Cholesterol metabolism and the pathogenesis of non-alcoholic steatohepatitis.

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Cholesterol metabolism and the pathogenesis of non-alcoholic steatohepatitis

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Abbreviations: ABC. ATP-binding cassette; ER: endoplasmic reticulum; FXR: farnesoid X-receptor; HMGCoAR: 3-hydroxy-3-methyl-glutaryl-CoA reductase; HNF: hepatocyte nuclear factor:; HSC: hepatic stellate cell; IL-1: interleukin-1; LDL: low density lipoproteins; LE: late endosome; LY: lysosome; LPS: lipopolysaccharide, LXR: liver X-receptor; miRNA: microRNA; NPC1: Niemann-Pick C1; NPC2: Niemann-Pick C2; NPC1L1: Niemann-Pick C1-like 1; oxLDL: oxidized low density lipoproteins; PM: plasma membrane; PP: pyrophosphate; PPAR: peroxisome proliferator-activated receptor; PXR: pregnane X receptor; SHP: small heterodimeric partner; SNP: single nucleotide polymorphism; SREBP: sterol regulatory binding protein; TLR-4: toll-like receptor-4; UFA: unsaturated fatty acids; VLDL. Very low density lipoproteins

Abstract

Emerging experimental and human evidence has linked altered hepatic cholesterol homeostasis and free cholesterol (FC) accumulation to the pathogenesis of non-alcoholic steatohepatits (NASH). This review focuses on cellular mechanisms of cholesterol toxicity involved in liver injury and on alterations in cholesterol homeostasis promoting hepatic cholesterol overload in NASH.

FC accumulation injures hepatocytes directly, by disrupting mitochondrial and endoplasmic reticulum (ER) membrane integrity, triggering mitochondrial oxidative injury and ER stress, and by promoting generation of toxic oxysterols, and indirectly, by inducing adipose tissue dysfunction. Accumulation of oxidized LDL particles may also activate Kupffer and hepatic stellate cells, promoting liver inflammation and fibrogenesis.

Hepatic cholesterol accumulation is driven by a deeply deranged cellular cholesterol homeostasis, characterized by elevated cholesterol synthesis and uptake from circulating lipoproteins and by a reduced cholesterol excretion. Extensive dysregulation of cellular cholesterol homeostasis by nuclear transcription factors sterol regulatory binding protein(SREBP)-2, liver X-receptor(LXR)-α and farnesoid X receptor(FXR) plays a key role in hepatic cholesterol accumulation in NASH. The therapeutic implications and opportunities for normalizing cellular cholesterol homeostasis in these patients are also discussed.

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1. Introduction

Nonalcoholic fatty liver disease (NAFLD) affects 30% of the general adult population and 70-80% of diabetic and obese patients[1]. NAFLD encompasses a histological spectrum, ranging from simple steatosis (SS) to steatosis plus necroinflammation (nonalcoholic steatohepatitis, NASH). While SS is considered to have a benign hepatological prognosis, NASH confers a 1.8-fold higher mortality, largely accounted for by liver-related complications, and is a leading cause of liver transplantation[2, 3]. Furthermore, both histological subtypes confer an increased risk of type 2 diabetes (T2DM) and cardiovascular disease (CVD)[3].

The pathogenesis of NASH is unclear: the original "two-hit" hypothesis theorized that a first "hit", namely hepatic steatosis, determined by metabolic factors (obesity, T2DM, dyslipidemia), sensitized the liver to subsequent "second hits", namely oxidative stress and proinflammatory cytokines, that cause hepatocellular injury and liver inflammation. The validity of this view has been recently challenged, and growing evidence suggests SS and NASH may actually be 2 separate diseases: in this "multi-parallel hit" model[4], the accumulation of "lipotoxic/pro-inflammatory" lipid species interacts with proinflammatory factors to yield NASH since the beginning, while in the other cases the liver develops steatosis and remains free from inflammatory and fibrotic changes [5]. The type of toxic lipids has been the subject of extensive research: experimental inhibition of hepatic TG synthesis[6] and human hypobetalipoproteinemia, which does not progress to cirrhosis despite massive steatosis[7], suggested that hepatic triglyceride (Tg) accumulation is not per se toxic, but rather protects the liver by buffering the accumulation of lipotoxic Tg precursors. Consistent with this view, subjects who are able to store excessive fat as neutral cholesterol esters and TG develop steatosis but not NASH and may be considered "good fat storers", while subjects who are unable to synthesize neutral lipids acccumulate toxic lipid species and develop progressive inflammation and fibrosis, leading to NASH. The search for the key toxic lipid species then focussed on free fatty acids (FFA), diacylglycerides, phospholipids (ceramides, sphingolipids), and most recently, free cholesterol (FC)[5]. Growing evidence connects altered cholesterol homeostasis

and hepatic FC accumulation to the pathogenesis of NASH. In the first National Health and Nutrition Examination Survey, higher dietary cholesterol consumption independently predicted a higher risk of cirrhosis [8], and epidemiological data connect an increased cholesterol intake to the risk and severity of NAFLD[9, 10]. In NAFLD patients, the development of NASH and fibrosis paralleled hepatic FC accumulation [11, 12] . Experimental induction of hepatic FC accumulation promoted steatohepatitis and fibrosis [13, 14, 15] , while correction of hepatic FC overload improved liver disease severity in NASH [16, 17, 18, 19, 20, 21, 22, 23].

We will review cellular mechanisms of cholesterol toxicity involved in liver injury and alterations in cholesterol homeostasis promoting hepatic cholesterol overload in NASH.

2. Mechanisms of cholesterol toxicity in NASH

Similar to atherosclerosis, where the vessel wall endothelium, the infiltrating macrophages and the fibroblasts promote atherosclerotic plaque formation, in the liver hepatocytes, the resident macrophages Kupffer cells and the hepatic stellate cells (HSC) are key mediators of liver injury and NASH.

Altered cholesterol metabolism has several toxic effects on each of these cells, promoting NASH through diverse mechanisms. With its large, rigid core, cholesterol molecule affects membrane organization and physical properties by modulating the coexistence of lipid-disordered and lipid-ordered phases, which is a critical determinant of membrane bilayer permeability and fluidity. Within this context, it is essential to maintain a physiological range of FC/phospholipid ratio in cellular membranes. Although the degree of saturation of the fatty acyl moieties of membrane phospholipids is the major determinant of the fluidity of lateral membrane domains, which consist of well-packed, detergent-resistant liquid-ordered rafts and more fluid, detergent-soluble liquid-crystalline regions[24], the interaction of the hydrophobic rings of cholesterol with these fatty acyl chains has important effects: in particular, the ability of cholesterol to pack tightly with saturated fatty acyl groups of membrane phospholipids allows the formation of liquid-ordered rafts. Thus,

cholesterol depletion causes marked disruption of these rafts, as in plasma membrane caveolae (see below) and, conversely, when the FC/phospholipid ratio rises above a physiological level, the liquid-ordered rafts may become too rigid, and the liquid-crystalline domains may begin to lose their fluidity. These events adversely affect certain integral membrane proteins with multiple membrane-spanning domains, undergoing conformational changes as part of their activity cycle, that require conformational freedom for proper function and that can be inhibited by a high FC/phospholipid ratio, including plasma membrane constituents like Na+-K+ ATPase, adenylate cyclase, alkaline phosphatase, rhodopsin, and transporters for glucose, organic anions, and thymidine[24].

Accordingly, different cellular organelles have distinct and tightly regulated cholesterol content and lipid composition: while the plasma membrane (PM) has a high cholesterol concentration, the endoplasmic reticulum (ER) and the mitochondria have a much lower cholesterol content (3–5% of the total cellular cholesterol)[25] and are highly sensitive to membrane fluidity loss induced by cholesterol enrichment.

2.1 Mitochondrial reduced glutathione (mGSH) pool depletion and oxidative injury

Mitochondrial membrane 2-oxoglutarate carrier is a mitochondrial transmembrane protein carrier which transports GSH, synthesized de novo in the cytosol, into the mitochondria[26]. Intact mitochondrial GSH (mGSH) stores are essential for controlling reactive oxygen species (ROS) generation by cell death stimuli such as tumour necrosis factor(TNF)-α and Fas ligand[25]. 2-oxoglutarate carrier is particularly sensitive to changes in mitochondrial membrane cholesterol content and fluidity: mitochondrial FC loading impairs GSH transport, depletes mGSH stores, promoting mitochondrial ROS generation and lipid peroxidation. The functional consequences are sensitization of hepatocytes to TNF-α, permeabilization of mitochondrial membrane, cytochrome c

release, hepatocyte necrosis and apoptosis(see glossary), which promote hepatic inflammation and NASH[27, 28](Figure 1, panel A-B).

2.2 Cholesterol-induced ER stress

The ER is a membranous organelle with critical cellular functions[29, 30]: folding and posttranslational modifications of nascent proteins to achieve their proper conformation; phospholipid synthesis; storage of calcium ions for cellular signalling; drug metabolism by enzymes such as cytochrome P450.

Several physiological, pharmacological, and pathological conditions, including ER FC accumulation, impair ER functions and protein folding capacity, resulting in a condition known as ER stress or unfolded protein response (UPR, see glossary), which plays a central pathogenetic role in obesity-associated disorders, including NAFLD[31, 32]. FC accumulation triggers ER stress by altering the critical FC-to-phospholipid ratio of the ER membrane, necessary to maintain its fluidity. The resulting stiffening of the ER membrane inhibits enzyme conformational changes and impairs ER activity, triggering ER stress and eventually cell apoptosis. Among the ER enzymes, the sarco(endo)plasmic reticulum Ca^{2+}-ATPase (SERCA) is particularly sensitive to minimal increases in ER cholesterol content, which impair the activity of this ATPase. As a result, the physiologically high intra-ER Ca^{2+} concentration falls and the ER folding capacity is impaired[33, 34](Figure 1 panel C-D).

The importance of ER FC accumulation the for triggering ER stress has been recently documented in hepatocytes[35, 36], where ER membrane cholesterol accumulation, rather than total cellular cholesterol overload, induced hepatic ER stress, while hepatocyte ER cholesterol lowering by two independent approaches (cyclodextrin or zaragozic acid) resolved UPR[36].

2.3 Generation of toxic oxysterols

Oxysterols are derived from hydroxylation of the side chain of cholesterol at different positions (C7α, C27, C24S, C22R and C25) of the molecule by autooxidation or by specific

monooxygenases, mainly belonging to the cytochrome P-450 family (Table 1). The addition of hydroxyl to the cholesterol molecule confers hydrophilic properties to oxysterols and increases their propensity for intracellular esterification, allowing faster transfer to endoplasmic reticulum or an alternative strategy to excrete cholesterol by extra-hepatic tissues[37].Oxysterol are difficult to measure in biological samples, due to several reasons. their plasma or tissue levels may be ~ 10 000 times lower than plasma cholesterol concentrations; additionally, oxysterols have a short half-life (1–62 h) and are unstable molecules, sensitive to auto-oxidation during isolation and extraction. Recently, new techniques, including isotope-dilution gas chromatography-mass spectrometry using deuterium-labelled internal standards, allowed a better characterization of these molecules and enhanced our understanding of their biological actions. Oxysterols serve important functions:

(a) as substrates for the formation of bile acids(BA)[37];

(b) as mediators of reverse cholesterol transport whereby excess cholesterol is returned from peripheral tissues to the liver for excretion[37];

(c) emerging evidence suggests oxysterols are natural ligands for nuclear transcription factors liver X-receptor(LXR)-α and sterol regulatory binding protein(SREBP)s, master regulators of hepatic lipid metabolism(Table 1-2, Figure 2), and may contribute to liver injury in NAFLD[38]. The oxysterols most extensively characterized in NAFLD include:

-7-hydroxycholesterol (7OHC): The main metabolic pathway of bile acid formation starts from the 7α-hydroxylation of cholesterol by CYP7A, a liver-specific microsomal cytochrome P450 with limited substrate specificity. The 7α-hydroxylation of cholesterol is the rate limiting step in the neutral pathway to bile acids. This enzyme is regulated at the transcriptional level by bile acids recirculating to the liver via the enterohepatic circulation, which modulate CYP7A1 gene transcription in a feed-back loop involving the nuclear receptors Small Heterodimer Partner (SHP, NR0B2), Liver Receptor Homolog-1 (LRH-1/CPF/FTF, NR5A2), and FXR (Table 1-2)[37]. In NASH, hepatic expression of CYP7A1 is reduced, resulting in an impaired activation of FXR, with potentially deleterious metabolic and proinflammatory effects (see section 3.6)[14].

