oxidation and secretion of fatty acids (FAs) all occurring in hepatocytes. FAs in the liver originate from the diet (15–30% of the liver FA pool), *de novo* lipogenesis (up to 30% of liver FA pools during feeding) and recycling of FAs released from adipose tissue during fasting[10].

Dietary triglycerides are broken down into FAs in the intestinal lumen and taken up by the enterocyte. There, the FAs can be temporarily stored in lipid droplets, or packaged as triglyceride or cholesterol ester in chylomicrons for secretion[11]. The chylomicrons deliver triglyceride to peripheral tissues, and the remaining remnant particles are delivered to the liver[12]. Their uptake into hepatocytes is not completely understood, but it is mediated by the LDL receptor and LDL receptor–related protein (LRP)[13–15]. Chylomicrons also transport dietary retinol that has been esterified in the intestine[16]. These retinyl esters are then hydrolysed to free retinol in hepatocytes, and most are transferred to HSCs for storage[17,18]. Retinoids bound to retinolbinding protein can also be taken up directly by HSCs from the circulation[18].

During the fed state, the liver has a crucial role in storing excess carbohydrates as lipid via *de novo* lipogenesis. Essentially, twocarbon units (acetylCoA) derived from glycolysis are used to synthesize longchain FAs in the cytoplasm. This process is regulated by

transcriptional factors, such as sterol regulatory element binding protein 1 (SREBP1)[19], carbohydrate response element binding protein (ChREBP)[20] and liver × receptors[20,21]. These transcription factors are activated by insulin, enabling the expression of lipogenic genes, such as acetylcoenzyme A carboxylase and fatty acid synthase. FAs derived from *de novo* lipogenesis can be esterified in a series of enzymatic reactions, culminating in formation of triglyceride, most likely primarily by diacylglycerol *O*acyltransferase (DGAT)2 (REFS 22–24) and stored in LDs.

The liver also has a major role in distributing lipids to other organs[5]. FAs in the liver can be converted to triglyceride and cholesterol ester to be secreted as verylowdensity lipoprotein (VLDL) particles[25]. The composition and biogenesis of LDs and VLDLs are different. VLDL formation begins with the synthesis of apolipoproteinB100 (apoB100)[25] and is followed by two stages of lipidation[26,27]. During translation, apoB100 is initially lipidated by microsomal triglyceride transfer protein[28], which helps transfer neutral and polar lipids to apoB100 to form preVLDL or a nascent lipo protein[29]. The endoplasmic reticulum (ER) luminal enzyme carboxyl esterase triacylglycerol lipase (also known as CES3 in mouse, CES1 in human) has been implicated in mobilizing triglyceride for secretion as part of VLDL[30]. Cell death activator CIDEB (CIDEB), highly expressed in the liver, localizes to the ER membrane and has been proposed to function in the initial lipidation process by associating with LDs and apoB100 (REF. 31). This function is further supported by the phenotype of *Cideb*knockout mice, which have circulating VLDLs with reduced triglyceride content[31]. The final maturation and lipidation of the nascent lipoprotein particle to VLDL are thought to occur elsewhere, possibly along the secretory pathway[32–34]. The mechanism by which VLDL is transported out of the liver remains mostly unclear, but apoB100 exits the ER in COPII vesicles[34], and VLDL particles are eventually secreted in the plasma to be taken up by other organs for energy[25]. VLDL export from the ER is mediated by TANGO1 and TALI (Mia2–cTAGE5 fusion) that enable the fusion of ER–Golgi intermediate compartment membrane to ER exit sites necessary for the subsequent export of VLDL particles[35,36].

Imbalances in hepatic lipid metabolism

Imbalances of the processes that maintain the normal homeostasis of lipid metabolism in the liver can lead to nonphysiological accumulation of triglyceride in hepatocytes, or steatosis[5]. Conceptually, excess triglyceride storage in LDs results from different processes: increased triglyceride synthesis combined with LD biogenesis or growth; decreased LD catabolism (including decreased fatty acid oxidation); or impaired triglyceride or VLDL secretion (FIG. 1).

Historically, steatosis has been classified pathologically as microvesicular steatosis (accumulation of small fat droplets with preserved cellular architecture) and macrovesicular steatosis (the formation of larger droplets that displaces the nucleus)[37]. In both of these situations, the LDs in hepatocytes are much larger (several microns in diameter) than the LDs normally found in the cytoplasm of most other culture cells (between 0.5–2 μm) (T.C.W. and R.V.F., unpublished observations) (FIG. 2). The mechanisms for generating larger LDs are discussed later. The factors promoting progression from simple steatosis to

steatohepatitis, fibrosis and cirrhosis in the setting of NAFLD are less clear, but two large longitudinal studies showed that fibrosis is the strongest predictor of liverrelated complications and diseasespecific mortality[38,39]. Fibrosis is related to activation of HSCs, leading to secretion of extracellular matrix, and this process is tied to lipid metabolism[4,18].

Cell biology of lipid droplets

The basic biology of LDs is only now being unravelled. Thus, there is still a large disconnect between our understanding of LD biology and how this knowledge relates to NAFLD disease progression and the potential for therapeutics. Here we review the basic cell biology of LDs, which is beginning to reveal how these organelles relate to NAFLD pathophysiology.

Today, we know that LDs are highly dynamic organelles with specific cellular machinery regulating each aspect of their biology. That was not always the case. LDs were first described by Richard Altmann and E. B. Wilson in 1890 and 1896, respectively, as fat droplets inside cells[40,41]. Because LDs were assumed to be inert forms of fat storage, investigation of their biology was limited for much of the next century. This aspect changed, however, in 1991 when Dean Londos and his laboratory discovered that LDs contained specific proteins, the most abundant of which he named perilipin[42]. It became apparent that perilipin regulates major aspects of lipid metabolism and LD cell biology, and a new view of LD biology began to emerge.

LDs are composed of a core of neutral lipids that is surrounded by a phospholipid monolayer and specific proteins that bind to their surfaces (FIG. 3a). The presence of LDs converts cells to emulsions, in which LDs are the dispersed, hydrophobic phase and the cytosol is the aqueous, continuous phase[43]. Depending on the type of cell and its metabolic state, the oil phase consists mainly of triglycerides and cholesterol esters, but other neutral lipids, such as ether lipids and waxes, are also found in LDs[44]. For example, the LDs of HSCs contain retinyl esters comprising ~70% of total body retinoid content[17]. At the interface of neutral lipids with water, molecules of both sides interact with molecules that are part of the other, nonpreferred phase. The energy penalty due to these unfavourable interactions is called surface tension and drives oil and water mixtures to evolve to minimize the surface area between phases[43]. To prevent the coalescence of droplets, cells coat the hydrophobic core of LDs with a surface monolayer of phospholipids[44], which act as surfactants and stabilize the dispersed particles. In particular, phosphatidylcholine, because of its ability to reduce surface tension, seems to have a key role in LD biology[45]. Other phospholipids are also important in stabilizing LDs, and the LD phospholipid surface and ER membrane lipid composition seem to be similar[46].

Across different cell types, the size and number of LDs differ considerably. Nearly all cells seem to form small droplets (300–800 nm diameter) that are designated initial LDs (iLDs). Numbers of iLDs in cells range from four to eight in yeast cells up to hundreds in cultured mammalian cells[44]. Later in LD formation, most cells convert some iLDs to larger socalled expanding LDs (eLDs, >1 μm diameter), giving rise to two distinct LD types within cells[47].

