

AperTO - Archivio Istituzionale Open Access dell'Università di Torino

Cholesterol metabolism and the pathogenesis of non-alcoholic steatohepatitis.

This is the author's manuscript

Original Citation:

Availability:

This version is available <http://hdl.handle.net/2318/130708> since

Published version:

DOI:10.1016/j.plipres.2012.11.002

Terms of use:

Open Access

Anyone can freely access the full text of works made available as "Open Access". Works made available under a Creative Commons license can be used according to the terms and conditions of said license. Use of all other works requires consent of the right holder (author or publisher) if not exempted from copyright protection by the applicable law.

(Article begins on next page)



UNIVERSITÀ DEGLI STUDI DI TORINO

This Accepted Author Manuscript (AAM) is copyrighted and published by Elsevier. It is posted here by agreement between Elsevier and the University of Turin. Changes resulting from the publishing process - such as editing, corrections, structural formatting, and other quality control mechanisms - may not be reflected in this version of the text. The definitive version of the text was subsequently published in [Prog Lipid Res. 2013 Jan;52(1):175-91. doi: 10.1016/j.plipres.2012.11.002. Epub 2012 Dec 1. Review].

You may download, copy and otherwise use the AAM for non-commercial purposes provided that your license is limited by the following restrictions:

- (1) You may use this AAM for non-commercial purposes only under the terms of the CC-BY-NC-ND license.
- (2) The integrity of the work and identification of the author, copyright owner, and publisher must be preserved in any copy.
- (3) You must attribute this AAM in the following format: Creative Commons BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/deed.en>), [doi: 10.1016/j.plipres.2012.11.002]

Cholesterol metabolism and the pathogenesis of non-alcoholic steatohepatitis

RUNNING TITLE: cholesterol and NASH

Giovanni Musso, M.D.¹, Roberto Gambino, Ph.D.^{2*}, Maurizio Cassader, Ph.D.²

¹Gradenigo Hospital, Turin, Italy

²Department of Medical Sciences, University of Turin, Italy

*equal first author

Corresponding author:

Giovanni Musso

Gradenigo Hospital, Turin

C.^{so} Regina Margherita 8

10132 Turin, Italy

E-mail: giovanni_musso@yahoo.it

Keywords: NAFLD, NASH, lipotoxic, free cholesterol, cholesterol homeostasis, ER stress

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 steatohepatitis (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.

Contents

1. Introduction

2. Mechanisms of cholesterol toxicity in NASH

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

2.2 Cholesterol-induced ER stress

2.3 Generation of toxic oxysterols

2.4 Cholesterol-induced hepatic Kupffer cells and HSC activation

2.5 Adipocyte cholesterol imbalance and adipose tissue dysfunction

3. Dysregulated hepatic cholesterol homeostasis in NASH

3.1 increased cholesterol synthesis

3.2 increased cholesterol-rich lipoprotein uptake

3.3 altered intracellular cholesterol transport and compartmentalization

3.3.1 Steroidogenic acute regulatory (StAR) transfer proteins

3.3.2 Niemann-Pick C1 (NPC1) and NPC2 proteins

3.3.3 Oxysterol-binding proteins and OSBP-related proteins

3.4 altered cholesterol absorption and secretion

3.4.1 Niemann-Pick C1 like 1 protein

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

3.5 altered intracellular cholesterol esterification and de-esterification

3.6 Nuclear regulators of cholesterol homeostasis

3.6.1 Sterol regulatory binding protein(SREBP)-2

3.6.2 Farnesoid X receptor

3.6.3 Liver X receptor(LXR)- α

4. Future perspectives

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 synthesis⁶ and human hypobetalipoproteinemia, which does not progress to cirrhosis despite massive steatosis⁷, 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 storsers”, while subjects who are unable to synthesize neutral lipids accumulate 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 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 ($\text{C7}\alpha$, 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]. Oxysterols 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 LXR α 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-ketocholesterol (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)- β 1, 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)- κ B, 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- β 1), 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(Figure 1-3)[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 maintained 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 developed 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, heterozygous 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 effectively 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: NPC1L1 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- α 1 (PPAR- α 1) 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 need 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 cell 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

Vertebrate 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: LXR α is highly expressed in the liver, adipose tissue and macrophages, whereas LXR β 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 transcription 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 LXR α 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, LXR α has a drawback: LXR α 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.

Disclosures.

No author has any present or past conflict of interest or financial relationship to disclose.

