Hepatic lipid droplet homeostasis and fatty liver disease

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Abstract

In cells, lipids are stored in lipid droplets, dynamic organelles that adapt their size, abundance, lipid and protein composition and organelle interactions to metabolic changes. Lipid droplet accumulation in the liver is the hallmark of non-alcoholic fatty liver disease (NAFLD). Due to the prevalence of obesity, the strongest risk factor for steatosis, NAFLD and its associated complications are currently affecting more than 1 billion people worldwide. Here, we review how triglyceride and phospholipid homeostasis are regulated in hepatocytes and how imbalances between lipid storage, degradation and lipoprotein secretion lead to NAFLD. We discuss how organelle interactions are altered in NAFLD and provide insights how NAFLD progression is associated with changes in hepatocellular signaling and organ-crosstalk. Finally, we highlight unsolved questions in hepatic LD and lipoprotein biology and give an outlook on therapeutic options counteracting hepatic lipid accumulation.

Keywords: lipid droplet, NAFLD, NASH, Steatosis, lipid metabolism, VLDL

1. Introduction

Lipids are a heterogeneous group of hydrophobic or amphipathic small molecules including an enormous amount of diverse species such as glycerolipids, glycerophospholipids, sphingolipids, sterol lipids or bile salts (*1*).They serve as building blocks of cellular membranes, precursors for hormones, and are important molecules for energy storage, cellular signaling and organ-crosstalk. The maintenance of lipid homeostasis requires coordination of lipid uptake, distribution, storage and utilization in cells and organs, but also on whole body level. Here, the central nervous system functions as key regulator that tightly interacts with metabolic active tissues such as the gastrointestinal tract, adipose, tissue and liver. The liver plays a major role in regulating the distribution of lipids within the body. In response to fasting and feeding, hepatocytes synthesize, store, and secrete lipids to maintain whole-body lipid homeostasis. External fatty acid uptake and internal *de novo* synthesis are balanced by rates of fatty acid

degradation and secretion of bile acids via mainly ATP-dependent transporters into canaliculi and lipoproteins via the secretory apparatus into the circulation. These processes are regulated by a combination of factors, such as the availability of substrates and metabolic hormones. Under physiological conditions, only small amounts of fatty acids are stored as lipids in cytosolic lipid droplets in the liver (*2*).

Lipid droplets and lipoproteins are two key components involved in hepatic lipid metabolism. Lipoproteins are macromolecules that transport lipids in plasma, whereas lipid droplets are lipid-rich organelles that function as energy storage depots within the cells. Lipid droplets also protect cells from lipotoxicity, an accumulation of lipids that impairs cellular membranes, induces mitochondrial dysfunction, and interferes with signaling pathways (*3-7*). Lipid droplets and lipoproteins share a common architecture: they consist of a core of mainly triglycerides and cholesteryl esters surrounded by a phospholipid monolayer (Figure 1A). The amphiphilic phospholipids serve as surfactants at the interphase between the hydrophobic lipid droplet core and the hydrophilic aqueous environment (the plasma or in the cell cytosol) (*8*). Proteins harboring hydrophobic domains decorate lipid droplet and lipoprotein monolayers: the lipoprotein proteome mainly consists of apolipoproteins and lipoprotein-associated proteins that regulate lipid metabolism, whereas the lipid droplet proteome is highly diverse. Proteomic- and microscopy-based studies have identified proteins involved in cellular trafficking, key signaling proteins, transcription factors, and histones on the surface of lipid droplets, in addition to proteins regulating lipid metabolism and flux (*9-11*). These differences between lipid droplets and lipoproteins relate to their different functions in hepatic lipid metabolism.

Imbalances between the rates of lipid droplet formation, lipid droplet mobilization, and lipoprotein or bile acid secretion can lead to excessive lipid accumulation, a pathological condition known as non-alcoholic fatty liver disease (NAFLD). This often occurs in obesity, when the adipose tissue exceeds its lipid storage capacity and lipids spill into the liver (*12*). This condition is characterized by the formation of many extremely large lipid droplets in hepatocytes (*13*). NAFLD encompasses different histopathological stages, ranging from clinical asymptomatic hepatic steatosis to non-alcoholic steatohepatitis (NASH), a condition accompanied by inflammation, fibrosis, and sometimes cirrhosis (*14*). However, not all obese humans develop

steatosis and only a small percentage of those individuals progress to NASH (*15*, *16*). The cellular processes underlying the development of NAFLD and progression to NASH are still poorly understood. Over the last decade, genome-wide association studies (GWAS) have identified several genomic sequence variations associated with increased risk of chronic liver disease (*17-21*). Obesity and dietary factors strongly amplify this risk in individuals carrying the identified genetic factors (*22*). Due to the increasing prevalence of obesity, NAFLD is becoming more common, with an incidence of 20%-30% in Western countries (*23*) and affecting in total about 1.8 billion people worldwide (*24*). Although potential therapies have entered Phase II and III clinical trials (*25*), no pharmacological therapies for NAFLD have been approved. Understanding the cellular and molecular processes leading to perturbation of liver lipid metabolism is necessary in order to identify novel pharmacological targets and treatment strategies.

Here, we review models of lipid homeostasis in hepatocytes; we focus on the role of lipid droplets and present recent findings describing the effect of steatosis on the subcellular organization. We discuss the effect of known genetic risk factors for NAFLD progression on hepatic lipid metabolism and lipid droplet biology, and recent insight into how signaling pathways and organ crosstalk affect lipid accumulation. Finally, we highlight unsolved questions concerning hepatic lipid droplet and lipoprotein biology.

2. Cell biology of hepatic lipid droplet homeostasis

2.1. Neutral lipid synthesis and lipid droplet biogenesis

Hepatic lipid stores incorporate fatty acids from different sources, depending on the metabolic state of the organism, as well as metabolite and hormone levels (hepatic lipid metabolism overview (Figure2). The majority of fatty acids originate from the breakdown of triglycerides in adipose tissue (lipolysis). Under fasting conditions this is almost the exclusive source of fatty acids, whereas, in the postprandial state, when plasma lipid and glucose levels are increased, a substantial portion of the hepatic fatty acid pool originates from LDL receptor-mediated endocytosis of chylomicron remnants, and from *de novo* lipogenesis using carbohydrates as substrates (*26*). In the fed state, insulin and elevated glucose levels stimulate hepatic lipogenesis through the activation of transcription factors. Sterol regulatory element binding protein 1C

(SREBP1c) and carbohydrate response element binding protein (ChREBP) act in synergy to induce the expression of lipogenic genes. Insulin increases SREBP1c transcription via an AKT signaling-dependent mechanism, and induces SREBP1c transcriptional activity via proteolysis (*27*). ChREBP activity is regulated by its cellular localization. Increased glucose levels prompt a phosphorylation-dependent relocalization to the nucleus, which induces gene expression (*28*). Recently, a mechanism was elucidated how ChREBP functions in adjusting the cellular response to glucose to intracellular energy stores. When lipid droplets accumulate in cells, MLX transcription factors, including ChREBP bind to LDs via highly conserved C-terminal amphipathic helices, thereby reducing their availability for nuclear transcriptional activity. In contrast, the absence of LDs results in increased expression of MLX target genes (*29*).