Specific cell types, such as adipocytes and hepatocytes, have even larger LDs (socalled giant LDs, up to tens of microns in diameter).

Neutral lipid synthesis and LD formation

Neutral lipids of LDs are synthesized by enzymes that reside primarily in the ER, and triglycerides are the neutral lipid most often found in hepatic steatosis[5,48]. Triglyceride synthesis is catalysed by DGAT1 and DGAT2 (REFS 49,50). Although both DGAT enzymes synthesize triglyceride, the enzymes are unrelated by sequence and seem to have distinct substrate specificities and subcellular localizations[48]. Current evidence suggests that DGAT1, an ER enzyme, is responsible for esterifying excess fatty acids that otherwise would cause toxicity to the ER membrane[51]. DGAT2, by contrast, seems to be more closely linked to *de novo* lipogenesis and to have a primary role in triglyceride storage[22–24]. Studies of selective DGAT1 and DGAT2 chemical inhibitors lend support to these respective functions. Two studies in HepG2 cells show that DGAT1 catalyses the reesterification of triglycerides via utilization of exogenously supplied FAs, whereas DGAT2 has a minor role in reesterification, but mediates esterification of newly synthesized FAs[23,24]. Both DGAT enzymes have emerged as drug targets (discussed later).

In addition to triglyceride, hepatocytes have a large capacity to synthesize and store cholesterol esters. Cholesterol ester synthesis is catalysed by the acyl CoA:cholesterol acyltransferase (ACAT) enzymes, ACAT1 and ACAT2 (also known as sterol *O*acyltransferase 1 and 2, respectively)[52–55]. Both ACAT1 and ACAT2 are expressed in human hepatocytes, although their relative contributions to cholesterol ester synthesis in the human liver are debated. In immunodepletion experiments, ACAT1 has been reported to be responsible for as much as 90% of cholesterol ester synthesis in adult human liver, with ACAT2 accounting for most of ACAT activity in fetal human liver[56,57]. However, ACAT2-knockout mice have almost no ACAT activity in the liver[58], and another study investigating adult human liver samples treated with ACAT2 inhibitor shows that ACAT2 accounted for >50% of ACAT activity[59]. The relative contribution of ACAT enzymes to liver cholesterol esters in humans, therefore, remains uncertain.

How neutral lipids, such as triglyceride, are packaged into LDs is beginning to be determined[60–62]. A current model posits that neutral lipids accumulate within the ER bilayer due to synthesis by enzymes, such as DGAT1 or ACAT1 or ACAT2 (REFS 60,63) (FIG. 3b). Once a critical concentration of neutral lipids is reached, there is phase separation to generate lipid lenses, and these grow and deform the ER bilayer membrane until they reach a critical size that triggers budding of iLDs into the cytoplasm[60] (FIG. 3c). At some point during this reaction, LDs acquire a number of specific proteins, such as perilipins[64], which target the organelles from the cytoplasm.

Although it is now generally accepted that the ER is the site of LD formation[65–68], many questions remain that have not been directly addressed. What bio physical processes mediate the formation of lipid lenses? At what size do LDs bud off into the cytoplasm? Do they really bud off, or do LDs remain connected with the ER, essentially forming a subdomain of this organelle? In addition, a number of proteins, including seipin, encoded by the lipodystrophy gene *BSCL2*, and fat storage inducing transmembrane protein 2 (FITM2, also

known as FIT2) have been implicated in LD formation[69–71]. Seipin is required for early steps of LD formation[72–74] and has been shown in multiple cell lines to be involved in the maturation of nascent LDs to mature iLDs[73]. FITM2 has been implicated in the budding of LDs toward the cytosol[68]. However, the molecular functions of seipin and FITM2 with respect to LD formation remain largely unclear.

LD growth

Once iLDs form, a subset of them can be converted to eLDs[47], which grow to larger sizes by localized triglyceride synthesis (FIG. 3d). This process is mediated by a class of triglyceride synthesis enzymes that relocalize from the ER to the surfaces of this LD population[47]. Among the enzymes that relocalize from the ER to LDs are glycerol3phosphate acyltransferase 4 (GPAT4) and DGAT2, which catalyse the first and last steps of triglycer ide synthesis, respectively. Interestingly, some triglyceride synthesis enzymes, such as DGAT1, always remain in the ER. DGAT1 contains multiple membranespanning domains with large ER luminal domains that anchor the protein in the ER[75]. By contrast, GPAT4 and maybe DGAT2 have most of their protein mass in the cytoplasm and are anchored by a membrane-embedded segment that does not seem to span the entire bilayer and might adopt a hairpin conformation[47]. This topology, lacking ER luminal domains, is compatible with localization to either the ER bilayer or the LD surface monolayer[76,77].

Protein targeting to LDs

The targeting of GPAT4, and probably other enzymes of the eLD pathway, occurs through membrane bridges between the ER and LDs, which provide physical continuity between the surfaces of the organelles[47,65]. How these bridges are established and maintained is unclear, but the ADP ribosylation factor 1 (ARF1)–COPI vesiclegenerating machinery has a crucial role[78–80]. When members of the ARF1–COPI machinery are depleted from cells, GPAT4 and other proteins fail to target to eLDs, and as a consequence, LDs remain a uniform size, failing to expand[78]. Current evidence suggests a model in which ARF1–COPI proteins perform this function not by generating transport carriers to the LD (analogous to its function in ER–Golgi trafficking), but by directly modulating the LD surface. In this model, ARF1–COPI buds off nanovesicles from the LD surface, removing mostly phospholipids, resulting in increased LD surface tension[43]. This increase in surface tension destabilizes the LD, enabling it to fuse with the ER and establishing a bridge (FIG. 3d). In addition to this targeting pathway from the ER, a number of proteins access LDs directly from the cytoplasm. How specificity of this targeting (versus targeting of other membranes) is achieved is poorly understood currently[81].

LD proteins

The protein composition of LDs varies among cell types. For liver tissue and hepatocytes, several studies reported on the LD proteome[82–85]. Those proteomic experiments were performed on the LD fraction and led to identification of a large number of proteins. Only a few candidates were found in common between these studies, such as PLIN2 and PLIN3, suggesting many nonspecific proteins were identified due to variation in LD purification protocols and contamination with other organelles. Interestingly, PLIN2 and PLIN3 proteins are upregulated in the setting of NAFLD (discussed later). LD surface proteins are not

limited to perilipins and neutral lipid synthesis enzymes. Indeed, many proteomic studies performed in nonliver samples and in various organisms show that LDs display a diverse set of proteins at their surface[84]. Among these are members of the CIDE family of proteins involved in LD growth[86] and enzymes involved in breakdown of neutral lipids, such as adipose triglyceride lipases[87].