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 enhances the generation of ROS in response to TNF- α and Fas ligand (FasL), causing cardiolipin (CL) peroxidation (CLOOH), which destabilizes the mitochondrial 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 Ca^{2+} concentration is necessary for maintenance of normal ER function and protein folding ability. This high Ca^{2+} concentration of ER is actively maintained by the sarco(endo)plasmic reticulum Ca^{2+} -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 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 (nonvesicular 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 in 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 2 α kinase (PERK), inositol-requiring kinase-1 α (IRE-1 α), 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 7 α hydroxylase (CYP7A1) catalyzes the 7 α -hydroxylation of cholesterol to 7 α -hydroxycholesterol (7OHC), the rate limiting step in the neutral pathway to bile acid synthesis, while the mitochondrial sterol 27-hydroxylase (CYP27A1) catalyzes 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 LXR α /SREBP-1c-mediated lipogenesis, further enhancing free fatty acid (FFA) accumulation and toxicity, and inflammatory response via NF- κ B 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- κ B-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)- β 1, 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- β 1 with negative regulatory function. The removal of this inhibitor sensitizes HSC to activation by TGF- β 1 and secretion of chemotactic factors monocyte chemotactic protein-1 (MCP-

1) and macrophage inflammatory protein 1 β (MIP1 β), 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.

REFERENCES

- 1 Chalasani N, Younossi Z, Lavine JE, Diehl AM, Brunt EM, Cusi K, et al. The diagnosis and management of non-alcoholic fatty liver disease: Practice guideline by the american association for the study of liver diseases, American College of Gastroenterology, and the American Gastroenterological Association *Hepatology* 2012; 55: 2005-23

- 2 Charlton MR, Burns JM, Pedersen RA, Watt KD, Heimbach JK, Dierkhising RA. Frequency and outcomes of liver transplantation for nonalcoholic steatohepatitis in the United States. *Gastroenterology*. 2011;141:1249-53

- 3 Musso G, Gambino R, Cassader M, Pagano G. Meta-analysis: Natural history of non-alcoholic fatty liver disease (NAFLD) and diagnostic accuracy of non-invasive tests for liver disease severity. *Ann Med*. 2011;43: 617-49

- 4 Tilg H, Moschen AR. Evolution of Inflammation in Nonalcoholic Fatty Liver Disease: The Multiple Parallel Hits Hypothesis. *Hepatology* 2010; 52:1836-1846

- 5 Neuschwander-Tetri BA. Hepatic lipotoxicity and the pathogenesis of nonalcoholic steatohepatitis: the central role of nontriglyceride fatty acid metabolites. *Hepatology* 2010; **52**: 774–88

- 6 Yamaguchi K, Yang L, McCall S, et al. Inhibiting triglyceride synthesis improves hepatic steatosis but exacerbates liver damage and fibrosis in obese mice with non-alcoholic steatohepatitis. *Hepatology* 2007; **45**: 1366–74

- 7 Peretti N, Sassolas A, Roy CC, et al.. Guidelines for the diagnosis and management of chylomicron retention disease based on a review of the literature and the experience of two centers. *Adv Clin Chem*. 2011;54:81-107

-
- 8 Ioannou GN, Morrow OB, Connole ML, et al. Association between dietary nutrient composition and the incidence of cirrhosis or liver cancer in the United States population. *Hepatology* 2009;50: 175–184.
- 9 Musso G, Gambino R, De Michieli F, et al. Dietary habits and their relations to insulin resistance and postprandial lipemia in nonalcoholic steatohepatitis. *Hepatology*. 2003;37: 909-16
- 10 Yasutake K, Nakamuta M, Shima Y, et al. Nutritional investigation of non-obese patients with non-alcoholic fatty liver disease: the significance of dietary cholesterol. *Scand J Gastroenterol*. 2009;44: 471-7.
- 11 Caballero F, Fernández A, De Lacy AM, et al. Enhanced free cholesterol, SREBP-2 and StAR expression in human NASH. *J Hepatology* 2009; 50:789-796
- 12 Puri P, Baillie RA, Wiest MM, et al. A lipidomic analysis of nonalcoholic fatty liver disease. *Hepatology*. 2007 ;46:1081-90.
- 13 Wouters K, van Bilsen M, van Gorp PJ et al. Intrahepatic cholesterol influences progression, inhibition and reversal of non-alcoholic steatohepatitis in hyperlipidemic mice. *FEBS Lett* 2010; 584: 1001–5.
- 14 Van Rooyen DM, Larter CZ, Haigh WG, et al. Hepatic free cholesterol accumulates in obese, diabetic mice and causes nonalcoholic steatohepatitis. *Gastroenterology*. 2011;141:1393-403.
- 15 Zhao L, Chen Y, Tang R, et al. Inflammatory stress exacerbates hepatic cholesterol accumulation via increasing cholesterol uptake and de novo synthesis. *J Gastroenterol Hepatol*. 2011; 26:875-83.
- 16 Deushi M, Nomura M, Kawakami A, et al. Ezetimibe improves liver steatosis and insulin resistance in obese rat model of metabolic syndrome. *FEBS Lett*. 2007;581:5664-70
- 17 Zheng S, Hoos L, Cook J, et al. Ezetimibe improves high fat and cholesterol diet-induced non-alcoholic fatty liver disease in mice. *Eur J Pharmacol*. 2008; 584: 118-24.