-27-hydroxycholesterol (27OHC): beside the main, or "classic", biosynthetic pathway of BA, an "alternative" (or acidic) metabolic pathway has been recently elucidated, where the first step is represented by hydroxylation at position 27, catalyzed by sterol 27-hydroxylase (CYP27A1), a mitochondrial cytochrome P-450 characterized by broad substrate specificity and by broad tissue and organ distribution, including vascular endothelium, fibroblasts and macrophages[37]. 27OHC not only acts as intermediate of BA synthesis, but could also modulate gene transcription in at least two manners, by preventing SREBP-2 activation or by upregulation of ABC transporter expression via LXRα receptor, eventually inhibiting cholesterol uptake and synthesis and promoting cholesterol excretion [37]. In NASH, hepatic expression of CYP27A1 is reduced, which may enhance SREBP-2-mediated cholesterol synthesis and uptake and reduce cholesterol excretion by ABC transporters, further aggravating hepatic cholesterol overload (see section 3.6)[14].

-7-chetocholesterol (7-KC): 7KC derives from auto-oxidation of cholesterol. Hepatic levels of 7KC are increased in animal models of NASH[38], and physiological concentrations of this oxysterol triggered hepatocyte apoptosis and directly activated hepatic macrophages and HSCs to secrete proinflammatory and profibrogenic cytokines transforming growth factor (TGF)-β1, monocyte chemoattractant protein (MCP)-1 and tissue inhibitor of matrix metalloprotease(TIMP)-1 in vitro, further promoting liver injury in NASH [38].

-25-hydroxycholesterol (25OHC): 25OHC derives from cholesterol hydroxylation catalyzed by a specific cholesterol 25-hydroxylase, which is not a member of the cytochrome P450 family and belongs to a family of enzymes that utilize diiron cofactors. 25-OHC concentrations are physiologically low in human hepatocytes, where it is converted into bile acids, but its levels have been found to be elevated in plasma of NAFLD patients[39].

25OHC potently modulates hepatic lipid metabolism and inflammatory response by enhancing hepatocyte LXR/SREBP-1c-mediated lipogenesis and by activating nuclear factor (NF)-κB, a key proinflammatory nuclear regulator, thereby promoting hepatic steatosis and inflammation[40].

25OHC also promotes secretion of proinflammatory cytokine IL-8 by hepatic macrophages, further contributing to liver inflammation in NASH[40]

The impact of current treatments on oxysterols is uncertain, and statins do not consistently affect oxysterol levels in NAFLD patients[39]. Therefore alternative approaches to modulate the action of toxic oxysterols are being evaluated, including the synthesis of antagonist oxysterols: in fact, different oxysterols have diverse and even opposite actions, and while 25OHC enhances lipogenesis and inflammation, 25-hydroxycholesterol-3-sulfate (25OHC3S), the sulphated derivative of 25OHC, synthesized from 25OHC by the cytosolic sulfotransferase SULT2B1b, had potent antilipogenic and anti-inflammatory effects in the liver[41]. Consistently, experimental hepatic SULT2B1b overexpression decreased lipogenesis, steatosis and plasma lipids in NAFLD[42]. Similarly, another synthetic oxysterol 22(S)-hydroxycholesterol (22(S)-HC) reduced LXR-mediated lipogenesis and intracellular lipid levels and improved insulin resistance in human hepatocytes[43]. Finally, administration of 27OHC to low density lipoprotein receptor knockout (LDLR-/-) mice fed a high fat/high cholesterol diet reduced lysosomal cholesterol accumulation in Kupffer cells and improved hepatic inflammation[44].

Collectively, these data highlight the potential for modulation of oxysterol action in the treatment of NAFLD.

2.4 Cholesterol-induced hepatic Kupffer cells and hepatic stellate cell (HSC) activation

Kupffer cells (KCs) represent 20-25% of the non-parenchymatous cells in the liver. Beside their essential role as phagocytes, KCs are the primary source of hepatic pro-inflammatory and profibrogenic cytokines (including TNF-α and TGF-β1), and recruit circulating inflammatory cells into the liver, thereby playing a key role in the pathogenesis of liver injury in NASH[45]. The role of cellular cholesterol overload in KCs activation has been demonstrated in mice fed a high fat/high cholesterol diet, where the accumulation of FC into the KCs was required for their activation to a

proinflammatory phenotype and NASH development[46]. As KCs lack cholesterol biosynthetic ability, they acquire cholesterol from circulating cholesterol-rich lipoproteins. The main pathway of cholesterol uptake by KCs is oxidized LDLs (oxLDLs) internalization through the scavenger receptors cluster differentiation protein(CD)36 and scavenger receptor A(SR-A): as plasma oxLDLs are markedly increased in metabolic syndrome and in NASH patients, particularly in the postprandial phase, scavenger-receptor-mediated uptake of these lipoproteins by may represent a major trigger for KC activation and liver inflammation[47]. Consistently, targeted inactivation of oxLDL uptake by KCs through genetic deletion of scavenger receptors or immunization with anti-oxLDL antibodies restored a quiescent KC phenotype and reversed hepatic inflammation[48,49, 50].

HSC are responsible for liver fibrogenesis in NASH. The ability of intracellular FC accumulation to directly activate HSCs through the toll-like receptor(TLR)-4-dependent pathway and trigger hepatic fibrogenesis has been well-documented in animal models of diet-induced and spontaneous intracellular cholesterol accumulation[51] (Figure 3). The exact mechanisms promoting HSC cholesterol overload are partially understood, but observational and experimental data support a role for oxLDLs uptake through the scavenger receptor lectin-like oxidized LDL receptor-1(LOX-1) in mediating HSC activation and hepatic fibrosis in NASH[52, 53] .

Collectively, these data indicate the inhibition of scavenger receptor-mediated uptake of oxLDL by KCs and HSCs is a potential therapeutic target for NASH.

2.5 Adipocyte cholesterol imbalance and adipose tissue dysfunction

Adipose tissue dysfunction plays a key role in the pathogenesis of obesity-related disorders, including NAFLD. Key features of dysfunctional adipocytes include resistance to the anti-lipolytic action of insulin and a proinflammatory adipokine pattern(enhanced TNF-α, IL-1, IL-6 and leptin and reduced adiponectin secretion), resulting in an increased release of toxic FFA and inflammatory cytokines into the portal blood to the liver[54, 55] .

Adipose tissue is a major site of cholesterol storage: in obese individuals half the whole body cholesterol is stored as FC in adipocytes, largely in the cytoplasmic lipid droplet and in lower percentage in PM[56], where it is concentrated in flask-like membrane invaginations called caveolae[57]. Caveolae are important signaling platforms, serving as a concentrating point for numerous signalling molecules, including insulin receptor and GLUT4, and depend for their stability and functionality on a high PM cholesterol content: decreasing PM cholesterol below a critical threshold increases PM fluidity and disrupts caveolae integrity and function[58](Figure 1,panel E-F).

Compared with small lean fat cells, hypertrophied adipocytes are characterized by intracellular cholesterol overload, largely contained in the lipid droplet: however, while the cholesterol/triglyceride ratio within the lipid droplet remains constant and independent of adipocyte size[59], hypertrophied adipocytes have a decreased PM cholesterol concentration, due to a dilution of PM cholesterol across an increased cell surface[60].The relative PM cholesterol depletion has 2 functional consequences[58]:

- the transcription factor SREBP-2, which is particularly sensitive to PM cholesterol content, senses PM cholesterol dilution as a true cholesterol depletion and activates transcription of its target genes (Table 3, Figure 2-3), further promoting cholesterol accumulation[58].

-PM cholesterol depletion disrupts caveolae integrity and function, impairs intracellular insulin signalling[61] and GLUT-4 translocation to PM[62, 63, 64] , and enhances secretion of proinflammatory cytokines(Figure1-3)[58, 65].

Importantly, these alterations were independently replicated by adipocyte PM cholesterol depletion through cholesterol synthesis inhibitors (i.e. statins) or selective PM cholesterol chelators (i.e. β-methylcyclodextrin), and reversed by PM cholesterol normalization, confirming that relative PM cholesterol depletion of hypertrophied adipocytes is important for adipose tissue dysfunction[57, 58, 63].

Mechanisms regulating cellular cholesterol sensing in adipocytes are being investigated: while cytosolic cholesterol pool derives mostly from circulating lipoprotein uptake, PM cholesterol is exquisitely sensitive to de novo cholesterol biosynthesis, and cholesterol synthesis inhibitors disrupted adipocyte caveolae integrity, impaired insulin signalling and adiponectin secretion in vitro and in vivo[61, 66]. The impact of these unwanted effects on liver disease and glucose metabolism in statin-treated NAFLD patients warrants further assessment[67].

3. Dysregulated hepatic cholesterol homeostasis in NAFLD

In hepatocytes, intracellular cholesterol homeostasis is mantained through a coordinate network involving cholesterol-sensors and nuclear transcription factors regulating cholesterol synthesis, esterification, uptake, intracellular transport and excretion(Table 1-2, Figure 2).

The 2 main sources of cellular cholesterol are de novo synthesis and uptake from plasma lipoproteins. To reach different cellular compartments, cholesterol must exit the ER, where it is synthesized, the cytosolic lipid droplets, where cholesterol esters are stored, and the endocytic compartments, where lipoprotein uptake occurs. Furthermore, cellular cholesterol must also reach the ER resident nuclear transcription factors SREBP-2 and LXR-α to regulate its synthesis and uptake in a feed-back loop (Figure 2, Table 1-2). In NAFLD these steps of cholesterol homeostasis are extensively deregulated, promoting cholesterol accumulation and toxicity.

3.1 increased cholesterol synthesis

HMG CoA reductase (HMGCoAR) is the rate-limiting enzyme in cholesterol biosynthesis. Hepatic HMGCR expression and activity are increased in NASH patients, paralleling both the extent of hepatic FC accumulation and the severity of liver histology[11, 68]. The increased HMGCoAR expression in NASH derives from enhanced gene transcription by SREBP-2, the principal transcriptional activator of HMGCoAR. Furthermore, HMGCoAR is also relatively

dephosphorylated in its active form, as a result of hepatic microRNA(miRNA)34a overexpression (see glossary)[6, 69].

Statins are known HMGCoAR inhibitors: although these agents improved transaminases and radiological steatosis in NAFLD patients, their effect on liver histology has been evaluated only in 1 small RCT, therefore preventing any definitive conclusion on the effect of these drugs on human disease[70]. Notably, statins seem to confer a significant cardiovascular protection in NAFLD: in the prospective Greek Atorvastatin and Coronary Heart Disease Evaluation (GREACE) study, enrolling hypercholesterolemic subjects with established CAD, statin-related CVD risk reduction was significantly greater (-68%) in patients with NAFLD than in those without NAFLD, with a number needed to treat of 5 NAFLD patients to save 1 CVD event[22].

Among the other enzymes involved in cholesterol biosynthesis, a polymorphism in squalene synthase, catalyzing the production of squalene from farnesyl pyrophosphate, was associated with NAFLD histological disease activity in a genome-wide association study[71]. Squalene synthase inhibitors are being devekoped as alternative, non-myotoxic cholesterol-synthesis inhibitors: unfortunately, the development of lapaquistat, the only drug evaluated in phase II/III trials, was terminated due to potential hepatotoxicity concerns[72].

3.2 increased cholesterol-rich lipoprotein uptake

Several membrane receptors contribute to cholesterol-rich lipoprotein uptake by hepatic and extrahepatic tissues, including the low density lipoprotein receptor (LDLR) for LDLs, the scavenger receptors CD36 and LOX-1 for oxLDLs, and the scavenger receptor class B type I (SR-BI). Beside mediating Kupffer and HSC activation by oxLDL(see above), dysregulated scavenger receptors contribute, together with LDLR, to hepatocyte cholesterol accumulation in NAFLD. Hepatocyte LDLR expression is increased in NAFLD, as a result of enhanced SREBP-2 activation[11, 14, 15, 66].

CD36 is a transmembrane receptor protein expressed on a broad range of cells, where it binds different ligands and is finely regulated by a network of nuclear receptors (Table 1). Hepatic CD36 expression is increased and correlates with the severity of steatosis in animal and human NASH[14, 73]. Furthermore, liver-specific CD36 overexpression induced, while deletion protected against the development of experimental hepatic steatosis and inflammation[46, 47, 74] (Table 1).

The SR-BI mediates the bi-directional selective transfer of cholesterol from HDL to hepatocytes and from hepatocytes to bile, with the net result of increasing reverse cholesterol transport and excretion[75]. Unlike ABC-transporters, SR-BI does not bind ATP or use ATP hydrolysis to drive transfer. SR-BI transcription is down-regulated in NASH as a result of increased SREBP-2 expression, thereby promoting whole-body cholesterol accumulation[14].