Giant LD formation

LDs in hepatocytes and adipocytes are often quite large (for example, >1–2 μm diameter) and are called giant or supersized LDs. These droplets arise from one of two processes. They can form by coalescence (for example, when there is insufficient phosphatidylcholine to cover the LD surface to lower surface tension to maintain a metastable state), or through proteinmediated, facilitated diffusion of neutral lipids from one LD to another[43] (FIG. 4). In this ripening process, the neutral lipids diffuse from the smaller to the larger LD, owing to a higher LaPlace in smaller LDs. The CIDE family of proteins seem to play a unique part in mediating this mechanism of LD growth[88]. Cell death activator CIDEA (CIDEA) and cell death activator CIDEC (CIDEC, also known as fatspecific protein 27 or FSP27), for example, are found at LD–LD contact sites, especially in adipocytes, and promote exchange of lipids between LDs[88]. CIDEB has also been proposed to mediate lipid transfer in the process of VLDL lipidation[31,86].

LD consumption

FAs can be mobilized from the LD neutral lipid pool for metabolic fuel. This step occurs either through the action of lipases that act directly on the LD surface or by delivering LDs to lysosomes where lysosomal acid lipase hydrolyses lipids (FIG. 5).

LD lipases.—Our current understanding about lipase action on LDs is derived chiefly from work in adipocytes. Patatinlike phospholipase domain containing (PNPLA)2 (also known as ATGL) catalyses the first step of triglyceride hydrolysis on the LD surface to generate diacylglycerol and free FA[87]. Diacylglycerol is then cleaved into monoacylglycerol and FA by hormonesensitive lipase, which also has broad substrate specificity (hydrolysing triglyceride, monoacylglycerol, and cholesterol ester)[89]. Monoacylglycerol is finally cleaved into glycerol and FA by monoglyceride lipase[90,91].

ATGL, hormonesensitive lipase and mono glyceride lipase are all expressed in liver, albeit at lower levels than in adipocytes[92], and whether these enzymes constitute the main enzymes for hepatic lipolysis remains uncertain. Hepatic ATGL overexpression increases FA oxidation and decreases triglyceride amounts[93]. Liverspecific ATGLknockout mice accumulate triglycerides in the liver, indicating a major role for this enzyme in hepatic lipid homeostasis[94]. On the other hand, other lipases might function in liver triglyceride metabolism. In particular, PNPLA3 is localized at LDs and shares 50% homology with ATGL[95]. In contrast to other lipases, PNPLA3 is upregulated by increased insulin levels and the lipogenic transcription factor SREBP1c[96]. In addition, PNPLA3 might be involved in the production of substrates for reesterification of FAs during lipolysis[97]. The physiological role of PNPLA3 is not completely understood, but a polymorphism in the protein is the predominant genetic risk factor for hepatic steatosis (discussed later).

A major role for triglyceride hydrolysis in liver is to provide substrates for VLDL assembly and secretion. Surprisingly, overexpression or knockdown of ATGL or hormonesensitive lipase does not affect hepatic VLDL secretion[93–98], suggesting that another lipase is involved in lipolysis for this purpose[92]. The chief candidate for providing substrates for VLDL secretion is the ER luminal triacylglycerol lipase[30], which is highly expressed in liver[99]. How a luminal lipase would access LD substrates is currently unclear. An attractive hypothesis is that it acts at the ER–LD membrane bridges, in which the ER lumen would have access to the cores of connected LDs to mobilize precursors.

Lipophagy.—In addition to lipolysis, lipids can be mobilized from LDs by lipophagy. During this process, small LDs are thought to be engulfed by a membrane bilayer with the activated LC3II protein to form an autophagosome that can be delivered to the lysosome[100,101]. Lipophagy of LDs during conditions of cell starvation seems to be required to maintain the flow of FAs to mitochondria for respiration[102]. Whether lipophagy has a prominent role during normal energy fluctuations remains uncertain. Even if lipophagy has a minor role in normal dietary conditions, it might be important in limiting lipid accumulation during transient dietary lipid overload.

Protein removal from LDs

When lipases digest the core of LDs, the LDs shrink in size, and as a consequence, the LD surface also shrinks[103]. What happens to the proteins on the LD surface under these conditions is not fully understood, but at least two pathways have been suggested: proteins might fall off due to macromolecular crowding[103] or be selectively extracted by chaperone-mediated autophagy, a type of autophagy that targets proteins for lysosomal degradation via chaperones[104]. At times of lipid deprivation and lipolysis, the protein composition of the LDs changes due to crowding and competition for binding to the LD surface[103]. In general, proteins with amphipathic helices that target the LD from the cytosol are more likely to be displaced from the shrinking surface, whereas proteins with more hydrophobic domains that localize to the LDs via ER bridges remain on the LD surface[103]. During chaperonemediated autophagy, proteins are thought to be recognized by the heat shock cognate 70 kDa protein, brought to the lysosomes, and recognized by lysosomeassociated membrane protein 2A (LAMP2A) for uptake and degradation[104]. Chaperonemediated autophagy can degrade LD-associated proteins, such as perilipin 2 and perilipin 3, which might be necessary for lipases and autophagic factors to access the cores of LDs[104].

Lipid droplets in NAFLD

Hepatic steatosis and LD formation

One factor leading to steatosis in hepatocytes is increased substrate availability for LD formation. Notably, obesity is often accompanied by insulin resistance, which increases FA release from adipocytes[105]. FAs from peripheral adipose stores are estimated to contribute to ~60% of the triglycerides stored in hepatocytes in patients with NAFLD[106]. In the case of alcoholic fatty liver disease (AFLD), chronic alcohol exposure in mice stimulates adipose tissue lipolysis with increased activity of ATGL and hormonesensitive lipase. These excessive free FAs can then be taken up by the liver, providing substrate for triglyceride

synthesis[107]. In mammals, increased hepatic mRNA levels of several FAbinding proteins promoting FA uptake into hepatocytes (including FABP4, FABP5 and CD36) have been associated with fatty liver disease[108–110].

The contribution of *de novo* lipogenesis to hepatic triglyceride production in healthy individuals is fairly small and accounts for ~5% of triglyceride incorporated into secreted VLDL[105]. By contrast, the contrib ution of *de novo* lipogenesis in patients with NAFLD is ~25%[105]. Despite insulin resistance associated with the metabolic syndrome, insulin paradoxically continues to stimulate *de novo* lipogenesis in patients with NAFLD via the SREBP pathway[110]. In the case of AFLD, ethanolfed mice show increased *de novo* lipogenesis, probably through increased expression of SREBP1 in the liver[111].

The formation of very large LDs in hepatocytes is the hallmark of steatosis. These large LDs in steatosis can arise from LDlocalized triglyceride synthesis[47] or from one of two known 'fusion' processes: ripening or coalescence. Ripening occurs when the contents of one LD diffuse into another LD[43]. A member of the CIDE family of proteins, CIDEC, promotes LD growth by this mechanism[88] and potentially contributes to giant LD formation[112,113]. By contrast, coalescence represents true fusion of LD organelles, which occurs more quickly[114]. Under normal conditions, coalescence of LDs is rare because phosphatidylcholine on the surface of LDs acts as a surface tensionlowering surfactant[45]. Coalescence of LDs and progression of NAFLD are more likely to occur in mouse hepatocytes if the phosphatidylcholine:phosphatidylethanolamine ratio of the LD membrane is decreased[115]. This feature has been confirmed in *Drosophila* cells in which knockdown of the ratelimiting enzyme in phosphatidylcholine synthesis, CTP:phosphocholine cytidylyltransferase, results in cells with fewer phosphatidylcholines and larger LDs[45]. Adequate levels of phosphatidylcholine are, therefore, required to prevent coalescence of LDs, and phosphatidylcholine levels are maintained in part by sensing of phosphatidylcholine deficiency. Moreover, livers of mice with NAFLD are often markedly deficient in phosphatidylcholine[116], and phosphatidylcholine supplementation improves liver weight and serum transaminases levels in a mouse model of NAFLD[117].