-
- 18 Nozaki Y, Fujita K, Yoneda M, et al. Long-term combination therapy of ezetimibe and acarbose for non-alcoholic fatty liver disease. *J Hepatol*. 2009;51:548-56.
- 19 Chan DC, Watts GF, Gan SK, et al. Effect of ezetimibe on hepatic fat, inflammatory markers and apolipoprotein B-100 kinetics in insulin-resistant obese subjects on a weight loss diet. *Diabetes Care*. 2010; 33:1134-9
- 20 Takeshita Y, Takamura T, Kita Y, et al. Efficacy of ezetimibe for the treatment of non-alcoholic fatty liver disease: a randomized controlled trial. *J Hep* 2011; 54: S346
- 21 Park H, Hasegawa G, Shima T, et al. The fatty acid composition of plasma cholesteryl esters and estimated desaturase activities in patients with nonalcoholic fatty liver disease and the effect of long-term ezetimibe therapy on these levels. *Clin Chim Acta*. 2010; 411: 1735-40.
- 22 Athyros VG, Tziomalos K, Gossios TD, et al. Safety and efficacy of long-term statin treatment for cardiovascular events in patients with coronary heart disease and abnormal liver tests in the Greek Atorvastatin and Coronary Heart Disease Evaluation (GREACE) Study: a post-hoc analysis. *Lancet*. 2010; 376:1916-22.
- 23 Yoneda M, Fujita K, Nozaki Y, et al. Efficacy of ezetimibe for the treatment of non-alcoholic steatohepatitis: an open-label, pilot study. *Hepatol Res* 2010;40:613–621.
- 24 Tabas I. Consequences of cellular cholesterol accumulation: basic concepts and physiological implications. *J Clin Invest* 2002; 110: 905-11
- 25 Montero J, Mari M, Colell A, et al. Cholesterol and peroxidized cardiolipin in mitochondrial membrane properties, permeabilization and cell death. *Biochim Biophys Acta*. 2010;1797: 1217-24
- 26 Coll O, Colell A, García-Ruiz C, et al. Sensitivity of the 2-oxoglutarate carrier to alcohol intake contributes to mitochondrial glutathione depletion, *Hepatology* 2003; 38; 692–702
- 27 Marí M, Caballero F, Colell A, et al. Mitochondrial free cholesterol loading sensitizes to TNF- and Fas-mediated steatohepatitis. *Cell Metab*. 2006; 4:185-98.
- 28 Mei S, Gu H, Yang X, et al. Prolonged Exposure to Insulin Induces Mitochondrion-Derived Oxidative Stress through Increasing Mitochondrial Cholesterol Content in Hepatocytes. *Endocrinology*. 2012; 153: 2120-9.

-
- 29 Fu S, Watkins SM, Hotamisligil GS. The role of endoplasmic reticulum in hepatic lipid homeostasis and stress signaling. *Cell Metab.* 2012;15: 623-34.
- 30 Zha BS, Zhou H. ER Stress and Lipid Metabolism in Adipocytes. *Biochem Res Int.* 2012;2012:312943.
- 31 Puri P, Mirshahi F, Cheung O, et al. Activation and dysregulation of the unfolded protein response in nonalcoholic fatty liver disease. *Gastroenterology.* 2008;134: 568-76
- 32 Gentile CL, Frye M, Pagliassotti MJ. Endoplasmic reticulum stress and the unfolded protein response in nonalcoholic fatty liver disease. *Antioxid Redox Signal.* 2011;15: 505-21
- 33 Fu S, Yang L, Li P, et al. Aberrant lipid metabolism disrupts calcium homeostasis causing liver endoplasmic reticulum stress in obesity. *Nature.* 2011;473:528-31
- 34 Park SW, Zhou Y, Lee J, et al. Sarco(endo)plasmic reticulum Ca²⁺-ATPase 2b is a major regulator of endoplasmic reticulum stress and glucose homeostasis in obesity. *Proc Natl Acad Sci U S A.* 2010;107:19320-5.
- 35 Li L, Hossain MA, Sadat S, et al. Lecithin cholesterol acyltransferase null mice are protected from diet-induced obesity and insulin resistance in a gender-specific manner through multiple pathways. *J Biol Chem.* 2011; 286:17809-20.
- 36 Hager L, Li L, Pun H, et al. Lecithin:Cholesterol acyltransferase deficiency protects against cholesterol-induced hepatic endoplasmic reticulum stress in mice. *J Biol Chem.* 2012 Apr 12. [Epub ahead of print] PMID:22500017
- 37 Garenc C, Julien P, Levy E. Oxysterols in biological systems: the gastrointestinal tract, liver, vascular wall and central nervous system. *Free Radic Res.* 2010;44: 47-73.