3.3 altered intracellular cholesterol transport and compartmentalization

Due to its water-insolubility, cholesterol requires specialized mechanisms to move across organelles in the cytosol, involving either vesicles or nonvesicular transport mechanisms. The latter is the major route for cholesterol movement and is mediated by diffusible carrier proteins, which have hydrophobic cavities to bind and transport cholesterol across the cytosol. Dysregulation of these carriers in NAFLD enhances FC accumulation and toxicity in specific cellular compartments.

3.3.1 Steroidogenic acute regulatory (StAR) transfer proteins

The steroidogenic acute regulatory lipid transfer (START) protein family is defined by the presence of a well-conserved 210 amino acid domain folding into a hydrophobic pocket to bind diverse lipid species. Among the 15 members of this family, the cholesterol-binding proteins are StARD1/D3 and StARD4/D5/D6[76], that bind cholesterol with a 1:1 stoichiometry and play an essential role in the delivery of cholesterol from late endosomes/lysosomes (LE/LY) to mitochondria(Table 1, Figure 2).

Compared to controls, hepatic StAR protein expression is 7- and 15-fold higher in patients with steatosis and NASH, respectively[11]. Little is known about the regulation of StAR proteins: StARD4 StARD5 are upregulated by SREBP-2 and by ER stress[77], respectively, both mechanisms being operative in NASH. Notably, StAR inhibition improved hepatocyte mitochondrial FC overload and injury[78], making StAR modulation a potential therapeutic target in NASH.

3.3.2 Niemann-Pick C1 (NPC1) and NPC2 proteins

Niemann-Pick C1 (NPC1) is an integral transmembrane protein localized to the LE/LY membrane, while NPC2 is a soluble lysosomal protein. Both proteins are required for delivery of LDL-derived cholesterol from LE/LY to the ER, the main regulatory site of cholesterol metabolism, and to other intracellular compartments[79](Table 1). In NPC1/2 deficient cells, LDL-cholesterol is internalized normally, but it is retained in LE/LY, with 2 functional consequences:

-failure to deliver cholesterol to the ER disrupts the feed-back inhibition of SREBP-2, which inappropriately upregulates cholesterol synthesis and uptake despite high cholesterol levels, aggravating hepatocyte cholesterol overload and toxicity(Figure 2).

-although cholesterol transport to ER is impaired, StAR-mediated FC delivery from LE/LY to mitochondria is preserved and upregulated by increased cholesterol availability in LE/LY[80], thereby aggravating mitochondrial injury[81].

In functional NPC1/2 deficiency, the amount of tissue cholesterol accumulation depends on the rate at which each tissue obtains cholesterol through the LDLR pathway: the liver is responsible for the uptake of 80% of LDL-cholesterol and is a major target organ, together with the central nervous system(CNS). Homozygous NPC1/2 deficiency leads to the rare autosomal-recessive Niemann-Pick type C (NPC) disease, characterized by liver, spleen, and CNS cholesterol accumulation, hepatosplenomegaly, liver failure, neurodegeneration and premature death. Intriguingly, heterogygous NPC1/2 deficiency leads to NAFLD, weight gain and metabolic syndrome

experimentally[82] and a recent European genome-wide study linked NPC1 gene to early-onset and morbid adult obesity[83].

3.3.3 Oxysterol-binding proteins and OSBP-related proteins

Oxysterol-binding proteins (OSBPs) and OSBP-related proteins (ORPs) are a large (>12 members) family of soluble cytosolic lipid-binding proteins, sharing a common lipid-binding OSBP-related domain. Initially identified for their oxysterol-binding capacity[84], these proteins were subsequently discovered to play key roles in numerous steps of lipid metabolism, including cholesterol delivery from LE/LY to ER, cholesterol shuttling between PM and ER, and intracellular signalling[85]. ORP8, a member of this family, plays a relevant role in lipid homeostasis and insulin sensitivity: its overexpression reduced, while silencing increased, hepatic cholesterol and triglyceride accumulation by modulating SREBP-2 and SREBP-1c expression[86]. ORP8 also enhances hepatic insulin sensitivity through an AKT-mediated mechanism[87], and its expression is reduced in obesity via a miRNA143-dependent pathway[83].

3.4 altered cholesterol absorption and secretion

3.4.1 Niemann-Pick C1 like 1 protein

Daily cholesterol intake in humans on a typical Western diet is 300–500 mg, and daily biliary secretion of cholesterol from the liver into the gut is 800–1200 mg, of which 50% is reabsorbed in the intestine and delivered to the liver, in a process resembling enterohepatic bile acid circulation[88]. NPC1-like 1 protein (NPC1L1) is expressed on the apical surface of enterocytes and on the canalicular membrane of hepatocytes and mediates active cholesterol absorption from gut lumen and from the bile, respectively(Table 1, Figures 1-2).. NPC1L1 is a N-glycosylated integral membrane protein with a sterol-sensing domain (SSD), which regulates NPC1L1 intracellular cycling by sensing membrane cholesterol content: when cells are enriched with

cholesterol[85], NPC1L1 localizes predominantly to the endocytic recycling compartment (ERC); when cells are deprived of cholesterol, NPC1L1 moves to the plasma membrane.

Genetic deletion or pharmacological inhibition of NPC1L1 by ezetimibe disrupt enterohepatic circulation of cholesterol, causing a substantial endogenous cholesterol loss and a feedback up-regulation of endogenous cholesterol synthesis, which however cannot fully compensate for the excessive cholesterol loss, as NPC1L1 deficiency or ezetimibe treatment significantly reduce blood cholesterol in rodents on low-cholesterol diets[89] and in pure vegetarians[90]. Preliminary experimental and human evidence suggest ezetimibe may effective reduce steatosis, necroinflammation and fibrosis in NASH[18, 19, 20]. Additional mechanisms beyond inhibition of cholesterol absorption may underlie the observed benefits of ezetimibe in NAFLD: NPLC1L1 down-regulates NPC2 expression, thereby promoting LE/LY FC overload[91]; ezetimibe restores hepatic microsomal triglyceride transfer protein (MTTP) expression, which is required for hepatic VLDL-Tg secretion[18, 92]; finally, ezetimibe may synergize with α-glucosidase inhibitors to enhance intestinal incretin glucagon-like peptide-1 secretion and peroxisome proliferators-activated receptor-α1 (PPAR-α1) activation[93, 94, 95]. As intestinal cholesterol absorption seems to be decreased in NAFLD patients[38, 96], the relative contribution of intestinal vs. hepatic NPC1L1 inhibition was evaluated to explain the observed benefits in NAFLD: selective hepatic NPC1L1 inhibition increased biliary cholesterol excretion, attenuated mitochondrial cholesterol overload, restored mGSH levels, reduced ROS generation, c-JUN N-terminal kinases (JNK) activation, and ER stress, eventually improving hepatic steatosis and insulin resistance [97, 98] .Collectively, these data indicate hepatic NPC1L1 inhibition is a crucial mediator of the benefits of ezetimibe in NASH.

Despite these premises, some issues needs to be addressed: experimental data suggest ezetimibe biliary excretion is reduced in NASH, which may reduce its disposition and bioactivity[99]; the impact of background cholesterol intake on ezetimibe effectiveness is unclear[100]; finally, the

potential synergism with statins on liver[101] and cardiovascular disease, the other major health-related issue in NAFLD, is unknown and require further investigation.

3.4.2 ATP-binding cassette (ABC) transporters and cellular cholesterol efflux

ABC transporters are a family of integral membrane proteins that actively transport a variety of small molecules across cell membranes. In mammals, at least four ABC-transporters mediate sterol efflux from cells: ABCA1, ABCG1, ABCG4, and ABCG5/ABCG8.

ABCA1 can be found in several celli lines, including macrophages and hepatocytes: in the latter, it localizes to the basolateral membrane, in contact with the sinusoidal vasculature, where it catalyzes the transfer of cellular cholesterol and phospholipids to lipid-free apolipoprotein I (apoA-I) to form HDL particles. Lipidation of apoA-I by ABCA1 is the rate-limiting step in reverse cholesterol transport and complete loss of ABCA1 function causes Tangier disease, characterized by HDL deficiency, sterol tissue accumulation, and atherosclerosis. While most studies on ABCA1 focused on its relationship with atherosclerosis, recent data suggest a reduced hepatocyte ABCA1 activity contributes to hepatic cholesterol accumulation and injury in NASH[14, 66]. Consistently, even minor (50%) loss of ABCA1 function in hepatocytes promoted FC overload and NASH, while ABCA1 overexpression improved liver disease experimentally[102].

ABGC5 and ABCG8 are 'half-transporters' that form an heterodimer (ABCG5/G8) on the apical membrane of enterocytes and on the canalicular membrane of hepatocytes, to efflux plant and animal sterols into the intestinal lumen and the bile, respectively, thereby opposing the activity of NPC1L1. The importance of ABCG5/G8 transporters in limiting cholesterol and phytosterols accumulation is exemplified by sitosterolemia, a rare autosomal recessive disorder characterized by ABCG5 or ABCG8 function loss, tissue accumulation of plant sterols (mainly β-sitosterol), hypercholesterolemia, tendon xantoma and premature atherosclerosis.

Recent animal and human data found a reduced ABCG5/G8 expression in NASH[14, 66], and the relevance of ABCG5/G8 for hepatic cholesterol homeostasis and NASH has been elucidated in transgenic mice: ABCG5/G8 knockout induced steatosis, hepatocyte apoptosis, ER stress and

hepatic insulin resistance, accelerating high-fat diet-induced NASH[103]. These experiments highlighted also the importance of intestinal cholesterol absorption for whole-body cholesterol balance: liver-specific ABCG5/G8 over-expression alone failed to induce a net cholesterol loss, as excreted biliary cholesterol was reabsorbed by intestinal NPC1L1. Conversely, hepatic ABCG5/8 over-expression plus NPC1L1 inhibition by ezetimibe induced hepatic cholesterol loss and reduced atherosclerosis, indicating that increased biliary cholesterol secretion must be coupled with decreased intestinal cholesterol absorption to promote net sterol loss from the body[104].

3.5 altered intracellular cholesterol esterification and de-esterification

Newly synthesized cholesterol is esterified and de-esterified according to cellular requirements by 2 ER enzymes, acyl-CoA cholesterol:cholesteryl transferase (ACAT)-2 and cholesteryl ester hydrolase (CEH), respectively.

In NAFLD, hepatocyte ACAT-2 activity is mildly increased[11, 14] , unchanged[66] or reduced[105], while CEH is 6-fold higher than in healthy controls[66]. The net effect of ACAT-2 and CEH activity on cellular cholesterol balance critically depends on a functioning cholesterol efflux machinery, represented by ABC transporters. In hepatocytes and macrophages, hydrolysis of CE is a required step for CE mobilization and excretion; consistently, hepatocyte-specific ACAT-2 deletion prevented cholesterol accumulation and steatosis[106], and transgenic CEH overexpression in hepatic macrophages reduced cellular cholesterol overload, improving hepatic insulin resistance and inflammation[107, 108]. However, if ABC transporter-mediated cholesterol efflux is impaired as in NAFLD[14, 66], inappropriate CEH activation enhances FC accumulation and toxicity (Table 1).

3.6 Nuclear regulators of cholesterol homeostasis

The processes described above are co-ordinately regulated by nuclear transcription factors, molecules that, upon ligand binding, bind to specific promoter regions, named response elements

(REs), of target genes and regulate transcription of their products. Three nuclear transcription factors regulating cholesterol metabolism have been linked to NAFLD: sterol regulatory element-binding protein(SREBP)-2, farnesoid X receptor (FXR), and liver X-receptor(LXR).

3.6.1 Sterol regulatory binding protein(SREBP)-2

Verterbate genomes contain 2 loci, named sterol regulatory element-binding factor (SREBF)-1 and SREBF-2, which code for SREBP-1, a key regulator of fatty acid synthesis, and for SREBP-2, a master regulator of cholesterol metabolism[109]: under low cellular cholesterol levels, SREBP-2 is activated and translocates from the ER to the nucleus, where it modulates transcription of target genes involved in cholesterol synthesis, uptake, secretion and transport, to increase intracellular cholesterol availability. Conversely, cellular cholesterol repletion retains SREBP-2 in the ER and prevent its activation(Figure 2, Table 2).