In addition to its role in preventing LD coalescence, normal phosphatidylcholine levels are required for VLDL secretion from hepatocytes[118]. Moreover, lysophosphatidylcholine acyltransferase 3 (LPCAT3) has a critical role in promoting VLDL production[119]. Mice lacking LPCAT3 in the liver have decreased amounts of arachidonoyl phosphatidylcholine in liver membrane fractions and VLDL particles. Despite normal morphology of the ER, the Golgi apparatus and mitochondria, these mice secrete less triglyceride from the liver and do so in smaller VLDL particles. When challenged with a highsucrose diet, they accumulate lipid droplets in the liver, as altered triglyceride secretion can lead to steatosis[119].

Increased LD formation can also contribute to hepatic steatosis, which occurs in a variety of circumstances. Activation of cell stress pathways generally increases the numbers of forming LDs in cells[120–122]. In mice, obesity due to genetic mutations or a highfat diet leads to the formation of massive amounts of LDs in hepatocytes. These processes are often accompanied by activation of ER stress[123], a known activator of a lipogenic gene expression

program in liver[124]. ER stress additionally probably triggers inflammatory responses, further promoting insulin resistance and lipogenesis[125].

Impaired LD consumption

Impaired mobilization of triglyceride and other neutral lipids (for example, from impaired lipolysis, lipophagy or FA oxidation) can also contribute to hepatic steatosis. Liverspecific ATGLknockout mice display hepatic steatosis[94]. Moreover, comparative gene identification-58 (CGI58, also known as ABHD5) activates ATGL[126], and deficiency of liver CGI58 in mice leads to steatohepatitis and fibrosis[127]. In humans, genetic mutations leading to deficiencies in ATGL or CGI58 are rare and lead to neutral lipid storage disease with myopathy or icthyosis (Chanarin–Dorfman syndrome), respectively[128,129]. Both diseases lead to abnormal lipid accumulation in most tissues, including the liver[130,131], and in the case of CGI58 deficiency, progression to cirrhosis and liver failure[130–132]. Furthermore, a role for CGI58 in diacylglycerol and triacylglycerol turnover independent of ATGL has been reported in mouse liver[133]. If CGI58 also performs such a function in humans, it could explain the higher risk of developing hepatic steatosis associated with CGI58 mutation than with ATGL mutation[134].

Studies have linked alterations in autophagy to hepatic lipid homeostasis. Ablation or inhibition of lipophagy promotes steatosis in cultured hepatocytes[100]. Moreover, adenovirus expression of *Atg7*, an autophagy gene, resulted in the reduction of hepatic triglyceride content and serum insulin levels in obese mice[135]. However, whether autophagic failure is a cause or a consequence of hepatic steatosis is still debated. Although the details are beyond the scope of this Review, autophagy might have an important role in fibrogenesis. HSCs seem to have increased levels of autophagy during their activation, and inhibition of autophagy inhibits their activation[136,137].

FA oxidation occurs in the liver during fasting or intense physical activity and alterations in FA oxidation have also been linked to NAFLD[37]. Mitochondria are the site of FA oxidation, and several reports show that LDs interact physically with mitochondria[138,139]. Proteins associated with LDs, such as PLIN2 and PLIN5, can alter FA oxidation[140,141]. PLIN5 is predominantly expressed in highly oxidative tissues, such as muscle and fasted liver, and promotes LD–mitochondria interaction[139]. In muscle, PLIN5 has been proposed to promote lipid storage and limit FA oxidation to protect against lipotoxic effects of high FA release, but this lipoprotective role of PLIN5 in liver has yet to be established[142]. By using labelled FAs, a study demonstrated that FAs are transferred from LD to mitochondria during starvation, and alteration of this process redistributes FA to neighbouring cells[102]. Even if it remains to be demonstrated in a context of NAFLD development, decreased LD–mitochondria interaction might favour hepatic steatosis.

LD–associated proteins and steatosis

Numerous LDassociated proteins have been implicated in the development of hepatic steatosis. Among these, perilipins and CIDE proteins are the most studied. PLIN1 is normally found in adipocytes and coats large LDs[143]. PLIN1 interacts with CIDEC and mediates exchange of lipids between droplets[144]. PLIN1 becomes expressed in the liver

during NAFLD[145] and could have a similar role in hepatocytes as it does in adipose tissue. PLIN1 also suppresses lipolysis by interacting with CGI58 and, therefore, might lead to increased steatosis by this mechanism[146]. PLIN2 is the most upregulated perilipin in rodents and humans with NAFLD[145] and promotes triglyceride accumulation and inhibits FA oxidation[140,147]. *Plin2*knockdown mice are protected from steatosis induced by a highfat diet, although this reduction in steatosis was associated with an increase in the expression of fibrogenesis genes[147,148]. *Plin*2 expressed in an adenoviral vector in rat hepatocytes is linked to decreased lipidrich VLDL production and secretion[140], suggesting that PLIN2 is a potential negative regulator of VLDL lipidation. In summary, PLIN2 activity results in decreased lipid secretion from hepatocytes and accumulation of triglycerides, leading to steatosis. PLIN3 and PLIN5 are also increased in livers of mice with steatosis[143]. PLIN5 binds ATGL and an ATGL activator, CGI58 (also known as ABHD5), in the livers of mice with acute hepatic steatosis, providing a link between PLIN5 and lipolysis. However, the probable complex mechanism of regulating triglyceride metabolism has yet to be elucidated[149]. As with NAFLD, the perilipins have been implicated in AFLD. PLIN1 probably has a similar role in the formation of larger LDs[150]. PLIN2 is the major LD protein associated with AFLD and is required for ethanolfed mice to develop steatosis[151]. AFLD and other LDassociated proteins have been reviewed in detail elsewhere[143,150].

The CIDE proteins are also an area of active research with regard to hepatic steatosis. CIDEA and CIDEC expression in the liver is upregulated in obese mice and associated with increased triglyceride storage in hepatocytes[86,152]. Thus, the CIDE family of proteins might have a similar role in hepatocytes as they do in adipocytes, promoting larger LDs and steatosis. CIDEB has a role in VLDL lipidation and how this process might relate to hepatic disease is under investigation[31].

Steatosis and insulin resistance

Insulin resistance often accompanies hepatic steatosis[153], but the causality behind this relationship is poorly understood (reviewed elsewhere[9,154,155]). Hyperinsulinaemia in insulin resistance drives hepatic steatosis, a process termed ‘selective insulin resistance’ because insulin continues to drive lipogenesis via the SREBP1 pathway[154,156] but fails to suppress gluconeogenesis.

Whether the accumulation of a specific type of lipid is sufficient and necessary for causing insulin resistance is unclear. Considerable evidence in multiple mouse models suggests that hepatic triglyceride accumulation per se does not lead to insulin resistance[154]. In fact, over-expression of DGAT in the liver, promoting triglyceride synthesis, does not result in insulin resistance[157]. In addition, whereas ATGL or CGI58 deficiency leads to increased hepatic triglyceride content, patients with ATGL deficiency have preserved wholebody insulin sensitivity[131], and CGI58knockdown mice are more sensitive to insulin[158].