-
- 38 Ferré N, Martínez-Clemente M, López-Parra M, et al. Increased susceptibility to exacerbated liver injury in hypercholesterolemic ApoE-deficient mice: potential involvement of oxysterols. *Am J Physiol Gastrointest Liver Physiol*. 2009;296:G553-62.
- 39 Ikegami T, Hyogo H, Honda A, et al. Increased serum liver X receptor ligand oxysterols in patients with non-alcoholic fatty liver disease. *J Gastroenterol*. 2012 May 9. [Epub ahead of print] PMID: 22569763
- 40 Xu L, Bai Q, Rodriguez-Agudo D, et al. Regulation of hepatocyte lipid metabolism and inflammatory response by 25-hydroxycholesterol and 25-hydroxycholesterol-3-sulfate. *Lipids*. 2010 ;45:821-32.
- 41 Ren S, Li X, Rodriguez-Agudo D, et al. Sulfated oxysterol, 25HC3S, is a potent regulator of lipid metabolism in human hepatocytes. *Biochem Biophys Res Commun*. 2007;360:802-8.
- 42 Bai Q, Zhang X, Xu L, et al. Oxysterol sulfation by cytosolic sulfotransferase suppresses liver X receptor/sterol regulatory element binding protein-1c signaling pathway and reduces serum and hepatic lipids in mouse models of nonalcoholic fatty liver disease. *Metabolism*. 2012 Jan 5. [Epub ahead of print] PMID: 22225954
- 43 Hessvik NP, Bakke SS, Smith R, et al. The liver X receptor modulator 22(S)-hydroxycholesterol exerts cell-type specific effects on lipid and glucose metabolism. *J Steroid Biochem Mol Biol*. 2012;128:154-64
- ⁴⁴Walenbergh S, Bieghs V, Hendriks T, et al. 27-Hydroxycholesterol: a potential treatment for non-alcoholic steatohepatitis in mice. *Hepatology* 2012; 56: 862A
- 45 You Q, Cheng L, Kedl RM, Ju C. Mechanism of T cell tolerance induction by murine hepatic Kupffer cells. *Hepatology*. 2008;48:978-90.
- 46 Leroux A, Ferrere G, Godie V, et al. Toxic lipids stored by Kupffer cells correlates with their pro-inflammatory phenotype at an early stage of steatohepatitis. *J Hepatol*. 2012 Mar 14. [Epub ahead of print] PMID: 22425624

47 Musso G, Gambino R, De Michieli F, et al. Association of liver disease with postprandial large intestinal triglyceride-rich lipoprotein accumulation and pro/antioxidant imbalance in normolipidemic non-alcoholic steatohepatitis. *Ann Med*. 2008;40:383-94.

48 Bieghs V, Wouters K, van Gorp PJ, et al. Role of scavenger receptor A and CD36 in diet-induced nonalcoholic steatohepatitis in hyperlipidemic mice. *Gastroenterology*. 2010;138:2477-86,

49 Bieghs V, Verheyen F, van Gorp PJ, et al. Internalization of modified lipids by CD36 and SR-A leads to hepatic inflammation and lysosomal cholesterol storage in Kupffer cells. *PLoS One*. 2012;7:e34378.

50 Bieghs V, van Gorp PJ, Walenbergh S, et al. Specific immunization strategies against oxidized LDL: A novel way to reduce non-alcoholic steatohepatitis in mice. *Hepatology*. 2012 Feb 15. doi: 10.1002/hep.25660

51 Teratani T, Tomita K, Suzuki T, et al. A high-cholesterol diet exacerbates liver fibrosis in mice via accumulation of free cholesterol in hepatic stellate cells. *Gastroenterology*. 2012 ;142:152-164

52 Kang Q, Chen A. Curcumin eliminates oxidized LDL roles in activating hepatic stellate cells by suppressing gene expression of lectin-like oxidized LDL receptor-1. *Lab Invest*. 2009; 89:1275-90

53 Musso G, Cassader M, De Michieli F, et al. Effect of lectin-like oxidized LDL receptor-1 polymorphism on liver disease, glucose homeostasis, and postprandial lipoprotein metabolism in nonalcoholic steatohepatitis. *Am J Clin Nutr*. 2011;94:1033-42.