Beside SREBP-2, the SREBPF-2 locus also generates the microRNA miRNA-33a, which is processed from an intron within the SREBF2 primary transcript[107]: miRNA-33a inhibits ABCA1-mediated cholesterol export, decreases NPC-1, and reduces the expression of several enzymes involved in mitochondrial fatty acid β-oxidation[110]. Therefore, the physiological activation of SREBP-2/miRNA33a under low cholesterol conditions promotes cholesterol synthesis and retention and increases availability of fatty acids to promote storage of neutral lipids CE and triglycerides. SREBP-2 is inappropriately activated despite cellular cholesterol overload in NAFLD, with hepatic SREBP-2 activity being 7- and 3-fold higher in NASH and steatosis patients than in controls[11, 14, 66]. Potential reasons for inappropriate SREBP2 activation in NAFLD include:

-direct stimulatory effect of hyperinsulinemia[14, 111] and proinflammatory cytokines[15, 112]: in conditions of chronic low-grade inflammation and hyperinsulinemia, like obesity and insulin resistance, the suppressive effect of elevated cellular cholesterol levels on SREBP-2 expression is overridden by high insulin and proinflammatory cytokine levels, which disrupt the physiological negative feed-back by cholesterol stores and activate directly SREBP-2[15] (Figure 2).

-downregulation of hepatic miRNA122: miRNA-122 (see glossary) is the most abundant miRNA in the liver, where it regulates the expression of nearly 200 genes[67], including SREBP-2, which is repressed by miRNA122. Hepatic miRNA 122 is strongly downregulated in NASH patients, and replicating miRNA-122 suppression significantly increased expression of SREBP-2 and its target genes in vivo and in vitro[67].

-genetic variation in SREBP-2 activity: a polymorphism in SREBP-2 recently predicted the incidence and severity of NAFLD and of coexisting glucose and lipid dysmetabolism in a cohort of initially lean, insulin sensitive subjects[113].

Collectively, these data highlight the importance of SREBP-2/miRNA33a complex for hepatic cholesterol metabolism, demonstrating its inappropriate upregulation may be crucial for hepatic cholesterol overload in NASH and suggesting its modulation may be a therapeutic target for NASH.

3.6.2 Farnesoid X receptor

Originally known for its function of bile acid sensor in enterohepatic tissues, farnesoid X receptor (FXR) has recently emerged as a master regulator of lipid and glucose homeostasis and of inflammatory and fibrogenic processes at hepatic and extrahepatic level. In the liver, FXR promotes cholesterol excretion by ABCG5/G8 transporters[114], triglyceride clearance by increasing fatty acid β-oxidation and suppressing lipogenesis, and has enhances insulin-sensitizing, anti-inflammatory and anti-fibrotic properties[115](Table 2). In NAFLD patients, hepatic FXR activity and bile acid biosynthesis are decreased and inversely related to the severity of liver histology[14, 66, 103] and, consistently, FXR-deleted mice on a high fat diet exhibit massive hepatic steatosis, necroinflammation and fibrosis, all reversed by FXR agonists[116, 117]. On this basis a novel class of potent, semi-synthetic bile acid FXR agonists is being evaluated for the treatment of obesity-related disorders, including NAFLD. One of these compounds, obeticholic acid (OCA), a semi-synthetic derivative of chenodeoxycholic acid, ameliorated liver fibrosis markers, insulin resistance, and weight gain in a phase IIa RCT enrolling diabetic NAFLD

subjects[118] and is currently evaluated in the multicenter, double-blind randomized "FXR Ligand NASH Treatment (FLINT)" trial (www.clinicaltrials.gov NCT01265498).

3.6.3 Liver X receptor(LXR)-α

There are two LXRs, LXR-α and LXR-β, with considerable sequence homology and the same ligand binding, but with different tissue distribution: LXRα is highly expressed in the liver, adipose tissue and macrophages, whereas LXRβ is expressed in many tissues[119, 120]. Originally identified for their function of nuclear cholesterol sensors activated in response to elevated intracellular oxysterols, LXRs were subsequently shown to be activated also by glucose[119]. To regulate transcription of its target genes, LXR must form an heterodimer with another nuclear receptor, the retinoid X receptor (RXR) which is ligand-activated by 9-cis retinoic acid.
LXR-α is a key regulator of whole-body cholesterol metabolism: upon activation, LXR-α increases the expression of ABCA1, ABCG5/G8 in macrophages, hepatocytes and enterocytes and downregulates intestinal NPC1L1 expression, thereby promoting reverse cholesterol transport and intestinal excretion[119]. Furthermore, LXR-α increases the hepatic trascription of cytochrome P450 7A1 (CYP7A1), the rate-limiting enzyme in bile acid synthesis, promoting cholesterol conversion to bile acids, and accelerates LDL-receptor degradation, thereby reducing hepatocyte cholesterol uptake[121]. Beside its key role in promoting net cholesterol loss from the body, LXR has also direct anti-inflammatory effects, and LXRα agonists improved hepatic inflammation and fibrosis by reducing cholesterol-induced activation of Kupffer and HSCs in animal models of NASH[13, 122, 123].
Despite these benefits, LXRα has a drawback: LXRα activation upregulates hepatic lipogenesis and inhibits VLDL catabolism, promoting hepatic steatosis, large VLDL triglyceride production and hyperlipidemia[120, 124, 125, 126, 127] (Table 2). Although the increased storage of neutral triglycerides may actually buffer the lipotoxic FC and free fatty acids and protect against the

development of NASH, the impact of resulting hyperlipidemia on CVD risk hampers the clinical application of LXR agonists for treatment of NAFLD at present.

4. Future perspectives

There is an unmet need for a safe and effective treatment for NASH: TZDs reverse necroinflammation and slow fibrosis progression, but promote weight gain; vitamin E improves NAFLD activity, but its impact on lipid and glucose metabolism is uncertain[70]. The pathogenesis of NASH is not fully elucidated, and emerging evidence suggest it can dissociate from insulin resistance and metabolic syndrome[128]. The data presented suggest NAFLD is associated with extensive derangement in numerous steps of hepatic cholesterol homeostasis, which converge to promote FC accumulation and toxicity, and eventually NASH. Pharmacological modulation of cholesterol homeostasis is therefore an attractive therapeutic option for NASH, not only for ameliorating liver disease, but also to reduce CVD risk, which is the other health-related burden in NAFLD[3, 22]. Current therapeutic options targeting cholesterol metabolism include inhibition of HMGCoAR by statins and of NPC1L1 by ezetimibe. Despite their established cardiovascular benefit, data on statin efficacy in NAFLD are sparse due to the feared hepatotoxicity of these drugs. However, current data demonstrated patients with hepatic steatosis do not seem at increased risk for statin hepatotoxicity[129]: on the basis of available evidenced, the Liver Expert Panel stated that statins can be safely used in patients with NAFLD and routine liver enzyme monitoring is not warranted in this population[130].

While the efficacy of these drugs warrants evaluation in large RCTs with histological end-points and clinical outcomes, other approaches targeting cholesterol-induced liver injury are being evaluated, including GLP-1 analogues, which reduced cholesterol synthesis and hepatic inflammation in methionine choline deficient (MCD) dietary models of NASH[131], hepatic thyroid receptor beta-agonists[132], antisense nucleotides modulating miRNA22a activity[109], strategies enhancing the cellular cholesterol-unloading and anti-inflammatory properties of HDL

particles[133], and agonists/antagonists of key nuclear regulators of cholesterol metabolism like FXR, SREBP-2. These approaches are currently evaluated in preclinical studies or phase II trials and promise to open up new avenues for the development of therapeutics for NASH and associated disorders.

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FIGURE LEGENDS

Figure 1: mechanisms of membrane cholesterol toxicity operating in hepatocytes (panel A-D) and adipocytes (panel C-F)

Panel A: intact mitochondrial glutathione (mGSH) stores are essential for controlling reactive oxygen species (ROS) generation by cell death stimuli such as tumour necrosis factor(TNF)-α and Fas ligand (FasL) in hepatocytes .GSH is exclusively synthesized in the cytosol and is transported into the mitochondria by the mitochondrial transmembrane 2-oxoglutarate protein carrier (2-OG).

Panel B: 2-OG carrier is particularly sensitive to changes in mitochondrial membrane cholesterol content and fluidity: when mitochondria are loaded with free cholesterol, mitochondrial GSH (mGSH) transport by 2-oxoglutarate (2-OG) carrier is impaired, resulting in mGSH depletion. GSH depletion enahnces the generation of ROS in response to TNF-α and Fas ligand (FasL), causing cardiolipin (CL) peroxidation (CLOOH), which destabilizes the mitochodrnial membrane lipid bilayer. Mitochondrial membrane destabilization and permeabilization result in cytochrome c (Cyt C) release and trigger of hepatocyte necrosis and apoptosis. Consistent with these model, the replenishment of mGSH by permeable precursors, such as GSH ethyl ester, protected mitochondrial cholesterol-loaded hepatocytes against TNF/Fas-mediated apoptosis. Furthermore, hepatocytes selectively depleted of mGSH reproduced the sensitization to TNF/FasL observed in the nutritional and genetic models of hepatic steatosis characterized by cholesterol accumulation.

Collectively, these data demonstrate that mitochondrial FC accumulation induces oxidative injury and sensitizes hepatocytes to the action of pro-inflammatory/proapoptotic agents

Panel C: the ER is a membranous organelle with critical cellular functions: folding and posttranslational modifications of nascent proteins to achieve their proper conformation; phospholipid synthesis; storage of calcium ions for cellular signalling; drug metabolism by enzymes such as cytochrome P450. A high intra-ER Ca2+ concentration is necessary for maintenance of normal ER function and protein folding ability. This high Ca2+ concentration of ER is actively maintained by the sarco(endo)plasmic reticulum Ca2+-ATPase (SERCA).

Panel D: FC accumulation triggers ER stress by altering the critical FC-to-phospholipid ratio of the ER membrane, necessary to maintain its fluidity when the FC:phospholipid ratio in the ER membrane reaches a critical threshold of stiffness, integral ER membrane proteins become inactive. Among the ER enzymes, the SERCA ATPase is particularly sensitive to minimal increases in ER membrane cholesterol content: the resulting stiffening of the ER membrane inhibits SERCA conformational changes and activity, leading to a fall in the physiologically high intra-ER Ca^{2+} concentration and impairing ER function and folding capacity(ER stress). ER stress or unfolded protein response (UPR, see glossary) plays a central pathogenetic role in obesity-associated disorders, including NAFLD.

The importance of FC accumulation the for triggering ER stress has been recently documented in hepatocytes where ER membrane cholesterol accumulation, rather than total cellular cholesterol overload, induced hepatic ER stress, while hepatocyte ER cholesterol lowering by two independent approaches (cyclodextrin or zaragozic acid) resolved ER stress.

The relevance of dysfunctional SERCA for free cholesterol-induced hepatocyte apoptosis and the pathogenesis of NAFLD and diabetes has recently emerged: in obese diabetic mice , where hepatic SERCA expression is dramatically reduced and normalization of SERCA activity in the liver alleviates ER stress, reduces lipogenesis, and improves glycemia and NAFLD .

Panel E: in adipocytes, cholesterol is largely contained in the cytoplasmic lipid droplet and in lower percentage in plasma membrane (PM), where it is concentrated in flask-like membrane invaginations called caveolae, which represent as much as 50% of the plasma membrane. These specialized membrane microdomains have a unique composition in that they are highly enriched in cholesterol and their coat proteins caveolins (-1, -2, and -3) and caveins (-1 and -2). Caveolae concentrate numerous signalling molecules, including insulin receptor(IR) and GLUT4, and depend for their stability and functionality on a high PM cholesterol concentration: lowering PM cholesterol below a critical threshold increases PM fluidity and disrupts caveolae integrity and function.

Panel F: compared with small lean fat cells, hypertrophied adipocytes from obese subjects are characterized by intracellular cholesterol overload, largely contained in the lipid droplet: however, while the cholesterol/triglyceride ratio within the lipid droplet remains constant and independent of fat cell size, hypertrophied adipocytes have a decreased PM cholesterol concentration, due to a dilution of PM cholesterol across an increased cell surface.The relative PM cholesterol depletion activates the transcription factor SREBP-2 further promoting cholesterol accumulation within the fat cell, and disrupts caveolae integrity and function, impairing intracellular insulin signalling and GLUT-4 translocation to PM, and enhancing secretion of proinflammatory cytokines including angiotensinogen, chemerin, TNF-α and IL-6.

These alterations were independently replicated by adipocyte PM cholesterol depletion through cholesterol synthesis inhibitors (i.e. statins) or selective PM cholesterol chelators (i.e. β-methylcyclodextrin), and reversed by PM cholesterol normalization, confirming that relative PM cholesterol depletion of hypertrophied adipocytes is an important determinant of adipose tissue function.