Other lipids, such as diacylglycerols and ceramides, have been implicated in insulin resistance because they can activate protein kinase C (PKC) isoforms[159,160] that can interfere with insulin signalling[154]. In some contexts, they might well promote insulin resistance. However, in other cases, increased levels of diacylglycerol have been associated

with normal hepatic insulin sensitivity[157,161]. In addition, mice with hepatic DGAT2 overexpression have increased levels of diacylglycerol and ceramides in the liver, but maintained normal insulin sensitivity in some but not all studies[157,162]. Buildup of a particular species of diacylglycerol (such as selected species of *sn*−1,2 diacylglycerol) or diacylglycerol accumulation in specific cellular locations, may predispose to insulin resistance due to activation of protein kinase C[163,164], but further studies are necessary to clarify this issue.

The relationship of LDs to hepatic insulin resistance is unclear, but, in general, LDs probably protect against lipotoxicity by storing lipids as triglycerides and protecting the ER from lipotoxic insults. Furthermore, when LD triglycerides are hydrolysed by ATGL, *sn*1,3 and *sn*−2,3 diacylglycerol are generated, and DGAT2 in the ER and at LD surfaces preferentially reesterifies *sn*−1,3 diacylglycerol to triglyceride[165]. This finding suggests that LDderived diacylglycerol is unlikely to activate PKC unless it undergoes isomerization to *sn*1,2 diacylglycerol.

Genetic risk factors for NAFLD

Genomewide association studies identified a mutation in the ATGL homologue *PNPLA3* (also known as adiponutrin) as a risk factor for hepatic steatosis prevalence and progression[166–169]. This mutation is a single nucleotide polymorphism (rs738409 C>G) encoding an isoleucinetomethionine variant at amino acid position 148 (Ile148Met) of the 481amino acid PNPLA3 protein[166–168]. This variant predisposes patients to the spectrum of NAFLD, progression of AFLD, as well as fibrogenesis associated with HCV infection[170]. The mechanism by which the Ile148Met variant leads to increased hepatic fat content is a subject of debate and remains unclear. Recombinant Ile148Met protein was initially shown to have decreased glycerolipid hydrolase activity, suggesting that the variant might result in decreased triglyceride hydrolysis at LDs[95,171,172]. However, mice with PNPLA3 deficiency do not have steatosis[173,174], implying that the variant does not result in simple loss of lipase function. In addition, overexpression of the Ile148Met variant in mouse liver resulted in hepatic steato sis[175], which would not be expected if the protein acted solely as a lipase. PNPLA3 has lysophosphatidic acid acyltransferase (LPAAT) activity when expressed in bacteria and, therefore, could be involved in the triglyceride synthesis pathway[176]. However, this activity was not observed in mouse liver expressing wildtype or mutant protein[175]. Moreover, PNPLA3Ile148Met knockin mice challenged with a highsucrose diet had increased hepatic steatosis and 40fold higher levels of PNPLA3 on LDs without changes in mRNA levels[177], again suggesting a gain of function rather than a loss. These authors entertained the possibility that PNPLA3 Ile148Met coats the LD sequestering CGI58, decreasing lipolysis, but their experiments had mixed results with respect to testing this hypothesis[177]. An alternative possibility is that, because the Ile148Met variant is found at increased abundance in LDs[177], it might affect the LD protein composition indirectly by crowding out other proteins[103], and altered composition might adversely affect the capacity for lipolysis.

The involvement of PNPLA3 in hepatic steatosis has been linked to its retinyl ester lipase activity in HSCs. Retinol is released from HSCs during the progression to a fibrotic state and

is associated with a loss of HSCassociated LDs[18,178]. Interestingly, this activity is affected in the Ile148Met variant. Moreover, PNPLA3 over expression reduces LD content in HSCs in contrast to the Ile148Met variant, suggesting a lossoffunction mutation[179]. Even if PNPLA3 does not have retinylester lipase activity in hepatocytes, this study opens new avenues on the role played by PNPLA3 in different liver cell types during steatosis development. At present, the precise role of PNPLA3 in hepatic steatosis and disease progression remains elusive and is an area of active research.

The next most significant genetic factor pre disposing patients to increased hepatic triglyceride content and elevated serum alanine aminotransferase is the transmembrane 6 superfamily member 2 (TM6SF2) rs58542926 C>T variant encoding Glu167Lys[180,181]. The TM6SF2 protein has 351 amino acids with multiple predicted transmembrane domains and localizes to the ER and ER–Golgi intermediate compartment in human liver cells[182]. When the human Glu167Lys variant is expressed in hepatoma cells, mRNA levels are similar to that of the wildtype, but protein levels are decreased by ~50%[180]. *TM6SF2* siRNA inhibition in human hepatic cell lines results in decreased secretion of triglyceriderich lipoprotein particles and increased LD content[182]. In mice, hepatic knockdown of *Tm6sf2* resulted in increased hepatic triglyceride content and low circulating levels of cholesterol and triglyceride levels, consistent with a defect in VLDL secretion[180]. In *Tm6sf2*knockdown mice fed a highsucrose diet, LDs were larger and more numerous[180]. Although another study did not demonstrate a hepatic phenotype in mice when *Tm6sf2* was overexpressed or knocked down, the authors suggested that transiently altering expression is probably different from a genetic variant in humans[181].

Yet another genomewide association study revealed that the rs641738 C>T variant in the locus coding for the membranebound lysophospholipid acyltransferase 7 (MBOAT7) is linked to an increased risk of alcoholrelated cirrhosis[183]. This variant was also associated with an increased risk of fibrosis[184]. MBOAT7 is an enzyme involved in the transfer of FAs between phospholipids and lysophospholipids, but the precise role of MBOAT7 in liver disease development remains unclear.

LDs in HCV infection

About half of patients chronically infected with HCV have steatosis, and the prevalence of steatosis is as high as 75% in patients with HCV genotype 3a infection[185]. Patients with HCV infection and >30% of hepatocytes with steatosis are more likely to have fibrosis, suggesting that patients with steatosis might represent a subgroup at increased risk of disease progression[185]. The development of steatosis in this population seems to be mediated by altered triglyceride lipolysis[186]. The role of LDs in the HCV life cycle and pathology have been extensively reviewed elsewhere[187–190]. HCV core protein represents the first 191 amino acids of the polyprotein and provides an anchor to the ER membrane. After two cleavages at the *N*terminus signal peptide, the core protein is produced and has access to the LD via the D2 domain[191,192]. This domain has two amphipathic αhelices that enable attachment of the core protein to LDs[192]. Overexpression of the core protein alone is sufficient to cause hepatic steatosis in mice[193]. The association between HCV core protein and LDs depends on DGAT1 (REFS 186,191), which also traffics the nonstructural protein

NS5A to the LD and facilitates the NS5A and core protein interaction[194]. This finding suggests that HCV core accesses LDs from the ER during formation, although this process has not been formally demonstrated experimentally. Whether HCV core requires ARF1–COPI proteins to traffic to LDs, such as some ERLD proteins, is unclear[78]. DGAT1-dependent localization of core to the LD also seems necessary for the steatogenic properties of HCV[191]; core protein expression results in increased triglyceride content in wildtype mouse liver, but not *Dgat1*knockout mouse liver[186]. Nonetheless, DGAT1 activity is not increased in core infected livers, implying that increased triglyceride synthesis is not a cause of steatosis[186]. Instead, the mechanism for HCVcoreinduced steatosis seems to involve decreased triglyceride turnover, an effect that depends on HCV core protein localization to the LD[186]. Furthermore, the effect of HCV core protein on lipolysis seems to be mediated by inhibition of ATGL through an unclear mechanism, while enhancing ATGL interaction with CGI58. It is speculated that ATGL cannot access the TG in LDs surrounded by HCV core protein[195].