Independent from their source, fatty acids are esterified into cholesteryl esters by acyl CoA cholesterol acyltransferases (ACATs 1 and 2), and into triglycerides by diacylglycerol-O-acyltransferases (DGATs 1 and 2). ACATs and DGAT1 belong to a family of membrane-bound O-acyl transferases that is characterized by several transmembrane domains. DGAT1 exclusively localizes to the endoplasmic reticulum and mainly catalyzes triglyceride formation using exogenously supplied fatty acids (*30*, *31*). DGAT2, on the other hand, has a hairpin structure which allows it to translocate from the endoplasmic reticulum to lipid droplets (*32*), inducing their expansion using preferentially *de novo* synthesized fatty acids as substrates (*33*).

Neutral lipid products of ACATs and DGATs are thought to accumulate between the leaflets of the endoplasmic reticulum bilayer (Figure3). Phase separation drives neutral lipid accumulation into a lipid lens that grows and finally buds (*34*). It remains unclear whether this neutral lipid accumulation and budding occurs spontaneously, or whether they depend on proteins or certain lipid species. In most cell types, budding occurs exclusively towards the cytosol, although luminal lipid droplet budding that incorporates lipid droplets into very low-density lipoproteins (VLDLs) has been observed in hepatocytes (*35*). Many questions about the generation of lipid droplets remain, including how the budding of the lipid lens towards the cytosol or endoplasmic reticulum lumen is regulated, and how the flux of neutral lipids towards either storage or secretion is controlled. Recent *in vitro* studies found that differences in the phospholipid composition between the two leaflets lead to tension asymmetry, which could favor budding towards the side

with lower monolayer tension (*36*). Several factors could drive this lipid asymmetry, such as (1) lipid-modifying enzymes acting on one leaflet of the bilayer, (2) the insertion of proteins at one side of the bilayer, or (3) an asymmetric refill of newly synthesized phospholipids, leading to better phospholipid surface coverage and less surface tension at one of the leaflets (*37*). The presence of specific proteins appears to favor the budding reaction. FIT2 (Fat storage-inducing transmembrane protein 2), an integral endoplasmic reticulum membrane protein is required for lipid droplet formation. FIT2 function is still poorly characterized. The protein has lipid-phosphate phosphatase activity (*38*, *39*) and might induce membrane asymmetries between the endoplasmic reticulum bilayers promoting lipid droplet budding. Another protein, Seipin, forms ring-shaped oligomers in the endoplasmic reticulum stabilizes the connection between growing lipid droplets and the endoplasmic reticulum. Seipin oligomers might thereby facilitate triglyceride transfer and lipid droplet budding (*40*, *41*). Ablation of Seipin function leads to loss of adipose tissue and extreme hepatic lipid accumulation. Mutations in Seipin have been identified in individuals with familial forms of lipodystrophy, a human genetic disease, where the ability to store fat in adipose tissues is completely or partially impaired (*42*), indicating that Seipin is crucial for lipid droplet formation. How the different proteins, phospholipid species and triglycerides act together to enable lipid droplet budding and whether the budding promoting factors differ between lipid droplet subpopulations, cell types and tissues is still topic of current research.

2.2. Interplay between neutral lipids and phospholipid metabolism

The liver continuously synthesizes large amounts of phospholipids to support lipid droplet and VLDL formation and secretion. The composition of lipid droplet and VLDL monolayers in human hepatocytes resembles the composition of the endoplasmic reticulum, which is mainly composed of phosphatidylcholine (50%), phosphatidylethanolamine (15-25%), and small amounts of phosphatidylinositol, phosphatidic acid, and lyso-phosphatidylcholine (*43*).

The geometry of phospholipids depends on the ratio of the size of the polar head groups to the volume the acyl chains occupy that is determined by their length and degree of saturation (*44*). Phosphatidylcholine and phosphatidylethanolamine differ in their geometrical and physical

properties because of the different sizes of their head groups (Figure1B). Due to its cylindrical shape, phosphatidylcholine provides good coverage of the lipid droplet surface area, making it a good surfactant important for lipid droplet stability. In contrast, phosphatidylethanolamine has a smaller head group, favoring a cone-shaped structure. Phosphatidylethanolamine is a poor surfactant and large amounts of cone-shaped phosphatidylethanolamines destabilize lipid droplets and induce fusion (*45*). Neutral lipids of the lipid droplet core can fill the space between phospholipids and intercalate into the monolayer, resulting in hydrophobic patches on the lipid droplet surface. These discontinuities in the phospholipid layer are known as packing defects (*46*). The amount of packing defects on lipid droplets is determined by parameters such as lipid droplet size, phospholipid composition, and availability (*8*). Phosphatidylcholine deficiency or low phosphatidylcholine/ phosphatidylethanolamine ratios lead to increased packing defects on the lipid droplet monolayer resulting in instable lipid droplets that tend to coalesce into large lipid droplets (*45*). In addition to determining lipid droplet stability, phospholipid availability and the phosphatidylcholine/ phosphatidylethanolamine ratio are crucial for VLDL secretion (*47*). Phosphatidylcholine-deficient VLDL particles are less stable and are partially degraded within the cells, resulting in decreased VLDL secretion and hepatic lipid accumulation (*48*). Imbalances in phospholipid levels and composition impair hepatic neutral lipid metabolism not only in mice but also in humans (*49*). Interestingly, patients with NAFLD and NASH have a lower phosphatidylcholine/ phosphatidylethanolamine ratio in the liver compared to healthy subjects (*50*) and patients with mutations in phosphatidylcholine-synthesis enzymes have severe fatty livers (*51*). The symptoms in these human disorders reinforce the importance of coordination of neutral lipid and phospholipid metabolism.

Considering the importance of phospholipid supply and composition to lipid droplet stability, it is not surprising that phosphatidylcholine synthesis in the liver is tightly regulated by metabolic state. Under conditions of increased lipid droplet formation or VLDL secretion, large amounts of phosphatidylcholine are required. This is achieved by regulating the level of CTP:phosphocholine cytidylyltransferase (CCT) – the rate-limiting enzyme of phosphatidylcholine synthesis – via a combination of transcriptional, translational, and post-translational mechanisms (*52*, *53*). The CCT isoform with the highest expression in the liver, CCTα, contains a lipid-sensing domain

consisting of an amphipathic helix with large hydrophobic residues. This domain recognizes large and frequent packing defects on the lipid droplet surface and in phosphatidylcholine deficient membranes (*46*). Recruitment of CCTα to these packing defects induces a conformational change that activates the protein. (*54*).