Mechanisms regulating cellular cholesterol sensing in adipocytes are being investigated: while cytosolic cholesterol pool derives mostly from circulating lipoprotein uptake, PM cholesterol is exquisitely sensitive to de novo cholesterol biosynthesis, and cholesterol synthesis inhibitors disrupted adipocyte caveolae integrity, impaired insulin signalling and adiponectin secretion in vitro and in vivo. The impact of these unwanted effects on liver disease and glucose metabolism in statin-treated NAFLD patients may be clinically relevant and warrants further assessment.

Figure 2: hepatocyte cholesterol homeostatic mechanisms involved in the pathogenesis of NAFLD

In hepatocytes, LDL particles are internalized via receptor-mediated endocytosis by LDL receptors (LDLR), and the resulting vesicles deliver their contents to early endosomes, where the acid lipase enzyme hydrolyzes LDL-derived cholesterol esters (CE) to free cholesterol (FCHOL). Then LDLR, devoid of their cholesterol cargo, returns to basolateral plasma membrane (PM), while cholesterol is transported from early to late endosomes (LE), which fuse with Golgi-derived vesicles containing hydrolytic enzymes and mature into lysosomes (LY). Intracellular cholesterol transport from LE/LY to cellular organelles and membranes is predominantly mediated by protein carriers (nonvescicular transport): the steroidogenic acute regulatory lipid transfer (StAR) proteins are essential for FCHOL delivery to mitochondria, while the Niemann-Pick C1 (NPC1) and the NPC2 are required for delivery to the ER, the main regulatory site of cholesterol metabolism, and to other intracellular compartments. Oxysterol-binding proteins (OSBPs)-related proteins (ORPs), and specifically ORP8, have been implicated in FCHOL delivery from LE/LY to ER and cholesterol shuttling between PM and ER. Functional deficiency of NPC1/2 carriers leads to LE/LY FCHOL accumulation, which is transported by StAR to mitochondria, and fails to downregulate sterol regulatory binding protein(SREBP)-2 in the ER, disrupting the negative feed-back on SREBP-2 regulated cholesterol synthesis and uptake and resulting is FCHOL accumulation and toxicity.

In the ER, cholesterol regulates expression of nuclear transcription factor SREBP-2, which is expressed as an inactive precursor protein. Under low intracellular cholesterol conditions, SREBP-2 binds its chaperone SREBP cleavage activating protein (SCAP) and translocates to the Golgi apparatus, where it is cleaved and activated by two proteases, site-1 serine protease and site-2 metalloproteinase. Conversely, in response to heightened cellular cholesterol levels, the SCAP changes conformation and binds to insulin-induced gene-1(Insig-1) and -2, which retains the SREBP-2/ SCAP complex within the ER.

The active SREBP-2 enters the nucleus, where it modulates transcription of key target genes involved in cholesterol homeostasis (Table 1-2)

In NASH, as a result of increased ER free cholesterol content, the ER membrane stiffens and prevents conformational changes of the, which is necessary for maintaining high intra-ER Ca^{2+} concentrations. As a result of the sarco(endo)plasmic reticulum Ca^{2+}-ATPase (SERCA) dysfunction, intra-ER Ca^{2+} levels fall and the normal functions and folding ability of the ER are impaired, triggering ER stress. In response to ER stress, three stress-sensing proteins found on the ER membrane: PKR-like eukaryotic initiation factor 2α kinase (PERK), inositol-requiring kinase-1α (IRE-1α), and activating transcription factor-6 (ATF-6) are activated and initiate a series of adaptive mechanisms, named unfolded protein response (UPR) . If ER stress conditions persist, however, ER triggers cell death through caspase-dependent (apoptosis) and caspase-independent (necrosis) mechanisms

A major pathway of cholesterol metabolism in hepatocytes is the synthesis of oxysterols and bile acids. Oxysterols are a class of oxygenated derivatives of cholesterol formed by autooxidation or by specific monooxygenases, mainly of the cytochrome P450(CYP450) family.

The microsomal sterol 7α hydroxylase (CYP7A1) catalyzes the 7α-hydroxylation of cholesterol to 7α-hydroxycholesterol (7OHC), the rate limiting step in the neutral pathway to bile acid synthesis, while the mitochondrial sterol 27-hydroxylase (CYP27A1) catalizes the "alternative" (or acidic) pathway of bile acids synthesis, leading to 27-hydroxycholesterol (27OHC).

A third, quantitatively minor pathway involves sterol 25-hydroxylase, which catalyzes cholesterol 25-hydroxylation to 25-hydroxycholesterol (25OHC).

Beside being early intermediates in the synthesis of bile acids, oxysterols are important modulators of nuclear transcription factors: 27-OHC represses SREBP-2-mediated cholesterol synthesis and stimulates LXR-α-mediated transcription of ABCA1 transporters, thereby promoting cholesterol excretion, while 25OHC activates LXRα/SREBP-1c-mediated lipogenesis, further enhancing free fatty acid (FFA) accumulation and toxicity, and inflammatory response via NF-κB activation, and has been recently implicated in liver injury in NASH

Conversely, 25-hydroxycholesterol-3-sulfate (25OHC3S), the sulphated derivative of 25OHC, synthesized by the cytosolic sulfotransferase SULT2B1b through sulfation of 25OHC, had potent and opposite effects on hepatic lipid metabolism and ameliorates steatosis and inflammation in experimental NASH

The synthesis of bile acid is activated by the farnesoid X receptor (FXR), which also promotes cholesterol excretion through ABCG5/G8 transporters and inhibits SREBP-1c-mediated lipogenesis and NF-κB-mediated inflammatory pathway.

In NASH, hepatic FXR expression and bile acid biosynthesis are decreased.

Figure 3: interactions among adipocyte, hepatocyte, hepatic stellate cell (HSC) and Kupffer cell in promoting cholesterol-mediated liver injury in NAFLD

Intracellular cholesterol accumulation induces a series of events in adipocytes and liver cells collectively promoting liver injury and NAFLD.

In hypertrophic adipocytes, cholesterol imbalance (and specifically, plasma membrane, PM, cholesterol depletion) upregulates SREBP-2, further promoting cholesterol accumulation, and disrupts caveolae integrity and function, impairs insulin receptor (IR)[36] and GLUT-4 translocation to plasma membrane

Free cholesterol (FCHOL) accumulation in obese adipocytes enhances secretion of proinflammatory cytokines, including angiotensinogen, chemerin, TNF-α and IL-6 which, together with elevated plasma insulin levels, downregulation of miRNA122 and possibly functional SREBF2 gene polymorphisms, disrupt the physiological negative feed-back by elevated cholesterol stores and activate directly SREBP-2 in hepatocytes.

In the liver, Kupffer cells accumulate FCHOL through uptake of oxidized LDLs by scavenger receptors CD 36 and SR-A and become activated, secreting proinflammatory adipokines (IL-1, TNF-α) and the profibrogenic cytokine Transforming Growth Factor(TGF)-β1, which activates hepatic stellate cells (HCS), promoting hepatic fibrosis.

The ability of intracellular free cholesterol overload to directly activate HSCs, without the mediation of KCs through the toll-like receptor(TLR)-4-dependent pathway and trigger hepatic fibrogenesis has been well-documented in mice on high-cholesterol diets and in Niemann–Pick type C1-deficient mice, the latter spontaneously accumulating intracellular free cholesterol .

HSC accumulate cholesterol through oxLDLs uptake by LOX-1 receptors and possibly by other yet poorly characterized receptor and non-receptor pathways.

High intracellular cholesterol levels up-regulates toll-like receptor(TLR)-4, which in turn down-regulates, through the adaptor molecule MyD88, the membrane receptor Bambi, a pseudoreceptor for TGF-β1 with negative regulatory function. The removal of this inhibitor sensitizes HSC to activation by TGF-β1 and secretion of chemotactic factors monocyte chemotactic protein-1 (MCP-

1) and macrophage inflammatory protein 1β (MIP1β), that recruit circulating macrophages to the liver to become Kupffer cells. Kupffer cells, in turn, secrete TGF-β and further activate HSC in a paracrine manner.

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Figure 1

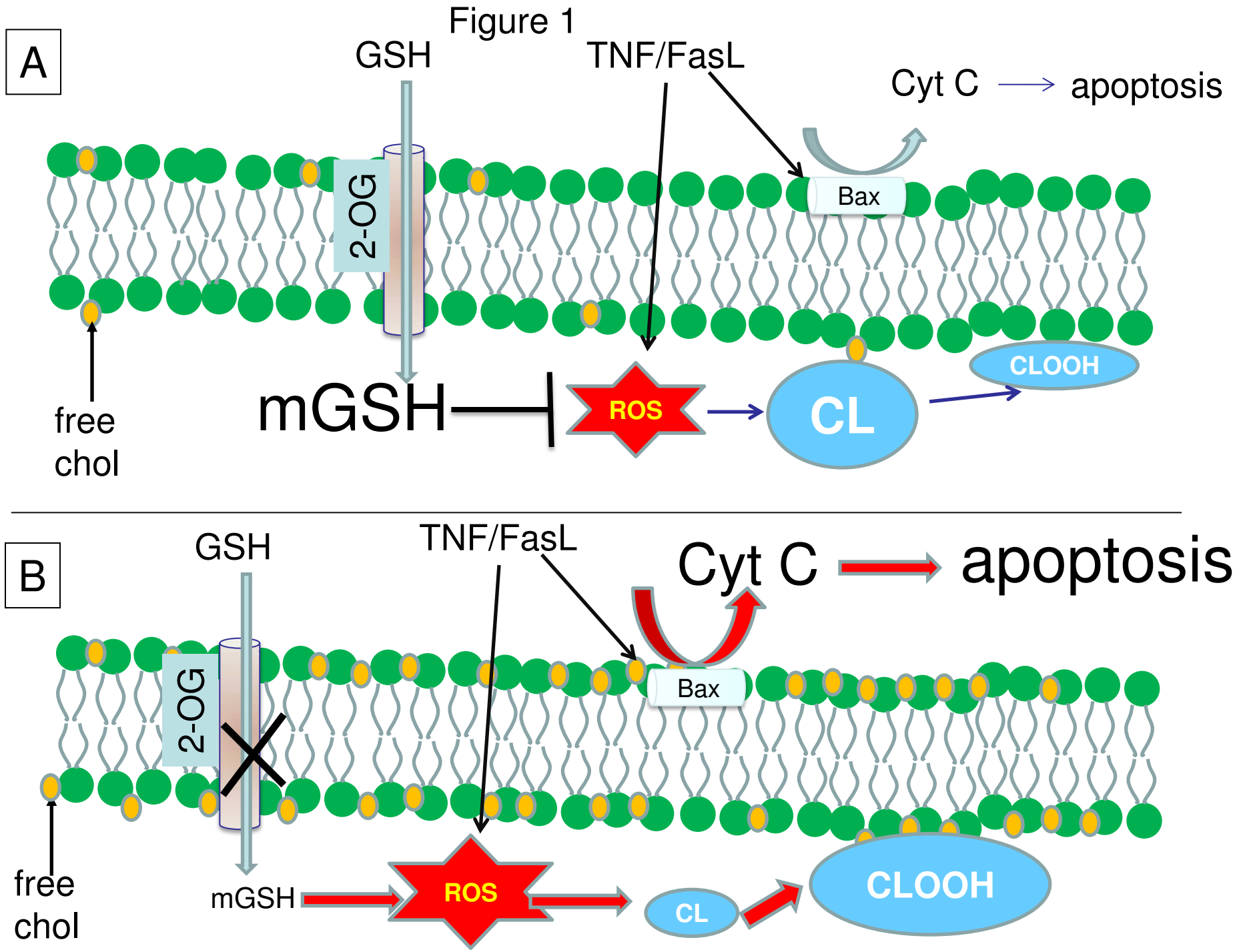
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Figure 1