LD–associated therapeutics

At present, the most effective treatment for NAFLD is to reduce body fat by weight loss and exercise, and there are no approved pharmacological therapies for NASH. As the underlying causes for the progression from simple steatosis to steatohepatitis and fibrosis and/or cirrhosis remains unclear, treatments could be designed to decrease lipid accumulation in the liver or to prevent the progression towards more advanced inflammatory disease. A consensus from a joint workshop with the Association for the Study of Liver Diseases and the FDA published in 2015 suggested outcome measures for studies investigating therapeutics that focus on the reversal of steatohepatitis with no progression to advanced fibrosis, or development of cirrhosis[196]. We suggest that therapeutic strategies might also include treatment or resolution of steatosis, analogous to the concept of lowering lipids to prevent atherosclerosis. Of note, the resolution of steatohepatitis almost never occurs without improvement in steatosis[196]. Here, we therefore focus on potential targets that reduce steatosis as it relates to triglyceride storage in LDs (FIG. 6).

An initial thought to improve hepatic lipid accumulation would be to decrease triglyceride synthesis, which could be achieved by targeting the triglyceride synthesis enzymes, DGAT1 or DGAT2. DGAT1 inhibition decreases liver triglyceride content in mice challenged with highfat diet[197], but, unfortunately, DGAT1 inhibitors administered to humans resulted in intolerable gastro intestinal adverse effects, most notably diarrhoea[198]. DGAT2 also seems a viable target, especially given its link to *de novo* lipogenesis[22] and eLDs[47]. Human DGAT2 inhibitor studies have not been reported, but the role of DGAT2 in hepatic steatosis has been studied in mice. One study used antisense oligonucleotides to decrease hepatic DGAT2 expression in obese mice on a highfat diet. Reductions in DGAT2 expression resulted in decreased steatosis and improved hyperlipidaemia[199]. Another study used DGAT2 antisense oligonucleotides in obese mice on a methionine and cholinedeficient diet (used to model NASH), which again decreased hepatic lipid content, but resulted in increased liver damage[200]. Similar results were found by inhibiting acylCoA desaturase (also known as stearoylCoA desaturase), a key enzyme involved fatty desaturation, pointing to a key role of triglyceride formation and storage in LDs as protective in hepatocytes by buffering them

from the potential lipotoxic effects of free FAs[201]. This finding highlights the complexity in choosing therapeutic targets, and the importance of studying the roles of DGAT1 and DGAT2, in steatosis and steatohepatitis.

Further understanding of the roles of LD formation, growth and catabolism as they relate to disease progression might provide additional opportunities for intervention. Particular proteins of interest include seipin, implicated in LD formation[70,73], GPAT4, important for LD growth[47], and CIDEC, involved in LD growth via ripening. Understanding the role of Ile148Met PNPLA3 variant, which seems to be a gainoffunction mutation, is paramount. As this mutation represents the most prevalent genetic risk factor for steatosis and disease progression, targeting this protein stands to benefit a large number of people. Strategies to increase lipolysis, possibly by increasing fat oxidation, are attractive avenues to lower hepatic lipid levels. Increasing secretion of triglycerides, although logical, might be problematic, as this strategy might be complicated by increases in atherogenic apoB-containing lipoproteins. In fact, any strategy to reduce triglyceride synthesis or storage needs to be evaluated to ensure that it does not trigger a lipotoxic stress pathway and generate non-salutary effects.

Conclusions

The latest discoveries have shed light on LD biology and the delicate balance of liver fat in human health and disease. Despite our growing knowledge and the burden of hepatic steatosis in multiple disease states, in particular NAFLD, there is a lack of treatment options beyond lifestyle changes and liver transplantation. Therein lies an opportunity to translate our growing understanding of LD regulation to develop therapies that target the process of abnormal lipid accumulation and progression of liver disease. The first step in accelerating this process is to address some key unanswered questions concerning the biology of LDs in the liver. First, there remain uncertainties about mechanisms for triglyceride packaging into newly formed LDs, LD budding and contact of eLDs with the ER during times of growth. Second, a better understanding of lipolysis in the liver is needed, since alterations in lipolysis can contribute greatly to NAFLD. Third, more studies are needed to define the molecular processes that lead to VLDL lipidation and secretion. The contribution of lipids from iLDs and eLDs for secretion and what proteins might mobilize these neutral lipids are open to discovery. A better understanding of these processes will ultimately lead to the design of molecular therapies aimed at decreasing NAFLD and its sequelae.

Acknowledgements

We thank S. Alexandrescu for the pathology images in Figure 2, and G. Howard for editorial assistance. R.V.F. acknowledges support from NIH National Institute of General Medical Sciences R01 GM099844, NIH National Institute of Diabetes and Digestive and Kidney Diseases R01 DK056084 and R01 DK101579. T.C.W. acknowledges support from NIH National Institute of General Medical Sciences R01 GM097194. T.C.W. is an investigator of the Howard Hughes Medical Institute. M.B. is supported by a fellowship from the Jane Coffin Childs Foundation.

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Key points

- Depending on the body's needs, the liver utilizes lipids to generate metabolic energy, secretes them as lipoproteins, or packages them for storage
- Unbalanced lipid storage and utilization result in supraphysiological triglyceride accumulation in hepatocytes, known as hepatic steatosis
- Hepatic lipids accumulate in organelles known as cytoplasmic lipid droplets
- Our expanding knowledge of lipid droplets and their associated protein machinery provide opportunities for molecular-based approaches for treating nonalcoholic steatosis and steatohepatitis