In vitro studies suggest that, under conditions of lipid droplet expansion, phosphatidylcholine deficiency occurs on cellular membranes and lipid droplets. Cellular sites of phosphatidylcholine deficiency might be species- and cell-type specific. In *Drosophila* S2 cells, CCTα is targeted to lipid droplets (*55*), whereas in hepatocytes and liver tissue CCTα translocates to nuclear membranes (*56*). However, organelle proteomic analysis indicates at least partial lipid droplet localization of CCTα also in steatotic liver (*57*). Additionally to regulating its activity by membrane binding, CCTα activity is post-prandially upregulated by the mammalian target of rapamycin (mTOR) pathway. Activation of mTOR complex 1 by nutrients leads to increased CCTα levels by post-transcriptional mechanisms and activation of CCTα by phosphorylation, thereby increasing phosphatidylcholine production and VLDL secretion (*58*). However, hepatic phosphatidylcholine synthesis can already be activated in advance of metabolic hormones and metabolites reaching the liver in response to feeding. Sensory food perception prior to food uptake can activate proopiomelanocortin neurons in the hypothalamus, which induces hepatic mTOR signaling and the endoplasmic reticulum stress response. mTOR and the stress response both increase hepatic CCTα expression and phosphatidylcholine synthesis. This research reveals an elegant mechanism of linking upregulating hepatic phosphatidylcholine synthesis to lipid droplet and VLDL formation in advance of metabolic needs (*59*). The control of CCTα activity at all those multiple levels in hepatocytes is a fascinating example how an enzyme adjusts its activity to changing metabolic states by a combination of several sophisticated mechanisms. It enables the hepatocytes to coordinate phospholipid synthesis with neutral lipid metabolism what is crucial for their metabolic flexibility.

Recently, it was found that in the liver nuclear lipid droplets contribute to the regulation of phosphatidylcholine synthesis. They are predominantly found in hepatocytes; their biogenesis is coupled to VLDL formation, and they derive from endoplasmic reticulum luminal lipid droplets that relocate to the nucleoplasm through leaks in the nuclear reticulum (*60*). These nuclear lipid

droplets appear to play a crucial role in the regulation of phosphatidylcholine synthesis, since they recruit CCTα and strongly contribute to its activation (*60*). The finding that nuclear lipid droplets are involved in this regulation of CCTα activity and that CCTα is favorably targeted to them compared to cytosolic lipid droplets, at least under certain conditions, raises new exciting questions, such as whether and how the protein and lipid composition of nuclear lipid droplets differ from cytosolic lipid droplets. Moreover, the physiological function of nuclear lipid droplets and their role in hepatocytes needs further investigation.

2.3. Lipid mobilization and degradation

In hepatocytes, lipolysis and autophagy together mobilize lipids for degradation via beta-oxidation or re-esterification for VLDL secretion (Figure3). Indeed, studies found that the majority of triglycerides for VLDL assembly derive from lipolysis and re-esterification of triglycerides stored in lipid droplets (*61*). In most tissues, when lipolysis is induced, lipid droplet-localized triglyceride lipase (ATGL) and its cofactor CGI-58 act sequentially with hormone sensitive lipase (HSL) and monoacylglycerol lipase (MGL) to cleave fatty acids from the glycerol backbone (*62*, *63*). However, the role of those lipases and their contribution to lipolysis in hepatocytes is unclear. Liver-specific ablation of either ATGL or CGI-58 in mouse models results in steatosis and fibrosis, indicating a major function for ATGL in liver, especially for channeling fatty acids into beta-oxidation (*64*). However, HSL is poorly expressed in human liver (*65*). Moreover, overexpression or knockdown of ATGL, HSL and MGL does not substantially affect hepatic VLDL secretion, indicating that their activities do not contribute substrates for hepatic VLDL production (*64*, *66*). Therefore, additional lipases such as endoplasmic reticulum localized TGH might mobilize lipids for VLDL formation (*67*, *68*).

In addition to the actions of the cytoplasmic and endoplasmic reticulum localized lipases, a subtype of macroautophagy called “lipophagy” plays a role in lipid droplet turnover and lipid mobilization in hepatocytes. Indeed, ablation of autophagy genes reduced triglyceride secretion and increased triglyceride storage in rat hepatocytes (*69*). When lipophagy is activated, a highly regulated network of proteins that includes the canonical autophagosomal proteins (such as ATG proteins and LC) interact to induce phagophore formation. This endoplasmic reticulum -derived

double-membrane structure selectively sequesters whole organelles such as complete lipid droplets and targets them for lysosomal degradation (*70*). Lipid droplets can either be completely engulfed or be consumed in a piecemeal fashion into autophagosomes (*71*). Studies have suggested that Rab proteins associated with lipid droplets mediate contacts between lipid droplets and autophagosomes (*72*). Rab proteins are believed to be organelle specific and maintain organelle identity (*73*). Many Rab proteins of the endosomal pathway have been associated with lipid droplets, opening up the question of recruitment specificity between lipid droplets and other organelles. How these Rab proteins or other adaptors then facilitate the recognition of lipid droplets by the phagophores is currently unknown.

Recent findings indicate that lipolysis and lipophagy are tightly coordinated and connected. In a hepatic cell line, lipolysis was found to act upstream of lipophagy, since ATGL activity on large lipid droplets is required to produce small newly formed lipid droplets by re-esterification that can subsequently be targeted by lipophagy (*74*). Regulation of lipolysis and lipophagy is crucial to prevent hepatic lipid accumulation in mice and humans.

Novel findings suggest that the expression of the most prominent genetic risk factor for hepatic steatosis, the single-nucleotide substitution I148M in PNPLA3 leads to a dysregulation of lipid catabolic processes and might impair the complex interplay between PNPLA3 and proteins regulating lipase activity. Recent studies have shed light on the molecular mechanisms underlying the development of steatosis induced by I148M and have added pieces to the complex puzzle of PNPLA3 function in hepatocytes. The I148M substitution ablates the triglyceride hydrolase activity that is normally present in purified PNPLA3 (*75*, *76*). However, the lipid droplet accumulation induced by expression of I148M is not caused by loss of catalytic activity, since genetic deficiency of PNPLA3 in mice fails to result in hepatic steatosis (*77*). In contrast, compelling evidence indicates that the I148M substitution mainly induces changes in protein levels and leads to an accumulation of PNPLA3 on the lipid droplet surface. Indeed, wildtype PNPLA3 is rapidly targeted for proteasomal degradation, whereas I148M evades ubiquitination and degradation (*78*). Wildtype PNPLA3 and I148M might both act as negative regulators of lipolysis by interacting with the ATGL cofactor CGI-58 on the lipid droplet surface (*79*). The extensive accumulation of I148M might sequester cellular CGI-58 pools and thereby impair the

activation of lipolysis. Although I148M is associated with a strongly increased risk of NAFLD and progression to NASH in humans (*80*), the endogenous function of PNPLA3 and the molecular mechanism underlying enhanced lipid accumulation in I148M carriers remain not fully understood. Considering the dramatic increase in NAFLD incidence for carriers of I148M sequence variant makes PNPLA3 an extremely interesting pharmacological target. Full elucidation of the role of PNPLA3 in liver lipid metabolism will be key of future research in order to develop new therapies.