54 Duval C, Thissen U, Keshtkar S, et al. Adipose Tissue Dysfunction Signals Progression of Hepatic Steatosis Towards Nonalcoholic Steatohepatitis in C57Bl/6 Mice Diabetes. 2010; 59: 3181–91

-
- 55 Musso G, Cassader M, De Michieli F, et al. Nonalcoholic steatohepatitis versus steatosis: Adipose tissue insulin resistance and dysfunctional response to fat ingestion predict liver injury and altered glucose and lipoprotein metabolism. *Hepatology*. 2012 Jun 8. doi: 10.1002/hep.25739.
- 56 Murphy S, Martin S, Parton RG. Lipid droplet–organelle interactions; sharing the fats. *Biochim. Biophys. Acta*. 2009; 1791:441–447.
- 57 Pilch PF, Meshulam T, Ding S, Liu L. Caveolae and lipid trafficking in adipocytes. *Clin Lipidol*. 2011 ; 6: 49–58.
- 58 Hailstones D, Sleer LS, Parton RG, Stanley KK. Regulation of caveolin and caveolae by cholesterol in MDCK cells. *J. Lipid Res*. 1998; 39:369–379.
- 59 Yu BL, Zhao SP, Hu JR. Cholesterol imbalance in adipocytes: a possible mechanism of adipocytes dysfunction in obesity. *Obes Rev*. 2010;11:560-7
- 60 Le Lay S, Krief S, Farnier C, et al. Cholesterol, a cell size-dependent signal that regulates glucose metabolism and gene expression in adipocytes. *J Biol Chem* 2001; **276**: 16904–16910.
- 61 Parpal S, Karlsson M, Thorn H, et al. Cholesterol depletion disrupts caveolae and insulin receptor signaling for metabolic control via insulin receptor substrate-1, but not for mitogen-activated protein kinase control. *J Biol Chem*. 2001;276:9670-8
- 62 Breen MR, Camps M, Carvalho-Simoes F, et al. Cholesterol depletion in adipocytes causes caveolae collapse concomitant with proteosomal degradation of cavin-2 in a switch-like fashion. *PLoS One*. 2012;7: e34516
- 63 Sánchez-Wandelmer J, Dávalos A, Herrera E, et al. Inhibition of cholesterol biosynthesis disrupts lipid raft/caveolae and affects insulin receptor activation in 3T3-L1 preadipocytes. *Biochim Biophys Acta*. 2009;1788:1731-9.

-
- 64 Karlsson M, Thorn H, Danielsson A, et al. Colocalization of insulin receptor and insulin receptor substrate-1 to caveolae in primary human adipocytes. Cholesterol depletion blocks insulin signalling for metabolic and mitogenic control. *Eur J Biochem.* 2004;271: 2471-9.
- 65 Bauer S, Wanninger J, Schmidhofer S, et al.. Sterol regulatory element-binding protein 2 (SREBP2) activation after excess triglyceride storage induces chemerin in hypertrophic adipocytes. *Endocrinology.* 2011; 152:26-35.
- 66 Khan T, Hamilton MP, Mundy DJ, et al. Impact of Simvastatin on Adipose Tissue: Pleiotropic Effects in Vivo *Endocrinology.* 2009; 150: 5262–5272.
- 67 Bellia A, Rizza S, Lombardo MF, et al. Deterioration of glucose homeostasis in type 2 diabetic patients one year after beginning of statins therapy. *Atherosclerosis* 2012;223: 197-203.
- 68 Min HK, Kapoor A, Fuchs M, et al. Increased hepatic synthesis and dysregulation of cholesterol metabolism is associated with the severity of nonalcoholic fatty liver disease. *Cell Metab.* 2012;15:665-74.
- 69 Cheung O, Puri P, Eicken C et al. Nonalcoholic steatohepatitis is associated with altered hepatic MicroRNA expression. *Hepatology* 2008; 48: 1810–20
- 70 Musso G, Cassader M, Rosina F, et al. Impact of current treatments on liver disease, glucose metabolism and cardiovascular risk in non-alcoholic fatty liver disease (NAFLD): a systematic review and meta-analysis of randomised trials. *Diabetologia.* 2012;55: 885-904
- 71 Chalasani N, Guo X, Loomba R, et al.. Genome-Wide Association Study Identifies Variants Associated With Histologic Features of Nonalcoholic Fatty Liver Disease. *Gastroenterology* 2010;139:1567–76
- 72 Stein EA, Bays H, O'Brien D, et al Lapaquistat acetate: development of a squalene synthase inhibitor for the treatment of hypercholesterolemia. *Circulation* 2011; 123: 1974–85.