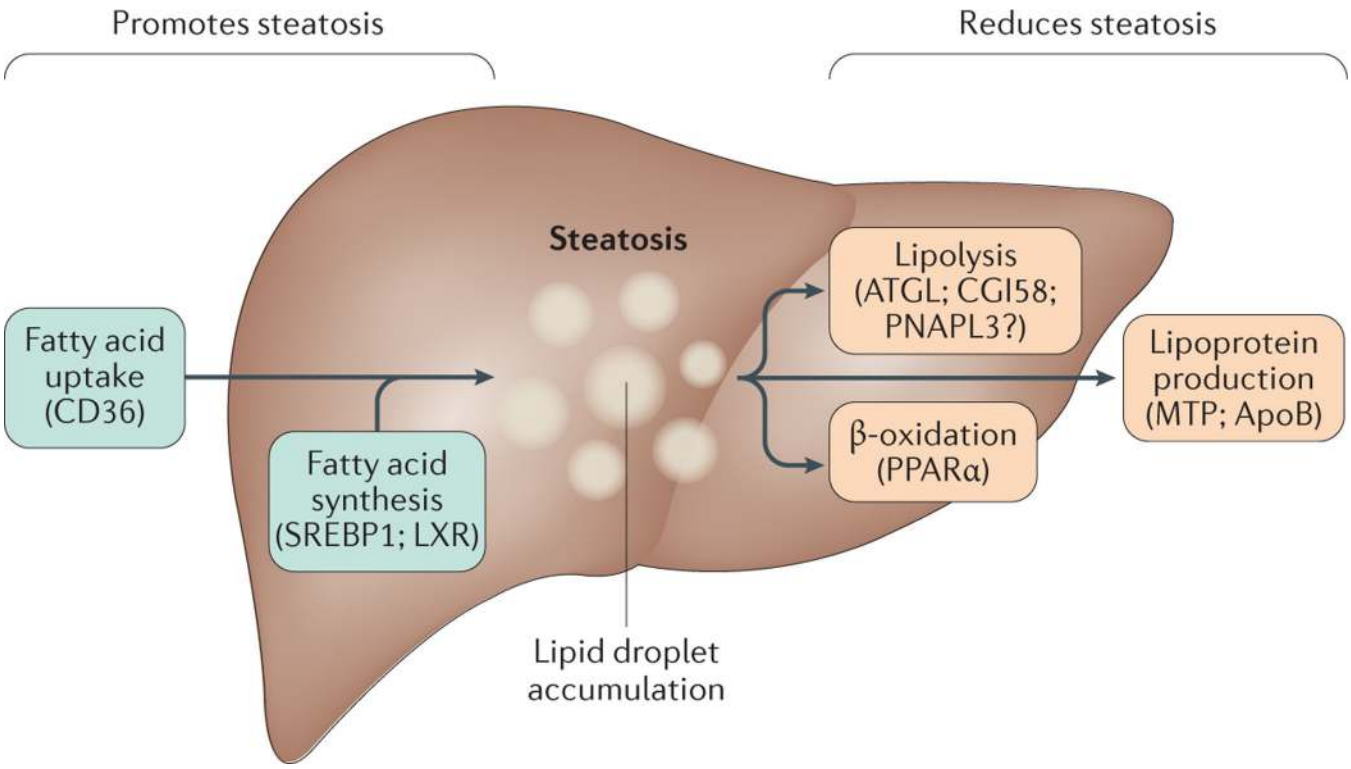

Figure 1 |. Hepatic steatosis results from an imbalance in lipid storage and lipolysis or secretion.
Hepatic steatosis can result from different processes: increased fatty acid (FA) uptake, *de novo* lipogenesis and triglyceride synthesis combined with lipid droplet (LD) biogenesis or growth; decreased LD catabolism (including decreased fatty acid oxidation); or impaired triglyceride or very-low-density lipoprotein (VLDL) secretion. Factors associated with these processes are listed in the figures, those that upregulate steatosis (green boxes) and those that downregulate steatosis (orange boxes).

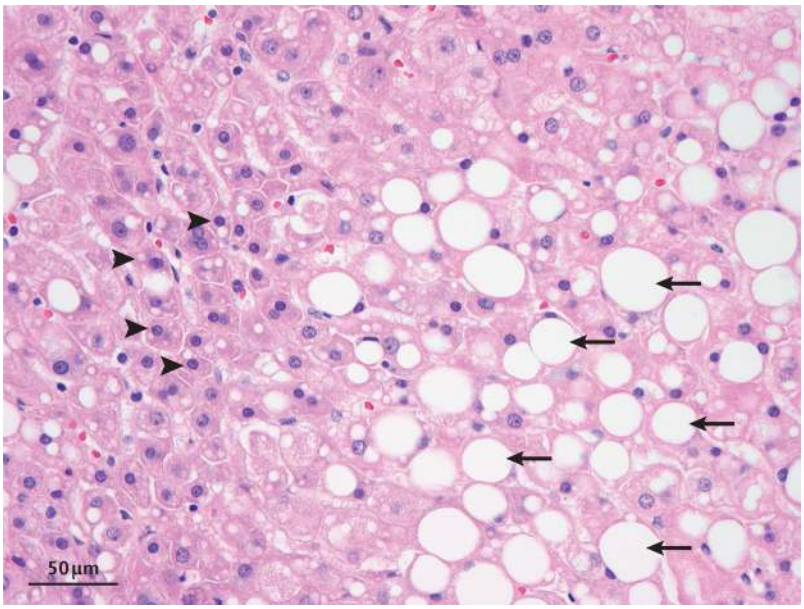

Figure 2 |. Histopathology of NAFLD showing areas of macrosteatosis and microsteatosis.
A haematoxylin and eosin-stained tissue sample from a human showing hepatocytes exhibiting microvesicular (arrowheads) and macrovesicular (arrows) steatosis. Hepatocytes with microvesicular steatosis have abnormal accumulation of lipid with preserved cellular architecture, including a non-displaced nucleus, whereas hepatocytes with macrovesicular steatosis have one large droplet that displaces the nucleus. We thank S. Alexandrescu at Boston Children's Hospital, USA, for this image.

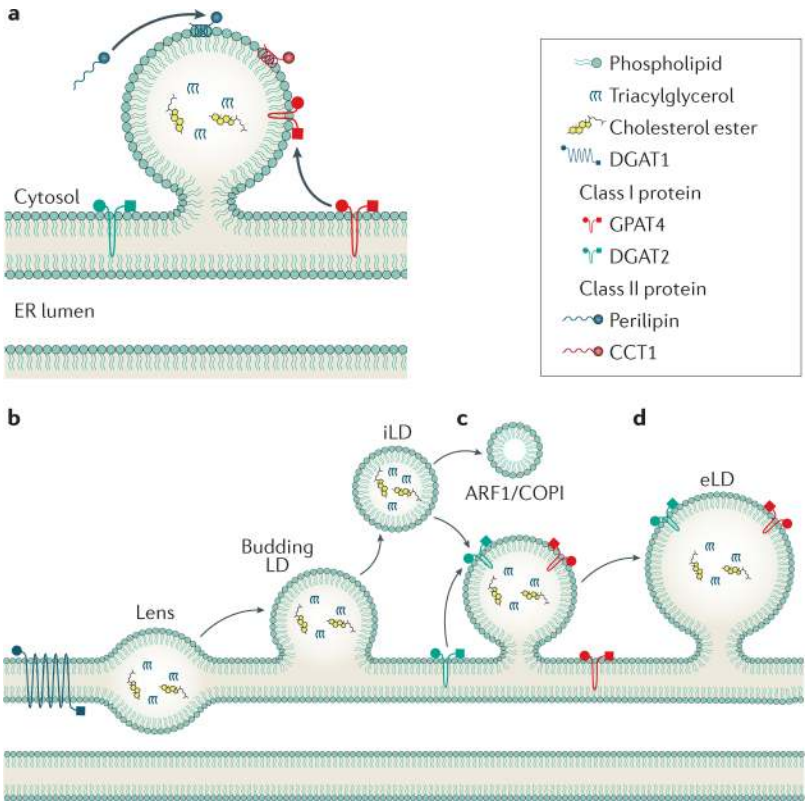

Figure 3 |. Lipid droplet formation and expansion.

a | Lipid droplets (LDs) consist of a neutral lipid core surrounded by a phospholipid monolayer. Proteins access the LD surface by relocalizing from the ER bilayer (class I) or from the cytosol (class II). **b** | LD formation begins with neutral lipid synthesis. The lipids accumulate in the ER bilayer to form a lens. **c** | Eventually, the bilayer deforms and causes the droplet to bud forming an initial LD (iLD). **d** | COPI can bud nano-LDs from the iLD, resulting in increased surface tension and reconnection of the iLD with the ER. This contact allows class I proteins to access the droplet, including GPAT4 and DGAT2. These enzymes are involved in triglyceride synthesis and result in LD growth, forming an expanding LD.