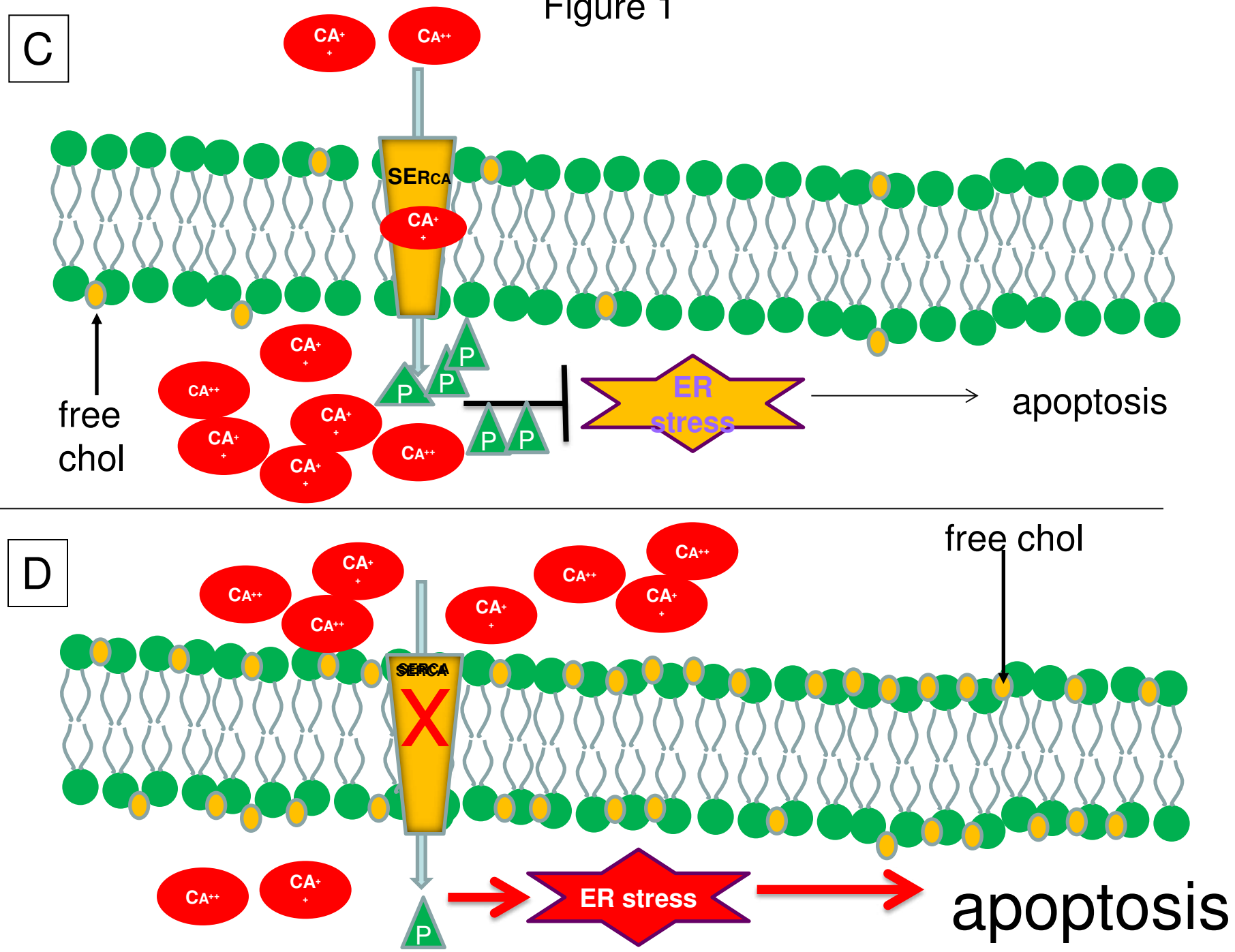
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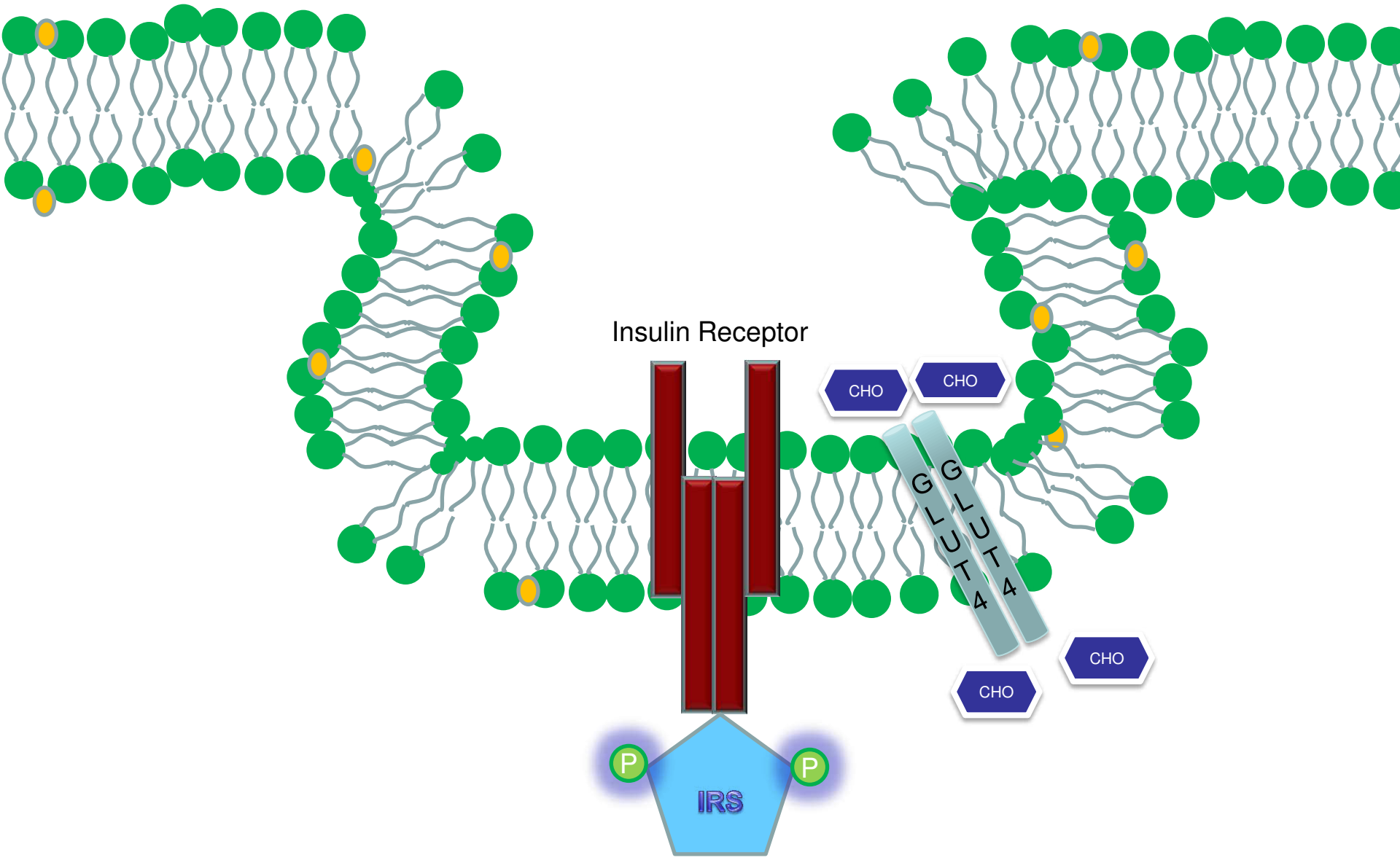
Figure 1



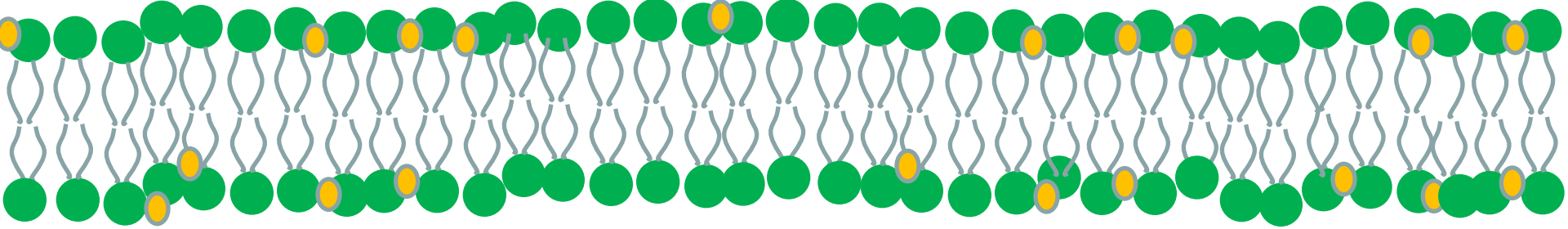


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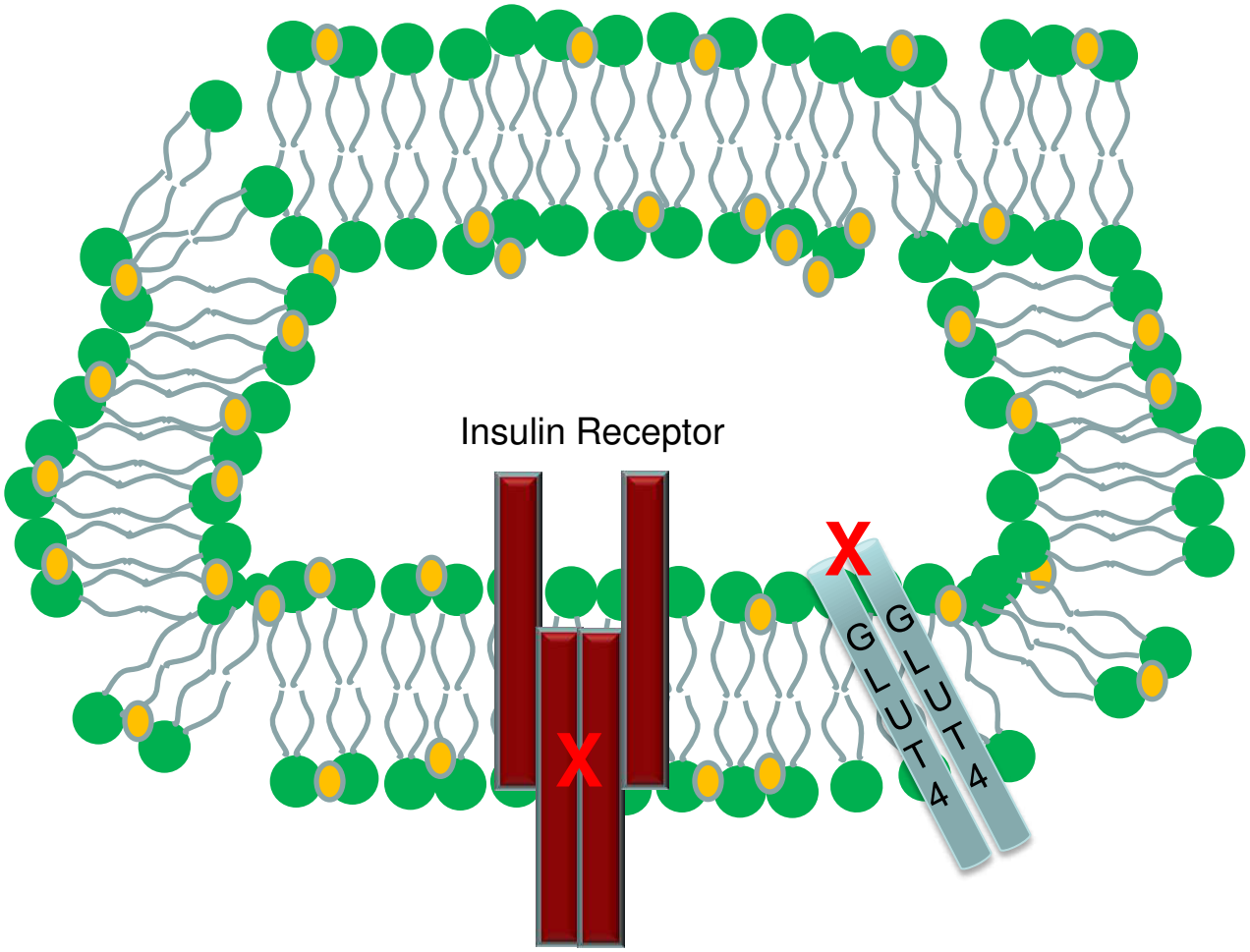
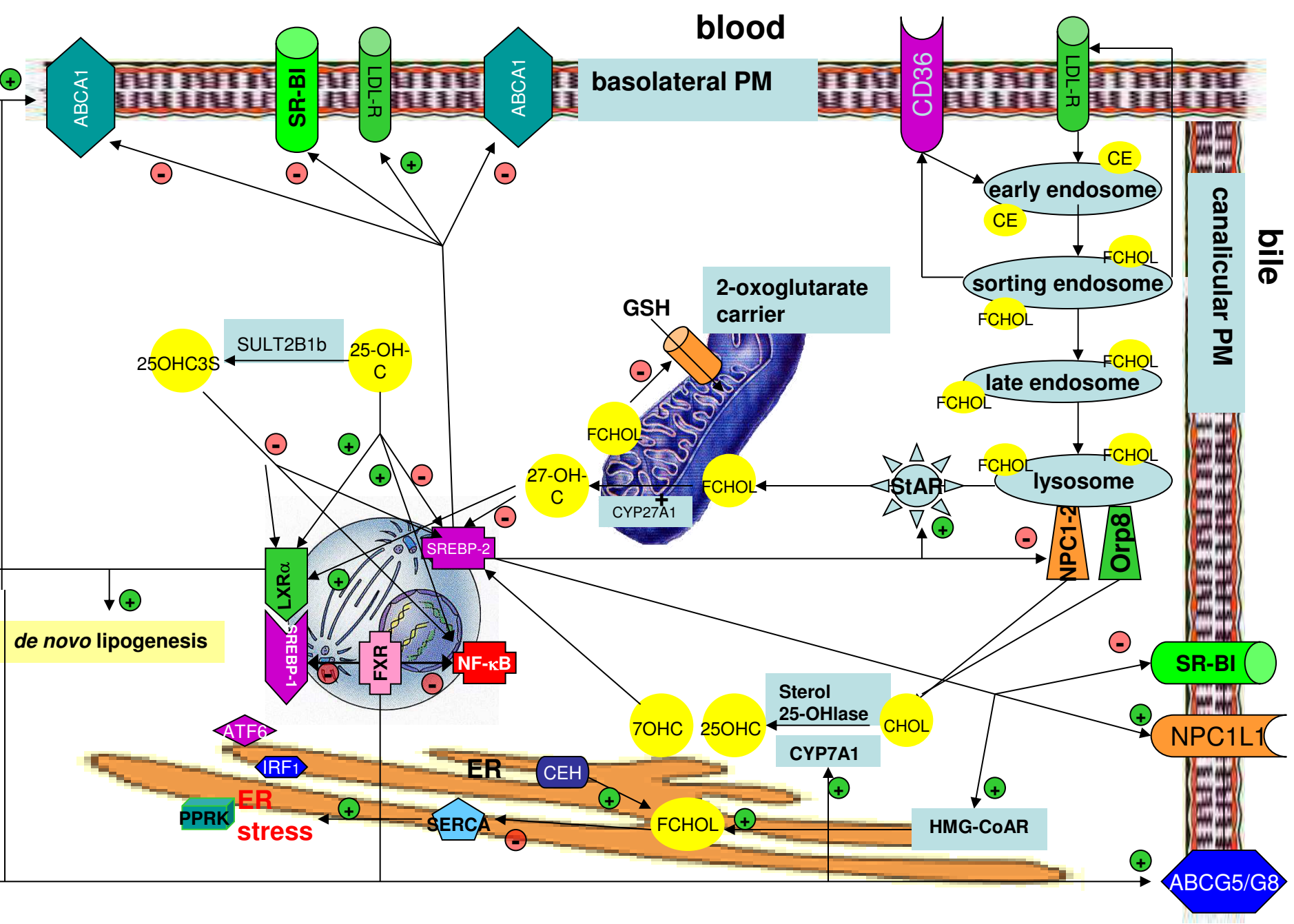
Figure 1