-
- 73 Miquilena-Colina ME, Lima-Cabello E, Sánchez-Campos S, et al. Hepatic fatty acid translocase CD36 upregulation is associated with insulin resistance, hyperinsulinaemia and increased steatosis in non-alcoholic steatohepatitis and chronic hepatitis C. *Gut* 2011; 60: 1394–1402.
- 74 Zhou J, Febbraio M, Wada T, et al. Hepatic fatty acid transporter Cd36 is a common target of LXR, PXR, and PPARgamma in promoting steatosis. *Gastroenterology*. 2008;134:556-67.
- 75 Wiersma H, Gatti A, Nijstad N, et al. Scavenger Receptor Class B Type I Mediates Biliary Cholesterol Secretion Independent of ATP-Binding Cassette Transporter g5/g8 in Mice. *Hepatology* 2009;50: 1263-72
- 76 Clark BJ. The mammalian START domain protein family in lipid transport in health and disease. *Journal of Endocrinology* 2012; 212: 257–275
- 77 Soccio RE, Adams RM, Maxwell KN, et al. Differential gene regulation of StarD4 and StarD5 cholesterol transfer proteins. Activation of StarD4 by sterol regulatory element-binding protein-2 and StarD5 by endoplasmic reticulum stress. *J Biol Chem*. 2005;280: 19410-8
- 78 Montero J, Morales A, Llacuna L, et al. Mitochondrial cholesterol contributes to chemotherapy resistance in hepatocellular carcinoma. *Cancer Res* 2008;68: 5246–56
- 79 Peake KB, Vance JE. Defective cholesterol trafficking in Niemann-Pick C-deficient cells. *FEBS Letters* 2010; 584: 2731–39
- 80 Charman, M., Kennedy, B.E., Osborne, N, et al. MLN64 mediates egress of cholesterol from endosomes to mitochondria in the absence of functional Niemann-Pick type C1 protein. *J Lipid Res*. 2009; 51: 1023–34.
- 81 Yu W., Gong J.S., Ko M., et al. Altered cholesterol metabolism in Niemann-Pick type C1 mouse brains affects mitochondrial function. *J. Biol. Chem*. 2005; 280: 11731–39
- 82 Jelinek D, Millward V, Birdi A, et al. Npc1 haploinsufficiency promotes weight gain and metabolic features associated with insulin resistance. *Hum Mol Genet*. 2011;20:312-21
- 83 Meyre D, Delplanque J, Chevre JC, et al. Genome-wide association study for early-onset and

morbid adult obesity identifies three new risk loci in European populations. *Nat. Genet.*, **2009**; 4:, 157-59

84 Oxysterol binding protein. Taylor FR, Kandutsch AA. *Chem Phys Lipids*. 1985;38: 187-94

85 Jansen M, Ohsaki Y, Rita Rega L, et al. Role of ORPs in sterol transport from plasma membrane to ER and lipid droplets in mammalian cells. *Traffic* 2011; **12**: 218–231

86 Zhou T, Li S, Zhong W, et al. OSBP-related protein 8 (ORP8) regulates plasma and liver tissue lipid levels and interacts with the nucleoporin Nup62. *PLoS One*. 2011;6:e21078.

87 Jordan SD, Krüger M, Willmes DM, et al. Obesity-induced overexpression of miRNA-143 inhibits insulin-stimulated AKT activation and impairs glucose metabolism. *Nat Cell Biol*. 2011;13:434-46.

88 Jia L, Betters JL, Yu L. Niemann-Pick C1-Like 1 (NPC1L1) Protein in Intestinal and Hepatic Cholesterol Transport. *Annu. Rev. Physiol*. 2011. 73:239–59

89 Labonte ED, Camarota LM, Rojas JC, et al. Reduced absorption of saturated fatty acids and resistance to diet-induced obesity and diabetes by ezetimibe-treated and *Npc1l1*^{-/-} mice. *Am. J. Physiol. Gastrointest. Liver Physiol*. 2008; 295:776–83

90 Clarenbach JJ, Reber M, Lutjohann D, et al. The lipid-lowering effect of ezetimibe in pure vegetarians. *J. Lipid Res*. 2006; 47:2820–24

91 Yamanashi Y, Takada T, Shoda J, et al. Novel function of Niemann-Pick C1-like 1 as a negative regulator of Niemann-Pick C2 protein. *Hepatology* 2012; 55; 953–64.

92 Yoneda M, Fujita K, Imajo K, et al. Induction of microsomal triglyceride transfer protein expression is a candidate mechanism by which ezetimibe therapy might exert beneficial effects in patients with nonalcoholic steatohepatitis. *J Gastroenterol*. 2011;46:415-6

93 Rudovich NN, Weickert MO, Machann J. et al. Combination of acarbose and ezetimibe prevents non-alcoholic fatty liver disease: a break of intestinal insulin resistance? *J Hepatol*. 2010;52:952-3.