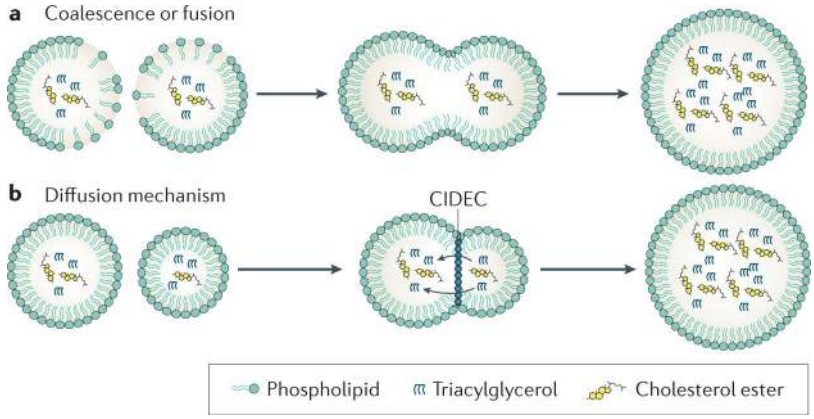

Figure 4 |. Giant lipid droplet formation.

Giant lipid droplets (LDs) form in one of two ways. **a |** Coalescence, which occurs for example during relative phosphatidylcholine deficiency, results in rapid fusion of two LDs. **b |** In a process called ripening, neutral lipids are transferred by the slower process of diffusion with a net transfer from smaller to larger LDs. Ripening seems to be facilitated by proteins such as CIDEC (also known as FSP27).

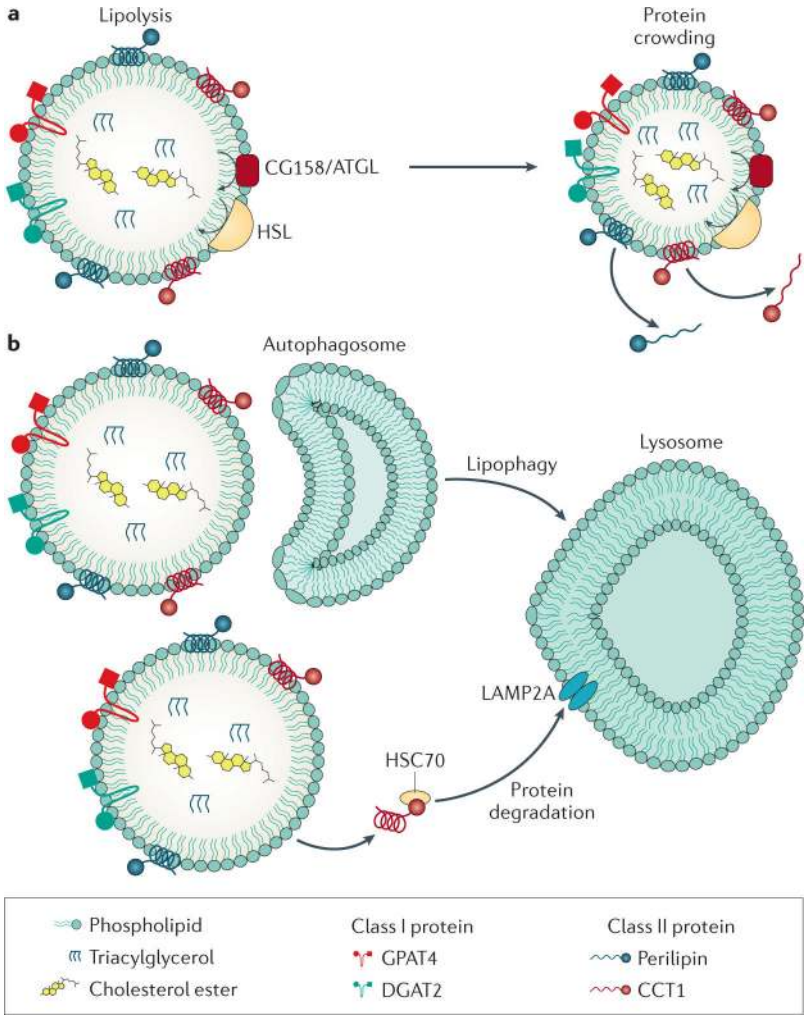

Figure 5 |. Lipid droplet consumption.

a | Lipid droplets (LDs) can be degraded by lipolysis. As the surface of the LD shrinks, there is protein crowding and some proteins, especially class II proteins, fall off. **b |** Other proteins are removed from the LD surface and brought to the lysosome by chaperone-mediated autophagy. Small LDs or parts of an LD can be engulfed by a membrane bilayer to form autophagosomes that can be delivered to the lysosome for degradation.

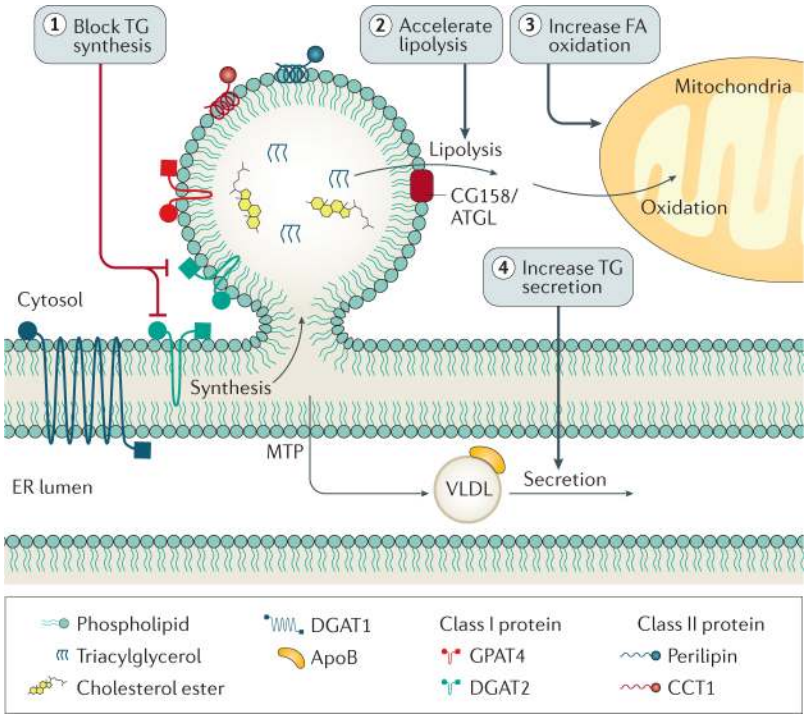

Figure 6 |. Possible therapeutic targets to decrease hepatic steatosis.

Interventions designed to decrease triglyceride (TG) synthesis, increase lipolysis, increase fatty acid (FA) oxidation, or increase very-low-density lipoprotein (VLDL) secretion could decrease hepatic TG content. ER, endoplasmic reticulum.