2.4. Organelle contacts to orchestrate hepatic lipid flux

Lipid synthesis and lipid droplet formation, as well as lipid mobilization and degradation involves multiple cellular compartments Therefore, to maintain hepatocellular lipid homeostasis and o coordinate lipid synthesis, storage, degradation and secretion, cellular organelles have to dynamically interact. Lipid droplets take a center stage in hepatic lipid metabolism. They form membrane bridges with the endoplasmic reticulum that enable protein and lipid trafficking (*81*). Lipid droplets are also found in close proximity with mitochondria and peroxisomes. This might help to channel released fatty acids into beta-oxidation and to protect mitochondria from toxic lipids (*82*). Moreover, studies using multispectral time-lapse microscopy, super-resolution microscopy, and electron microscopy have detected lipid droplets in close proximity to the endoplasmic reticulum, endosomes, Golgi apparatus, and lysosomes (*83-85*). Interaction of lipid droplets with these other compartments relies on tethering proteins, which form the structural basis of the contact sites. These proteins are also sites of regulatory control. They have been implicated in signaling and lipid transport, and they coordinate with effector proteins that mediate metabolite exchange and inter-organelle communication (*86*, *87*).

Proteomic organelle profiling experiments have revealed strong changes in the subcellular organization and interactions of organelles involved in lipid metabolism. A proteomic comparison of lean and obese mice indicated increased contacts between lipid droplets, mitochondria, plasma membrane, and the endoplasmic reticulum in steatosis (*57*). Close proximity of lipid droplets with the plasma membrane and endoplasmic reticulum might promote channeling of fatty acids towards lipid storage after their uptake through the plasma membrane and conversion

into triglycerides in the endoplasmic reticulum. Increased mitochondrial contacts might favor efficient trafficking of fatty acids released from lipid droplets by lipases for beta-oxidation that is upregulated in high fat diet. In steatotic liver, the known lipid transfer and organelle tethering proteins ESYT2 and VPS13A/D relocate to lipid droplets (*88*, *89*). Therefore, these tethering proteins are potential candidates to regulate metabolically induced contact site formation. Whether they also function in the regulation of lipid droplet contacts in steatosis and whether they can transfer lipids between lipid droplets and other membranes remains unknown.

Under these conditions, strengthening interactions of organelles involved in lipid synthesis storage and degradation, might help counteract the effects of lipid overload and re-program the cells towards increased lipid storage and usage. However, unexpectedly also a co-flotation of Golgi apparatus membranes with lipid droplets in the liver of severely steatotic mice was observed. This was caused by increased association of Golgi apparatus membranes with the lipid droplets. Immunofluorescence revealed Golgi apparatus fragmentation in steatotic liver. Those Golgi apparatus fragments wrapped around lipid droplets, forming direct contacts with the lipid droplet monolayer (*57*). One possibility is, that this close apposition might enable lipid exchange between lipid droplet and the Golgi apparatus for lipidation of lipoproteins.

Indeed, it is thought that after co-translational lipidation of newly translated apoB100 by microsomal triglyceride transfer protein (MTP) nascent apoB100 is further lipidated by fusion with intra-luminal lipid droplets in the endoplasmic reticulum and during its trafficking across the Golgi after their export from the endoplasmic reticulum (*90-92*). VLDL export from the endoplasmic reticulum towards the Golgi apparatus is mediated by a specific pathway designed for large cargos involving the receptor TANGO1 and its interacting protein TALI, a fusion protein of Mia2–cTAGE5. This receptor complex interacts with ApoB and recruits the nascent VLDL particles towards endoplasmic reticulum exit sites for COPII dependent budding of “mega carriers” (*93*). Indeed, several Golgi apparatus proteins have been identified to participate in lipid transfer to nascent VLDLs, indicating a role for this compartment in VLDL assembly. Golgi apparatus localized ADP-ribosylation factor-related protein 1 (ARFRP1), GTP binding protein ARF-1 (ADP ribosylation factor-1) and phospholipase D activities are required for efficient lipidation (*94*, *95*). TM6SF2, an endoplasmic reticulum and Golgi apparatus localized transmembrane

protein, is required to mobilize neutral lipids for VLDL assembly but is not required for lipoprotein secretion. Interestingly, the reported missense mutation TM6SF2(E167K) is associated with NAFLD in humans and extensive lipid accumulation in diet induced obesity mouse model. This points to a crucial role for the Golgi apparatus in lipoprotein lipidation. The underlying mechanism and the lipid trafficking routes for this process are however still unclear.

Interestingly, the misplacement of the Golgi apparatus in steatotic mice had severe consequences on the secretory function of the liver. Isolated primary hepatocytes exhibited a strong reduction in secretory capacity (*57*), raising the alternative hypothesis that the loss of the normal Golgi apparatus structure might be a pathological process. The key question is whether the reorganization of the secretory apparatus induced by lipid accumulation is also happening in humans with NAFLD. Indeed, several findings in humans support this hypothesis: in patients with NASH the increase in VLDL secretion does not reach the extent of triglyceride synthesis (*96*) and in obese humans intrahepatic lipid was found to be negatively correlated with VLDL-triglyceride secretion rates (*97*). The reduction of secretory function induced by lipid accumulation might provide an explanation why hepatic lipid accumulation cannot be compensated by increased triglyceride secretion. A secretion defect induced by lipid accumulation might additionally be an important factor promoting disease progression, since it might lead to a vicious cycle promoting progression of steatosis. These results indicate that cellular changes in pathologies might in some cases not only be on the level of protein expression and organelle composition, but also on the level of organelle contacts and subcellular dynamics. So far little is known how organelles change their composition, interactions and what the functional consequences are. These aspects need to be taken into consideration for understanding cellular processes in NAFLD and NASH progression.

3. Hepatic and systemic regulation of lipid metabolism

3.1 Dysregulation of hepatic signaling pathways in NAFLD

In NAFLD, activity of key signaling pathways regulating metabolic homeostasis is altered. Systems biology approaches have revealed activity changes of key signaling nodes for metabolic control and a global rewiring of hepatic phosphorylation networks in mouse models for NAFLD (*98*). Most

prominently, an impairment of hepatic insulin action in steatotic liver reinforces the dysregulation of hepatic lipid metabolism. In livers of patients with NAFLD, insulin is unable to suppress hepatic gluconeogenesis despite continuing to activate lipogenesis, a condition termed ‘‘selective insulin resistance” (*99*). This leads to a paradox: under conditions of exogenous lipid overflow, *de novo* lipogenesis is strongly upregulated, contributing to the pathogenesis of NAFLD (*26*). The molecular basis for this phenomenon is still unclear.

A widely discussed hypothesis for “selective insulin resistance” is an impairment of signaling at a point downstream of the insulin signaling cascade. Here, the pathway splits into two branches: one that regulates carbohydrates and one that regulates lipid metabolism. IRS1 signaling through SREBP1c remains active and induces lipogenesis, whereas IRS2-mediated suppression of gluconeogenesis via FOXO1 deactivation is lost (*100*). Some researchers have suggested that differential hepatic distribution and alterations of IRS1 and IRS2 expression play important roles in this process (*101*, *102*). In addition to altered expression, changes in the subcellular localization of key transcription factors induced by toxic fatty acids might lead to selective insulin resistance. Normally, activation of the insulin signaling cascade leads to FOXO1 phosphorylation, which in turn causes nuclear FOXO1 to relocate to the cytoplasm, preventing it from stimulating gluconeogenic gene expression (*103*, *104*). However, palmitate-treated HepG2 cells show impaired nuclear export of FOXO1 by insulin, leading to a loss of phosphorylation by Akt kinase and increased expression of gluconeogenic genes (*105*).