-
- 94 Moritoh Y, Takeuchi K, Hazama M. Combination treatment with alogliptin and voglibose increases active GLP-1 circulation, prevents the development of diabetes and preserves pancreatic beta-cells in prediabetic db/db mice. *Diabetes Obes Metab.* 2010;12:224-33.
- 95 Nagai K, Matsumaru K, Takahashi Y, et al. Effective therapy using voglibose for nonalcoholic steatohepatitis in a patient with insufficient dietary and exercise therapy: exploring other treatment possibilities. *Case Rep Gastroenterol.* 2011; 5:336-43.
- 96 Simonen P, Kotronen A, Hallikainen M, et al. Cholesterol synthesis is increased and absorption decreased in non-alcoholic fatty liver disease independent of obesity. *Journal of Hepatology* 2011; 54:153–59
- 97 Nomura M, Ishii H, Kawakami A, et al. Inhibition of hepatic Niemann-Pick C1-like 1 improves hepatic insulin resistance *Am J Physiol Endocrinol Metab* 2009; 297: E1030–38.
- 98 Tang W, Jia L, Ma Y, et al. Ezetimibe restores biliary cholesterol excretion in mice expressing Niemann–Pick C1-Like 1 only in liver. *Biochimica et Biophysica Acta* 2011; 1811: 549–555
- 99 Hardwick RN, Fisher CD, Street SM, et al. Molecular mechanism of altered ezetimibe disposition in nonalcoholic steatohepatitis. *Drug Metab Dispos.* 2012;40:450-60.
- 100 Jia L, Ma Y, Liu G, et al . Dietary cholesterol reverses resistance to diet-induced weight gain in mice lacking Niemann-Pick C1-Like 1. *J. Lipid Res.*2010; 51:3024–33
- 101 Van Rooyen DM, Larter CZ, Yeh MM, et al. Free cholesterol is a lipotoxic mediator in metabolic syndrome NASH: lipidomic analyses, dietary and pharmacologic interventions in obese, diabetic mice. *Hepatology* 2011; 54: S4: 1154A
- 102 Yang Y, Jiang Y, Wang Y, et al. Suppression of ABCA1 by unsaturated fatty acids leads to lipid accumulation in HepG2 cells. *Biochimie.* 2010;92:958-63
- 103 Su K, Sabeva NS, Liu J, et al. The **ABCG5** ABCG8 sterol transporter opposes the development of fatty liver disease and loss of glycemic control independent of phytosterol accumulation.. *J Biol Chem.* 2012 Jun 19. [Epub ahead of print] PMID: 22715101

-
- 104 Basso F, Freeman LA, Ko C, et al. Hepatic ABCG5/G8 overexpression reduces apoB-lipoproteins and atherosclerosis when cholesterol absorption is inhibited. *J Lipid Res.* **2007**;48:114-26
- 105 Savard C, Tartaglione EV, Kuver R, et al.. Synergistic interaction of dietary cholesterol and dietary fat in inducing experimental steatohepatitis. *Hepatology.* 2012 Apr 16. doi: 10.1002/hep.25789. [Epub ahead of print]
- 106 Alger HM, Brown JM, Sawyer JK, et al. Inhibition of acyl-coenzyme A:cholesterol acyltransferase 2 (ACAT2) prevents dietary cholesterol-associated steatosis by enhancing hepatic triglyceride mobilization. *J Biol Chem.* 2010;285:14267-74
- 107 Bie J, Zhao B, Song J, et al. Improved insulin sensitivity in high fat- and high cholesterol-fed Ldlr-/- mice with macrophage-specific transgenic expression of cholesteryl ester hydrolase: role of macrophage inflammation and infiltration into adipose tissue. *J Biol Chem.* 2010 ;285:13630-7.
- 108 Bie J, Zhao B, Marqueen KE, et al.. Macrophage-specific transgenic expression of cholesteryl ester hydrolase attenuates hepatic lipid accumulation and also improves glucose tolerance in ob/ob mice. *Am J Physiol Endocrinol Metab* 302: E1283–E1291, 2012
- 109 Bommer GT, MacDougald OA. Regulation of Lipid Homeostasis by the Bifunctional SREBF2-miR33a Locus. *Cell Metabolism* 2011; 13: 241-47
- 110 Gerin I, Clerbaux LA, Haumont O, et al. Expression of miR-33 from an SREBP2 Intron Inhibits Cholesterol Export and Fatty Acid Oxidation. *J Biol Chem.* 2010;285:33652-61
- 111 Xie X, Liao H, Dang H et al. Down-regulation of hepatic HNF4alpha gene expression during hyperinsulinemia via SREBPs. *Mol. Endocrinol.* 2009; **23**: 434–43
- 112 Ye Q, Chen Y, Lei H, et al. Inflammatory stress increases unmodified LDL uptake via LDL receptor: an alternative pathway for macrophage foam-cell formation. *Inflamm. Res.* 2009; 58:809–818