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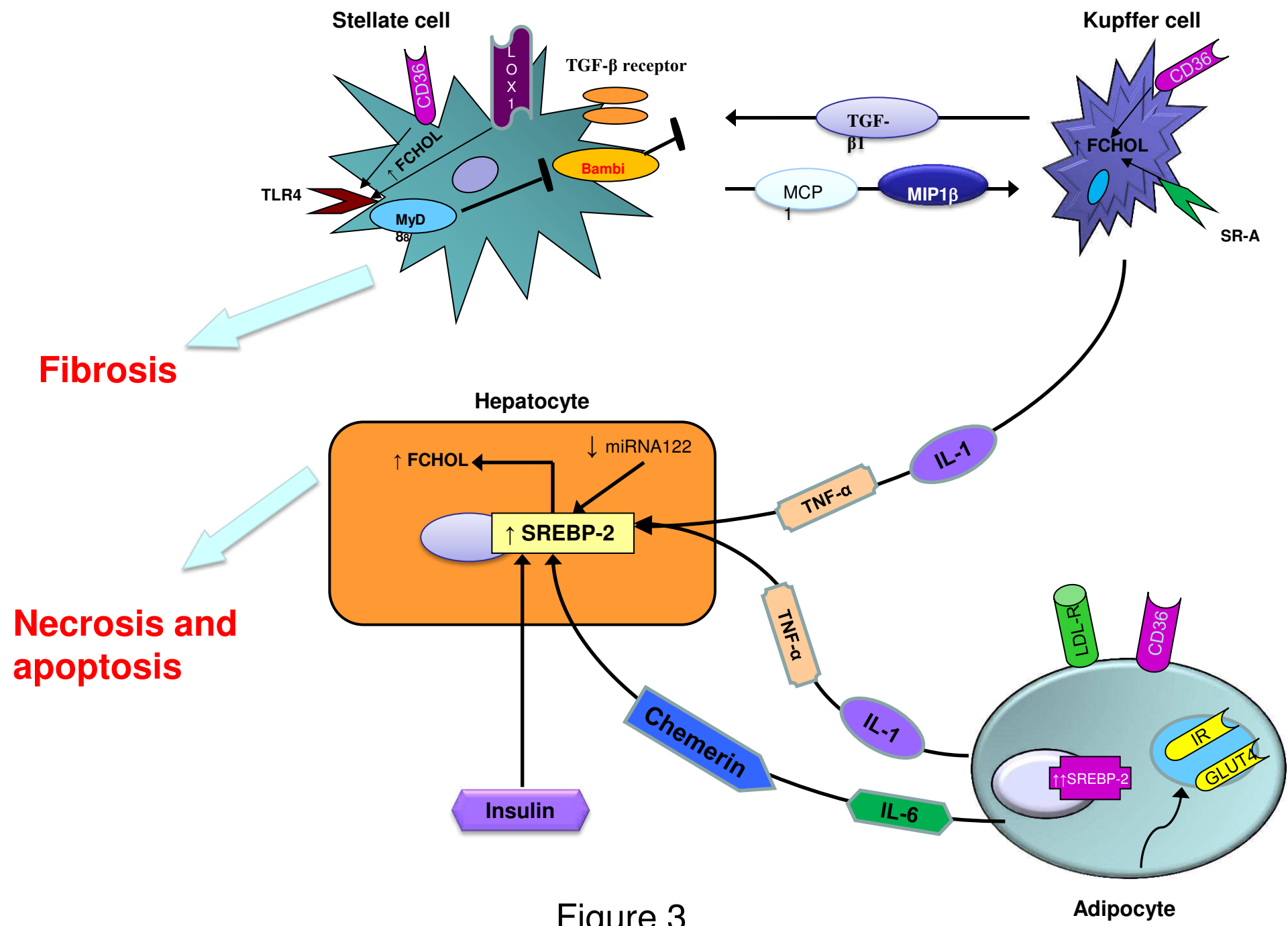
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CD36
↑FCHOL
SR-A
Fibrosis
Hepatocyte
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TNF-α
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Necrosis and
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Insulin
LDL-R
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GLUT4
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Figure 3

Table 1. Mechanisms of cellular cholesterol homeostasis and their potential implications in NAFLD pathogenesis

Cholesterol synthesis				
Factor	**Localization**	**Regulators**	**Function**	**Biological effect**
HMGCoAR	Hepatocyte ER	SREBP-2 ↑ HMGCoAR gene transcription miRNA34a: ↑ HMGCoAR dephosporylation and activation	catalyzes the conversion of HMGCoA to mevalonate, rate-limiting step in cholesterol biosynthesis	HMGCoAR expression and activity in hepatocytes are inappropriately elevated in NAFLD and correlate with the severity of liver histology[68]
Squalene synthase	Hepatocyte ER	SREBP-2 ↑ squalene synthase gene transcription	catalyzes the synthesis of squalene from farnesyl-PP, downstream the mevalonate step	Opposite from HMGCoAR inhibition, squalene synthase inhibition causes the accumulation of isoprenoid intermediates (isopentenyl-PP, farnesyl-PP), precursors for signaling molecules regulating protein synthesis, mitochondrial respiration (ubiquinone, coenzyme Q10), and glycosylation
Cholesterol-rich lipoprotein uptake				
Factor	**Localization**	**Regulators**	**Function**	**Biological effect**
LDL receptor	Transmembrane receptor in hepatocytes and macrophages	SREBP-2 ↑ LDL receptor expression	Catalyzes the uptake of circulating LDLs	LDL receptor is inappropriately overexpressed in NAFLD and correlates with the severity of liver histology[11, 14, 15, 68, 112]
Cluster differentiation protein 36 (CD36)	Hepatocytes, macrophages, HSC, adipocytes	Insulin ↑ CD36 translocation to PM LXRα, PXR, PPAR-γ:↑ CD36 GENE its transcription in the liver[74]	Transmembrane multifunctional receptor for different ligands: oxLDLs, VLDL, collagen type I and IV, free fatty acids	Hepatocyte CD36 membrane expression is increased and correlates with steatosis and insulin resistance in NAFLD patients[73]. The hepatic CD36 deletion reversed NASH [48, 49, 74]
lectin-like oxidized LDL receptor-1(LOX-1)	HSCs, adipocytes, endothelial cells	PPAR-γ, curcumin: ↓ LOX-1 expression Wnt signaling: ↑ LOX-1 expression [52]	Scavenger receptor for oxLDLs in HSCs	Upon oxLDL-binding, LOX-1 triggers HSC-mediated fibrogenesis([52] SNP in LOX-1 correlated with the risk of severity of NASH in humans [53]

Factor	Localization	Regulators	Function	Biological effect
scavenger receptor class B type I (SR-BI)	Hepatocytes (both sinusoidal and canalicular membrane), macrophages	SREBP-2: ↓ SR-BI gene expression	mediates the bi-directional, ATP-independent transfer of cholesterol from HDL to hepatocytes and from hepatocytes to bile, increasing reverse cholesterol transport and excretion into bile	SR-BI is down-regulated in animal models of NASH[14]
Intracellular cholesterol transport				
Factor	**Localization**	**Regulators**	**Function**	**Biological effect**
Steroidogenic acute regulatory protein (StAR)	Endosomes and lysosomes of hepatocytes and steroidogenic cells	SREBP-2: ↑ StARD4 expression ER stress: ↑ StARD5 expression[77]	↑ cholesterol transport from LE/LY to outer membrane of mitochondria and from the outer to the inner membrane of mitochondria	Hepatic StAR expression was increased 7- and 15-fold in steatosis and NASH patients, respectively[11], thereby enhancing mitochondrial free cholesterol accumulation and toxicity
Niemann-Pick C1 (NPC1) and NPC2 proteins	LE/LY of a broad range of tissues	miRNA33a: ↓ NPC1 gene expression[109] NPC1L1: ↓ NPC2 at posttranslational level	Delivery of late endosome/lysosome free cholesterol to ER and other cellular compartments.	Heterozygous NPC1/2 deficiency leads to fatty liver, obesity and metabolic syndrome[82] NPC1 gene is linked to to early-onset and morbid adult obesity in Europeans [83]
Oxysterol-binding proteins (OSBPs) and OSBP-related proteins (ORPs)	Cytosol of hepatocytes and of broad range of tcells	miRNA143: ↓ ORP8 in obesity[87]	Ccholesterol and oxysterol-binding proteins involved in nonvesicular cholesterol delivery from LE/LY, cholesterol transport between the ER and PM and inctracellular signalling.	ORP8 ↓ hepatic cholesterol and triglyceride accumulation by inhibiting activation of SREBP-2 and SREBP-1c ORP8 ↑ hepatic insulin sensitivity through an AKT-mediated mechanism[86, 87]
Cholesterol absorption and secretion				
Factor	**Localization**	**Regulators**	**Function**	**Biological effect**
Niemann-Pick C1-like 1 (NPC1L1)	Hepatocytes c(analicular membrane), enterocytes (apical membrane)	SREBP-2, HNF-1α : ↑ gene transcription LXR-α, PPAR-α, PPAR-δ: ↓ gene transcription	NPC1L1 mediates the uptake of free cholesterol from the bile by hepatocytes and from gut lumen by enterocytes INPC1L1 ↓ NPC2 activity, promoting LE/LY cholesterol overload [91]	NPC1L1 inhibition by ezetimibe reduces hepatic and intestinal free cholesterol absorption, reduces cellulat cholesterol overload, mitochondrial ROS generation and ER stress, improving NASH in animals and humans[17-21, 92-95]