Researchers do not believe that lipid droplet accumulation – the hallmark of NAFLD development – is the cause of hepatic insulin resistance. However, sequestering toxic lipids into lipid droplets might counteract the development of insulin resistance by preventing the induction of endoplasmic reticulum stress, dysfunction of mitochondria, and impairment of signaling pathways (*4*). However, according to the current hypothesis in the field, cellular levels of numerous bioactive lipid species such as DAGs, ceramides, and free fatty acids such as palmitate and their acyl-CoA derivatives are associated with hepatic insulin resistance (*106*, *107*). These lipids might induce insulin resistance by activating members of the protein kinase C (PKC) family, which interferes with the insulin signaling cascade on the phosphorylation level (*108*). According to one model, DAG accumulation in membranes induces targeting of cytosolic PKCs to the plasma

membrane, leading to the activation of kinase activity by a conformational change (*109*). Interestingly, our hepatic organelle proteome revealed lipid droplet localization of the novel PKC isoform PKC□ and the PKC effector kinase PKD3 (*57*). This might be caused by DAG accumulation in lipid droplets, since lipidomic analysis indicated that in steatotic liver the main portion of DAG accumulates in lipid droplets and not in membranes (*110*). In steatotic liver and lipid-loaded hepatocytes, PKD3 and PKC□ are both activated and suppress insulin signaling (*111*). However, whether lipid droplet-targeted PKC□ and PKCD3 are active and whether this lipid droplet-bound pool contributes to insulin resistance still needs to be determined. It also remains unclear which lipid species and subspecies influence the development of insulin resistance and at which cellular compartment those species are bioactive in mediating insulin resistance.

3.2. Organ crosstalk regulating hepatic lipid metabolism

Signals from all organs of the body affect hepatic lipid metabolism and thereby determine NAFLD progression. Those signals reach the liver in form of circulating metabolites, metabolic hormones, or extracellular vesicles. Compelling evidence suggests, that in obesity signals from different organs act together with hepatic factors to induce selective insulin resistance in the liver. In obesity, decreased insulin sensitivity in the brain and fat results in increased white adipose tissue lipolysis, thereby increasing the delivery of fatty acids and glycerol to the liver. These metabolites might then act as dominant inducers of gluconeogenesis and triglyceride synthesis in hepatocytes thereby overruling hepatic insulin action on the suppression of gluconeogenesis (*112*). Therefore, alterations in organ crosstalk between the brain, adipose tissue, and liver might be important drivers for enhanced hepatic glucose output, whereas hepatic insulin induces increased *de novo* lipogenesis. As an alternative to metabolite-mediated effects, hormonal effects can be transmitted by vagal efferents from the brain to the liver, as hypothalamic leptin signaling reduces steatosis via the dorsal vagal complex (*113*). In addition to brain-mediated metabolic control, studies have shown that the “gut–liver axis” promotes NAFLD onset and progression. Incretins, such as GLP-1, as well as metabolites produced by the gut microflora, influence hepatic fat deposition and modulate hepatic inflammation and fibrosis. Thus, NAFLD develops as a consequence of multiple organs adapting to lipid overload, leading to pathological reactions that collectively promote hepatic steatosis and NAFLD (*114*).

Bile acids synthesized in liver from cholesterol via a multi-enzyme pathway not only potentiate the absorption of lipids and vitamins in the intestine, they are also endogenous ligands that activate the nuclear receptor farnesoid X receptor (FXR) and G protein-coupled bile acid receptor 1 (GPBAR1/TGR5) to regulate cholesterol and triglyceride metabolism, but also glucose metabolism and energy expenditure (*115-118*). Bile acids act on the liver itself but also control gene expression in distant tissues. They activate a cross-organ feed-back loop to involving FXR, thus stimulating expression of FGF15 and FGF19 in enterocytes to suppress their own synthesis (*119-121*). TGR5 is expressed in the liver, immune cells, BAT, enteroendocrine cells and neurons in the intestine and brain (*122*). Activation of TGR5 by bile acids increases energy expenditure and induces GLP-1 secretion from the intestine (*123, 124*). In addition to circulating metabolites, bile acids and metabolic hormones, extracellular vesicles might be important players in organ crosstalk and NAFLD progression. Extracellular vesicles carry microRNAs, proteins, and lipids and are thought to mediate communication between neighboring cells and to distant organs (*125*). Extracellular vesicles differ in size and cargo and shell composition depending on their secretion mechanism, tissue origin, and state. After binding to surface receptors on the plasma membrane of target tissues, extracellular vesicles are internalized and deliver their cargo into the cell, thereby activating signaling cascades. Adipose tissue might be an important source of extracellular vesicles acting on hepatic lipid metabolism. Different types of adipose tissues shed extracellular vesicles that differ in their microRNA content and mediate different metabolic effects. Brown adipose tissue-derived exosomes, 40-120 nm large extracellular vesicles, might improve glucose tolerance and lower plasma insulin and FGF21 levels by affecting protein expression in liver (*126*), whereas visceral white adipocytes from obese mice were reported to shed exosomes mediating inflammatory and fibrotic signaling pathways (*127*). Exosomes are thought to mediate effects mainly by transferring microRNA. The effects of protein or lipid transfer to target cells are less understood. Recently, a study identified a novel pathway of lipolysis-independent lipid release from white adipose tissue. Adipocytes release lipid-filled exosomes that are taken up by nearby macrophages that then accumulate lipid droplets. This type of lipid release is strongly increased in obesity, raising the questions (1) to what extent lipid-carrying vesicles contribute to plasma lipid levels and (2) are exosomes capable of transferring

lipids to more distal organs such as the liver (*128*). A limitation of these studies is the challenge of purifying extracellular vesicles to complete homogeneity, making it difficult to distinguish effects induced by extracellular vesicles from effects caused by contaminations such as cell fragments and intracellular proteins and vesicles. Moreover, different isolation and purification strategies might be selective for certain extracellular vesicles subspecies.

4. Therapeutic options and outlook

Considering the lack of liver-targeted pharmacologic therapies for NAFLD, lifestyle interventions including a reduction of caloric intake in combination with exercise remain the optimal therapeutic strategy according to clinical practice guidelines. Reducing lipid accumulation is the premise for the resolution of steatohepatitis. General caloric restriction is the most effective way to reduce liver fat; the effects of modifying diet composition seem to be small (*129*) Data on the influence of eating patterns – such as different intermittent fasting schemes – on NAFLD development and progression in humans are limited. Some studies indicate additional metabolic benefits of intermittent fasting compared to general caloric restriction (*130*). Interestingly, mouse studies have found a reduction of hepatic steatosis and inflammation under intermittent fasting. Under those conditions, levels of certain hepatic DAG species were reduced and were correlated with decreased PKCε activation and increased insulin sensitivity (*131*).

In humans, sustained weight loss is in many cases difficult to achieve and maintain. Therefore, developing pharmacotherapies that target NAFLD progression would be of great clinical value. Several pharmacotherapies counteracting hepatic lipid accumulation are under development. Logically, enzymes involved in endoplasmic reticulum triglyceride synthesis such as DGATs are attractive pharmacological targets. For instance, SCD1 and DGAT2 – which are required for the formation of large lipid droplets, the hallmark of NAFLD – might present potent targets. DGAT2 relocates to a subset of lipid droplets that subsequently expand (*33*). SCD1 is responsible for the formation of monounsaturated fatty acids from saturated fatty acids, the preferred substrates for DGATs, and a reduction of SCD1 activity leads to reduced hepatic triglyceride storage and smaller lipid droplets (*132*). An alternative strategy of reducing hepatic fat content is activating autophagy. In human livers of lean and obese subjects, apoptosis signal-regulating kinase 1

(ASK1) expression increased autophagy and correlated negatively with liver fat content and NASH scores, introducing ASK1 as a novel therapeutic target for NAFLD. Deactivating ASK1 by pharmacological inhibition or genetic ablation enhanced lipid droplet accumulation and liver fibrosis in mice. Consistently, in human livers of lean and obese subjects, ASK1 expression increased autophagy and correlated negatively with liver fat content and NASH scores (*133*). In summary, multiple pharmacological therapies are under development, raising the hope that in the near future, the availability of therapeutic options will decrease the burden of NAFLD and NASH, as well as associated complications.

5. Conclusion

Overall, the field of lipid metabolism is making progress in understanding the cellular mechanisms of hepatic lipid storage and how imbalances in lipid storage, secretion, and degradation lead to steatosis. Over the last few years, new therapeutic targets have been identified and novel drugs have entered clinical trials. However, many key questions (Figure3) remain unanswered such as (1) how lipid accumulation interferes with signaling pathways and mediates insulin resistance. (2) What are the mechanisms for lipid droplet formation in the cytosol, the endoplasmic reticulum lumen and in the nucleus? (3) What is the mechanisms underlying lipoprotein secretion and lipidation? (4) What are the trafficking routes and how is lipid flux regulated between membranes, lipid droplets and lipoproteins? (5) How are the lipid catabolic pathways coordinated and which proteins are involved in lipid mobilization? All those crucial questions wait to be answered. New technological advances, for example in the fields of lipidomics, proteomics, and super-resolution microscopy might offer exciting possibilities to address those questions. A basic understanding of these cellular processes will help us identify new pharmacological targets that interfere with pathological processes.

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Figure1. Lipid geometry and protein binding on lipid monolayers. (A) Lipoproteins and lipid droplets consist of a lipophilic core containing triacylglycerides and cholesteryl esters sourrounded by a phospholipid monolayer. Lipoproteins, (here VLDL), are decorated by a defined set of apolipoproteins that bind to the surface via amphipathic alpha-helices and beta-sheets. In contrast, the lipid droplet protoeme is highly divers and dynamic. Proteins are either targeted to lipid droplets from the ER via membrane bridges (class I) or directly from the cytosol (class II). Their lipid droplet binding domains include short hairpins, amphipathic helices and lipid anchors. (B) Phospholipid geometry is defined by the head group size, the number and length of acyl-chains and saturation degree. Examples for different phospholipid shapes are indicated. (C) Lipid droplet packing defects. Cylindrical lipids such as phosphatidylcholine (red) enable tight packing. Conically shaped (blue) (e.g. phosphatidylethanolamine) favor packing defects that increase the exposure of neutral lipids on the lipid droplet surface.

Figure2. Neutral lipid homeostasis in the liver. Hepatocytes use different sources of lipids dependent on the metabolic state. Hepatocytes take up large amounts of fatty acids released from adipose tissue. The uptake is especially high under fasting conditions. In the post-prandial state, dietary lipids arrive from the gut in chylomicron remnant particles which are internalized via the LDL receptor. Moreover, increased circulating glucose and insulin levels activate de novo lipogenesis on transcriptional level via ChREBP and SREBP1c. Regardless of their origin, fatty acids are esterified into triglycerides and cholesteryl ester and stored in intracellular lipid droplets. triglycerides and cholesteryl ester stored in lipid droplets are mobilized by lipases. They can be re-esterified and packed into VLDL particles for secretion. Alternatively they can be channeled into mitochondrial beta-Oxidation. The resulting acetyl-CoA is used for energy generation via tricarboxylic acid cycle and respiratory chain or as a building block for e.g. cholesterol synthesis. Under a carbohydrate restricted diet, extendet fasting or starvation but in metabolic dysregulation in diabetes acetyl-CoA is metabolized to ketone bodies serving as energy supply of mainly the brain. Under physiological conditions only small amounts of lipid droplets accumulate in hepatocytes, since lipid uptake and synthesis are tightly balanced by lipid degradation and secretion. Intra-hepatic signaling pathways and crosstalk from other organs such as white and brown adipose tissues, the intestine and the brain regulate hepatic lipid metabolism.

Figure3. Key questions in hepatocellular lipid metabolism. Fatty acids are esterified to triglycerides and cholesteryl ester by enzymes residing in the endoplasmic reticulum that are then thought to form a lipid lens between the two endoplasmic reticulum leaflets. Lipid droplets bud by a poorly understood mechanism **(1)**. The proteins Seipin and FIT2 have been linked to budding of cytosolic lipid droplets. However, in hepatocytes also endoplasmic reticulum -luminal lipid droplets have been observed that are thought to fuse with nascent VLDL particles. Factors driving the budding reaction towards one or the other side of the endoplasmic reticulum are not known **(2)**. Cytosolic lipid droplets carry a variety of proteins on their surface. These can either be targeted to lipid droplets from the endoplasmic reticulum during initial droplet formation, via endoplasmic reticulum -lipid droplet bridges (class I) or from the cytosol (class II). How the composition of the lipid droplet proteome is regulated remains poorly characterized **(3)**. Lipid droplets residing within the nucleoplasm derive from their endoplasmic reticulum lumenal counterparts, their function, targeting and escape from the nuclear reticulum is still a topic of active research **(4)**. In the liver, the formation of lipid droplets is also tightly coupled to VLDL lipidation. VLDL are initially thought to be formed by co-translational lipidation of the nascent apolipoprotein B100 and subsequent fusion with luminal lipid droplets. Mechanistic insights of this process are still missing **(5)**. Vesicles carrying VLDLs bud from the endoplasmic reticulum in a COP-II dependent mechanism involving the proteins TANGO1 and TALI. Prior their secretion VLDLs pass the Golgi apparatus where they are thought to be further lipidated. The mechanism as well as the neutral lipid sources are unclear **(6)**. Lipid droplets can be degraded via lipolysis or lipophagy. The coordination and functional roles of the two processes remain to be fully elucidated **(7)**. In lipophagy, lipid droplets are sequestered by a phagophore, which subsequently forms the autophagosome and targets the lipid droplets for lysosomal degradation. In lipolysis, lipid droplet stored TGs can be sequentially cleaved into glycerol and fatty acids. The lipid droplet residing lipase ATGL hydrolyzes the first fatty acid from the glycerol backbone. According to a recent model lipid droplet accumulation of the genetic variant PNPLA3(I148M) sequesters the ATGL activator CGI-58 by competitive binding and reduces lipolysis. Only the rapid ubiquitination and proteasomal degradation of wildtype PNPLA3 might enable sufficient ATGL activity. The detailed mechanism is still unclear **(8)**. Endoplasmic reticulum localized lipases such as TGH are

thought to mobilize lipids for re-esterification and incorporation into VLDL particles. Individual contributions and specificity of the different lipases, as well as subcellular coordination and recruitment of lipid droplets warrants further research **(9)**.