Factor	Localization	Regulators	Function	Biological effect
ATP-binding cassette transporter A1(ABCA1)	Hepatocytes, enterocytes, macrophages	miRNA33a: ↓ ABCA1 gene transcription[109] OSBPs, UFA : ↓ ABCA1 activity by accelerating protein degradation[102] LXR-α. ↑ ABCA1 gene transcription apoA-1: ↑ ABCA1 protein stability	In hepatocytes, ABCA1 catalyzes cholesterol excretion to apoA-I to form HDL	Complete ABCA1 loss of function causes Tangier disease Hepatocyte ABCA1 expression is reduced in NASH patients[68] Modulation of its activity affects the development of NASH in cellular and animal models[102]
ATP-binding cassette transporter G5/G8 (ABCG5/G8)	Hepatocytes, enterocytes, macrophages	LXR-α, HNF-4α, FXR: ↑ gene Transcription[110, 111, 116]	ABGC5 and ABCG8 form an heterodimer (ABCG5/G8) on the apical membrane of enterocytes and on the canalicular membrane of hepatocytes to excrete sterols into the intestinal lumen and the bile	ABCG5/G8 expression is reduced in animal and human NASH[14, 68]
Intracellular esterification and de-esterification				
Factor	**Localization**	**Regulators**	**Function**	**Biological effect**
acyl-CoA cholesterol:cholesteryl transferase (ACAT)-2	ER of hepatocytes, enterocytes and macrophages	HNF1α, UFA, cholesterol : ↑ ACAT-2 activity	Rate-limiting enzyme for free cholesterol esterification to cholesteryl ester	It promotes cholesterol excretion in the presence of an intact ABC transporter machinery. In NAFLD, hepatocyte ACAT-2 activity is mildly increased[11, 14] unchanged[68] , or reduced[105]
cholesteryl ester hydrolase (CEH)	ER of hepatocytes, enterocytes and macrophages	PPAR-α, PPAR-γ: ↓ CEH gene transcription LXR-α: ↑ CEH gene transcription	ER enzyme that hydrolyzes cholesteryl ester to free cholesterol	It promotes cholesterol excretion in the presence of intact ABC transporters; it enhances free cholesterol accumulation if cellular cholesterol export machinery is impaired In NAFLD patients, hepatocyte CEH activity is inappropriately elevated[68]

Intracellular metabolism to oxysterols and bile acids				
Factor	**Localization**	**Regulators**	**Function**	**Biological effect**
Sterol 7α hydroxylase (CYP7A1)	microsomal cytochrome P450 expressed only in hepatocytes	Bile acids, insulin, SHP: ↓ transcription of CYP7A1 LXRα, FXR, HNF-4α: ↑ transcription of CYP7A1	CYP7A1 catalyzes the 7α-hydroxylation of cholesterol to 7α-hydroxycholesterol (7OHC), the rate-limiting step in the neutral pathway to bile acid synthesis	CYP7A activity is reduced and its activity is inversely related to histological severity in NASH [14, 68, 105] If excess 7OHC is excreted by hepatocytes through ABCG5/G8 transporters , it can directly activate hepatic macrophages and HSCs to secrete proinflammatory and profibrogenic cytokines[38]
Sterol 27-hydroxylase (CYP27A1) and in macrophages	mitochondrial cytochrome P-450 with broad tissue distribution.:hepatocytes, endothelium, fibroblasts, macrophages	Bile acids, insulin, LPS, IL-1, : ↓ transcription of CYP27A1 HNF-1α: ↑ transcription of CYP27A1	CYP27A1 catalizes the "alternative" (or acidic) pathway of bile acids synthesis, leading to 27-hydroxycholesterol (27OHC) 27OHC : ↓ SREBP-2-activation and ↑ LXR-α-mediated transcription of ABC transporters, thereby promoting cholesterol excretion	CYP27A1 activity is reduced and its activity is inversely related to histological severity in NASH [14, 68, 105] 27OHC administration reduced cholesterol accumulation in Kupffer cells and improved hepatic inflammation in NASH[44]
Sterol 25-hydroxylase	microsomal diiron cofactor enzyme, not a cytochrome P450 family, with broad tissue distribution.	LPS/TLR-4 pathway ↑ 25OHC synthesis	It catalyzes cholesterol 25-hydroxylation to 25-hydroxycholesterol (25OHC). 25OHC enhances lipogenesis via LXRα/SREBP-1c activation and inflammatory response via NF-κB activation, leading to NASH[39, 41]	Although a quantitatively minor precursor of bile acids, 25OHC is a potent regulator of lipid metabolism and inflammatory response. 25OHC levels are increased in NAFLD patients and correlate with insulin resistance[38] 25OHC promotes lipogenesis and inflammation, leading to NASH experimentally[39-41]
Hydroxysteroid sulfotransferase (SULT2B1b)	Cytosolic enzyme Broad tissue distribution.	cAMP ↑ SULT2B1b expression	Catalyzes 25OHC sulfation to 25-hydroxycholesterol-3-sulfate (25OHC3S)	25OHC3S had opposite effects than 25OHC on lipid metabolism and inflammation and improved NAFLD in cellular and mouse models([39-41] Enhancement of on of SULT2B1b activity may counteract effects of toxic oxysterols

Abbreviations: ABC. ATP-binding cassette; ER: endoplasmic reticulum; FXR: farnesoid X-receptor; HMGCoAR: 3-hydroxy-3-methyl-glutaryl-CoA reductase; HNF: hepatocyte nuclear factor:; HSC: hepatic stellate cell; IL-1: interleukin-1; LDL: low density lipoproteins; LE: late endosome; LY: lysosome; LPS: lipopolysaccharide, LXR: liver X-receptor; miRNA: microRNA; NPC1: Niemann-Pick C1; NPC2: Niemann-Pick C2; NPC1L1: Niemann-Pick C1-like 1; oxLDL: oxidized low density lipoproteins; PM: plasma membrane; PP: pyrophosphate; PPAR: peroxisome proliferator-activated receptor; PXR: pregnane X receptor; SHP: small heterodimeric partner; SNP: single nucleotide polymorphism; SREBP: sterol regulatory binding protein; TLR-4: toll-like receptor-4; UFA: unsaturated fatty acids; VLDL. Very low density lipoproteins;

Table 2 Nuclear regulators of cholesterol homeostasis potentially involved in NAFLD pathogenesis

SREBP-2			
Cells	**Modulators**	**Molecular targets**	**Biological effect**
Hepatocytes, adipocytes	Activators: IL-1b, IL-6, SAA [15], Insulin[111], PM cholesterol depletion[60], Inhibitors: miRNA122[69], 7-OHC, 27-OHC[109]	↑ LDL receptor ↑ HMG-CoAR, ↑ squalene synthase[60]	↑ extracellular cholesterol uptake ↑ cholesterol synthesis
		↑ NPC1L1	↑cholesterol reabsorption from the intestine and bile
		↑StARD4[77]	↑ cholesterol transport to mitochondria
		↓ scavenger receptor class B type I (SR-BI)[14]	↓ reverse cholesterol transport and elimination into bile
adipocytes		↑ secretion of proinflammatory adipokines (angiotensinogen, TNF-α, IL-6, chemerin[60, 65]	Chronic low-grade nflammation Insulin resistance

miRNA33a			
Cells	**Modulators**	**Molecular targets**	**Biological effect**
Hepatocytes, adipocytes	**Same as SREBP-2**	↓ ABCA1[109]	↓ cholesterol efflux
		↓ NPC-1[109]	Cholesterol accumulation in LE/LY
		↓ mitochondrial trifunctional protein hydroxyacyl-CoA dehydrogenase/ 3-ketoacyl-CoA thiolase/enoyl-CoA hydratase βsubunit (HADHB); ↓carnitine palmitoyltransferase 1A (CPT1A); ↓ carnitine O-octanoyltransferase (CROT) [110]	↓ mitochondrial fatty acid β-oxidation

Farnesoid X receptor (FXR)			
Cells	**modulators**	**Molecular targets**	**Biological effect**

Hepatocytes, Adipocytes, macrophages, enterocytes	Activators: Natural bile acids (chenodeoxycholic acid), semisynthetic bile acids (obeticholic acid), PUFA (arachidonic, linolenic, or docosahexaenoic), synthetic agonists(GW4064, 6α-ECDA, fexaramine)	↑ CYP7A1, ↑ ABCG5/G8[115]	↑ bile acid synthesis and cholesterol excretion into bile
		↓ SREBP-1c-mediated lipogenesis (SHP-dependent and -independent mechanisms) [115] ↑ PPAR-α-mediated activation of fatty acid β-oxidation [115] ↓ ApoC-III and apoA-1 synthesis ↑ ApoC-II synthesis and VLDL-receptor expression ↓ hepatic lipase activity[115]	↓ liver triglyceride ↓ secretion and ↑ clearance of VLDL, thereby reducing plasma triglycerides ↓ synthesis and uptake of HDL-C, with a net reduction in plasma HDL-C levels
		↓ hepatic expression of gluconeogenetic enzymes PEPCK, G6-Pase and fructose-1,6-biphosphatase ↑ IRS-1 phosphorylation and coupling with the PI-3K activity[115]	↓ hepatic gluconeogenesis ↑ hepatic insulin sensitivity
		↑Intestinal expression of antibacterial factors such as angiogenin, inducible nitric oxide synthase (iNOS) and IL18[115]	Maintains gut integrity and impermeability to bacterial endotoxins
		↓ hepatic stellate cell expression of collagen α(1)I, α-smooth muscle actin, tissue inhibitors of metalloproteinase 1 and 2 and TGF-β1 genes[117]	↓ hepatic fibrogenesis[117, 118]
Liver X receptor(LXR)-α			
Cells	**modulators**	**Molecular targets**	**Biological effect**
Hepatocytes, enterocytes,	Activators: glucose[119]	↑ transcrioption of CYP7A1, the rate-limiting enzyme of bile acid synthesis	↑ cholesterol conversion to bile acids

macrophages	natural oxysterols 22(R)OHC, 24(S)OHC, 27OHC, 24(S), 25OOC Synthetic agonists GW3965, T0901317 Inhibitors: natural oxysterol 25OHC3S , Synthetic oxysterol 22(S)OHC [40-42]	↓NPC1L1 [114, 120]	↓ cholesterol reabsorption from the intestine and bile
		↑ macrophage, intestinal and hepatic ABCG5/G8[120]	↑ cholesterol reverse transport and excretion into bile and gut lumen
		↑ macrophage and hepatic ABCA1[120]	↑ cholesterol efflux to acceptor apoa-I to form HDL-C
		↑ hepatic CEH[120]	↑ FC availability for ABC transporters→↑ cellular free cholesterol efflux
		↑ CD36 hepatocyte expression[74]	↑ uptake of plasma oxLDLs and FFAs
		↑ LDLR ubiquitination and degradation[121]	↓ uptake of plasma LDL
		↑ activation of sterol regulatory element-binding protein-c (SREBP-c), carbohydrate response element-binding protein (ChREBP) and their target enzymes fatty acid synthase(FAS), acyl coenzyme A carboxylase (ACC), and stearoyl CoA desaturase 1 (SCD-1) [124, 125]	↑ hepatic lipogenesis, steatosis and large VLDL secretion
		↑ secretion of angiopoietin-like protein 3 (Angptl3), an inhibitor of lippprotein lipase (LPL)[126]	↓ VLDL catabolism
		↓ apoA-V secretion[127]	↓ VLDL catabolism
Macrophages Kupffer cells		↓ activation and secretion of proinflammatory cytokines[13, 124]	↓ hepatic inflammation in NASH
		Maintains gut integrity and impermeability to bacterial endotoxins[122]	↓ endotoxin-mediated liver injury

Hepatic stellate cells		↓ HSC activation and fibrogenesis[123]	↓ hepatic fibrosis in NASH

Abbreviations: ABC. ATP-binding cassette; CYP7A1: Sterol 7α hydroxylase; 27OHC: 27-hydroxycholesterol; 7OHC: 7-hydroxycholesterol; 22(R)OHC: 22(R)-hydroxycholesterol, 24(S): 24(S)-hydroxycholesterol,

27OHC: 27-hydroxycholesterol 24(S), 25OOC: 24(S), 25-epoxycholesterol 25OHC3S:25-hydroxycholesterol-3-sulfate , 22(S)OHC: 22(S)-hydroxycholesterol;

SAA: serum amyloid A protein; LE: late endosome; LY: lysosome;

PM: plasma membrane; IL: interleukin; HMGCoAR: 3-hydroxy-3-methyl-glutaryl-CoA reductase; NPC1, NPC2: Niemann-Pick C1, C2; NPC1L1: Niemann-Pick C1-like 1; oxLDL: oxidized low density lipoproteins; PPAR: peroxisome proliferator-activated receptor; SHP: small heterodimeric partner; StARD4: Steroidogenic acute regulatory protein D4; LE/LY: late endosomes/lysosomes; PEPCK: phosphoenol-pyruvate carboxykinase; IRS-1: insulin-receptor substrate-1; TGF: transforming growth factor; CEH: cholesteryl ester hydrolase; LDLR: low density lipoprotein receptor; FFA: free fatty acids; HSC:hepatic stellate cells; VLDL: very low density lipoproteins