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A

VLDL

ApoB100

ApoC-II

ApoE

Lipid droplet

Lipid anchor
(class II protein)

Short hairpin
(class I protein)

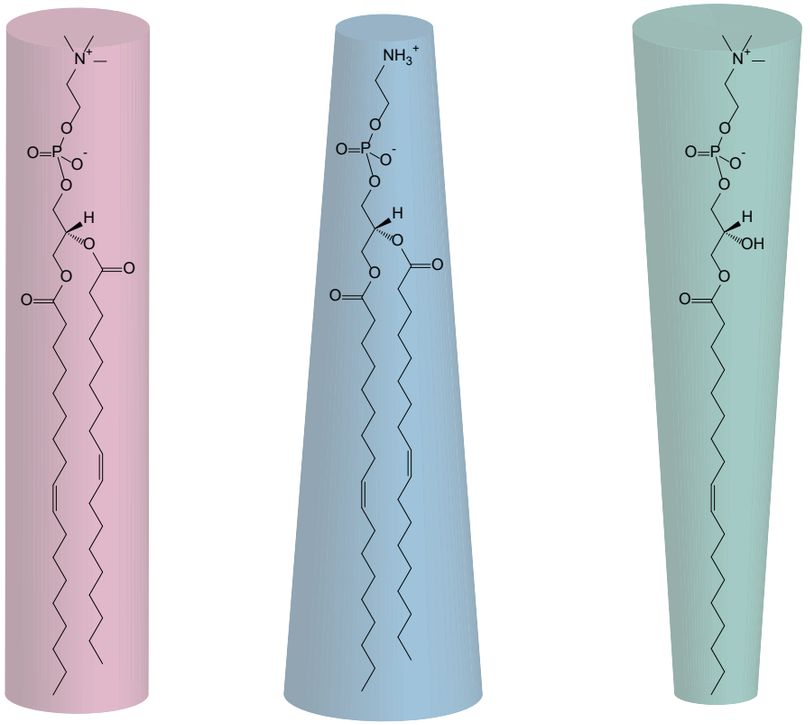
Amphipathic helix
(class II protein)

B

phosphatidyl-choline (PC)
phosphatidylinositol

phosphatidyl-ethanolamine (PE)
phosphatidic acid
diacylglycerols

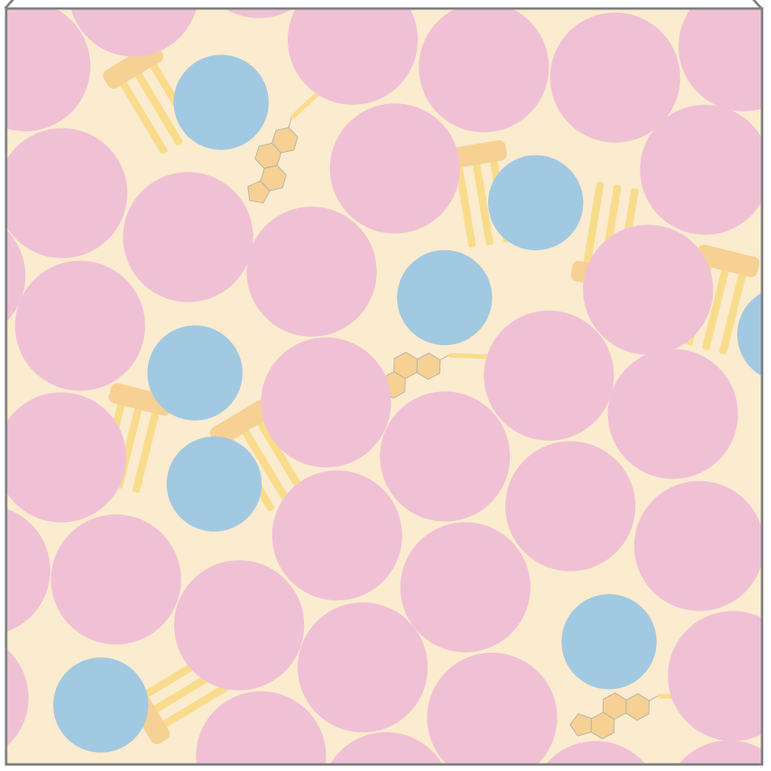
lyso-phospholipids
polyphosphoinositides

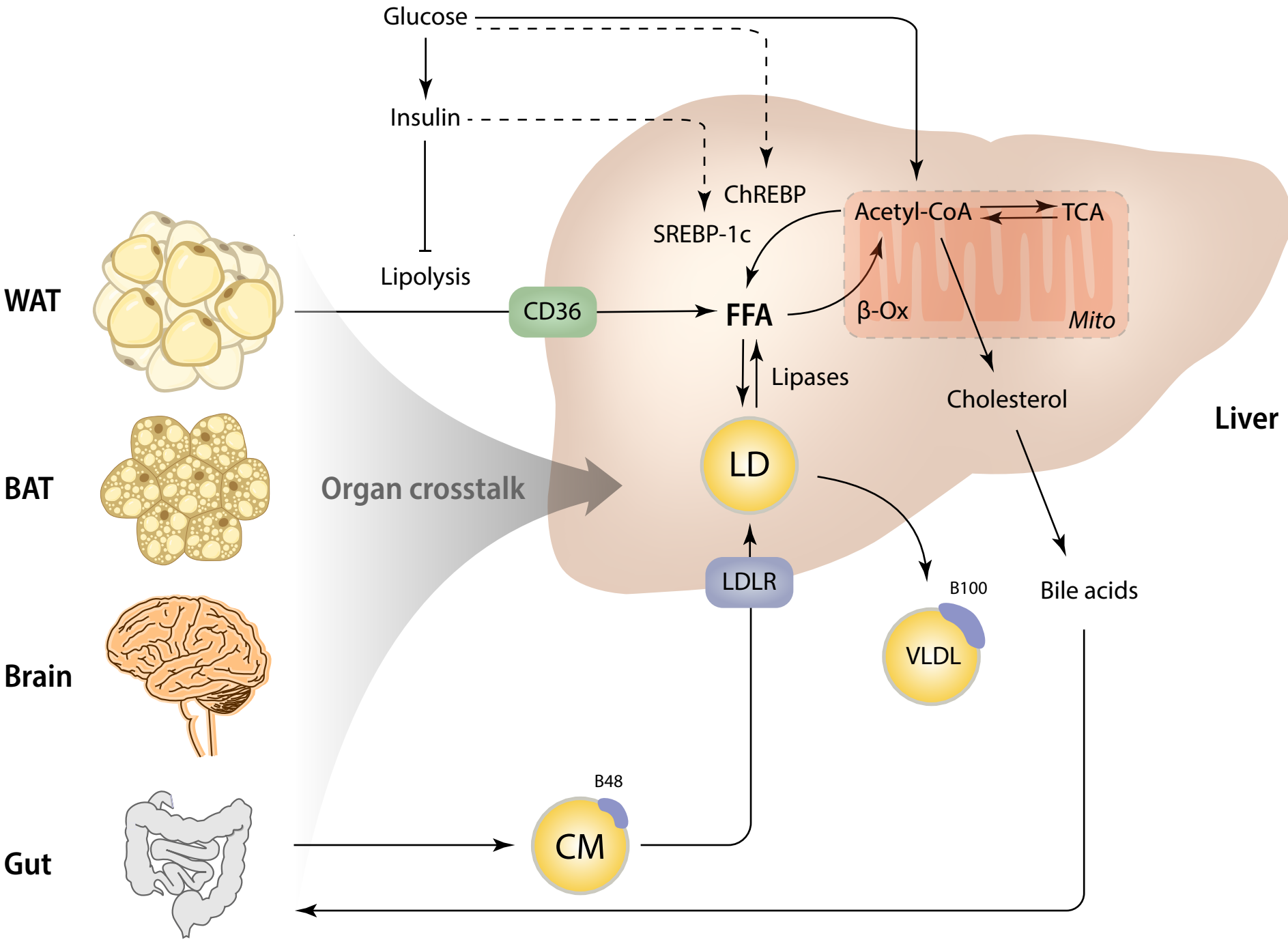


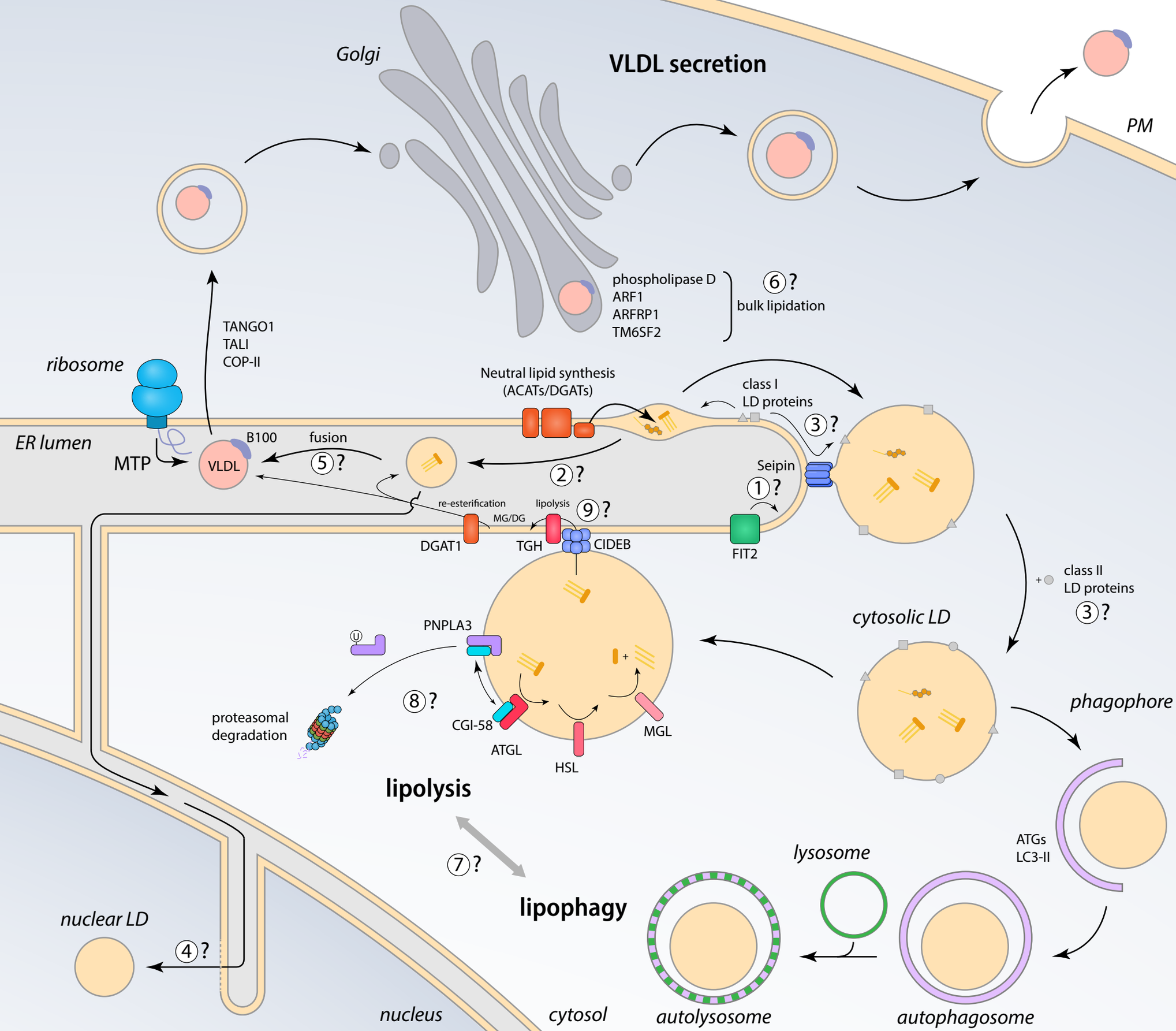
cylindrical *conical* *inverted conical*

C

Packing defects




Glucose
Insulin
Lipolysis
ChREBP
SREBP-1c
Acetyl-CoA
TCA
β-Ox
Mito
WAT
CD36
FFA
Lipases
Cholesterol
Liver
BAT
Organ crosstalk
LD
LDLR
B100
VLDL
Bile acids
Brain
B48
CM
Gut


Golgi
VLDL secretion
PM
phospholipase D
ARF1
ARFRP1
TM6SF2
6 ?
bulk lipidation
TANGO1
TALI
COP-II
ribosome
ER lumen
MTP
VLDL
B100
fusion
5 ?
Neutral lipid synthesis
(ACATs/DGATs)
2 ?
class I
LD proteins
3 ?
Seipin
1 ?
re-esterification
MG/DG
lipolysis
9 ?
DGAT1
TGH
CIDEB
FIT2
class II
LD proteins
3 ?
cytosolic LD
PNPLA3
Ub
proteasomal
degradation
8 ?
CGI-58
ATGL
HSL
MGL
lipolysis
phagophore
ATGs
LC3-II
7 ?
lipophagy
lysosome
nuclear LD
4 ?
nucleus
cytosol
autolysosome
autophagosome