-
- 113 Musso G, Cassader M, Bo S, et al. Sterol regulatory binding factor (SREBF)-2 predicts incidence and severity of NAFLD and of associated lipid and glucose dysmetabolism. *Diabetes*. Accepted 2012 in press
- 114 Li T, Matozel M, Boehme S, et al. Overexpression of cholesterol 7 α -hydroxylase promotes hepatic bile acid synthesis and secretion and maintains cholesterol homeostasis. *Hepatology*. 2011;53:996-1006.
- 115 Adorini L, Pruzanski M, Shapiro D. Farnesoid X receptor targeting to treat nonalcoholic steatohepatitis. *Drug Discov Today*. 2012 May 29. [Epub ahead of print] PMID: 22652341
- 116 Kong B, Luyendyk JP, Tawfik O, et al. Farnesoid X Receptor Deficiency Induces Nonalcoholic Steatohepatitis in Low-Density Lipoprotein Receptor-Knockout Mice Fed a High-Fat Diet. *J Pharmacol Exp Ther*. 2009; 328: 116–122,
- 117 Fiorucci S, Rizzo G, Antonelli E, et al. A farnesoid x receptor-small heterodimer partner regulatory cascade modulates tissue metalloproteinase inhibitor-1 and matrix metalloprotease expression in hepatic stellate cells and promotes resolution of liver fibrosis. *J. Pharmacol. Exp. Ther*. 2005; 314: 584–595.
- 118 Sanyal AJ, Mudaliar S, Henry RR, et al. A new therapy for non-alcoholic fatty liver disease and diabetes? Int-747-the first FXR hepatic therapeutic study. *Hepatology* 2010; 50: 389A
- 119 Mitro, N., Mak, P.A., Vargas, L, et al. The nuclear receptor LXR is a glucose sensor. *Nature* 2007; 445, 219–23
- 120 Liu Y, Qiu de K, Ma X. Liver X receptors bridge hepatic lipid metabolism and inflammation. *J Dig Dis*. 2012;13: 69-74
- 121 Zelcer N, Hong C, Boyadjian R, et al. LXR regulates cholesterol uptake through Idol-dependent ubiquitination of the LDL receptor. *Science* 2009;325:100–4.

-
- 122 Liu Y, Han X, Bian Z, et al. Activation of liver X receptors attenuates endotoxin-induced liver injury in mice with nonalcoholic fatty liver disease. *Dig Dis Sci*. 2012;57:390-8
- 123 Beaven SW, Wroblewski K, Wang J, et al. Liver X receptor signaling is a determinant of stellate cell activation and susceptibility to fibrotic liver disease. *Gastroenterology*. 2011;140:1052-62.
- 124 Lima-Cabello E, García-Mediavilla MV, Miquilena-Colina ME, et al. Enhanced expression of pro-inflammatory mediators and liver X-receptor-regulated lipogenic genes in non-alcoholic fatty liver disease and hepatitis C. *Clin Sci (Lond)*. 2011;120:239-50.
- 125 Higuchi N, Kato M, Shundo Y, et al. Liver X receptor in cooperation with SREBP-1c is a major lipid synthesis regulator in nonalcoholic fatty liver disease. *Hepatol Res*. 2008;38:1122-9
- 126 Inaba T., Matsuda M, Shimamura M., et al., “Angiopietin-like protein 3 mediates hypertriglyceridemia induced by the liver X receptor,” *Journal Biol Chem* 2003; ,278: 21344–51.
- 127 Jakel H, Nowak M, Moitrot E et al., “The liver X receptor ligand T0901317 down-regulates APOA5 gene expression through activation of SREBP-1c,” *Journal of Biol Chem* 2004; ,279: 45462–69
- 128 Yilmaz Y. NAFLD in the absence of metabolic syndrome: different epidemiology, pathogenetic mechanisms, risk factors for disease progression? *Semin Liver Dis*. 2012;32: 14-21
- ¹²⁹ Browning JD. Statins and hepatic steatosis: perspectives from the Dallas Heart Study. *Hepatology* 2006; 44: 466-71
- ¹³⁰ Cohen DE, Anania FA, Chalasani N. An assessment of statin safety by hepatologists. *Am J Cardiol* 2006; 97(8A): 77C-81C.
- ¹³¹ Nakade Y, Yamamoto T, Banno F, et al. Effect of Glucagon-Like Peptide-1 (GLP-1) Analogue on Hepatic Cholesterol Metabolism in Nonalcoholic Steatohepatitis (NASH). *Digestive Disease Week 2012*; poster Tu1813.

¹³² Musso G, Gambino R, Cassader M Emerging molecular targets for the treatment of nonalcoholic fatty liver disease. *Annu Rev Med.* 2010;61: 375-92.

¹³³ Otocka-Kmiecik A, Mikhailidis DP, Nicholls SJ, Davidson M Rysz J, Banach M. Dysfunctional HDL: A novel important diagnostic and therapeutic target in cardiovascular disease? *Progress in Lipid Research* 2012; 51: 314–324

Figure 1

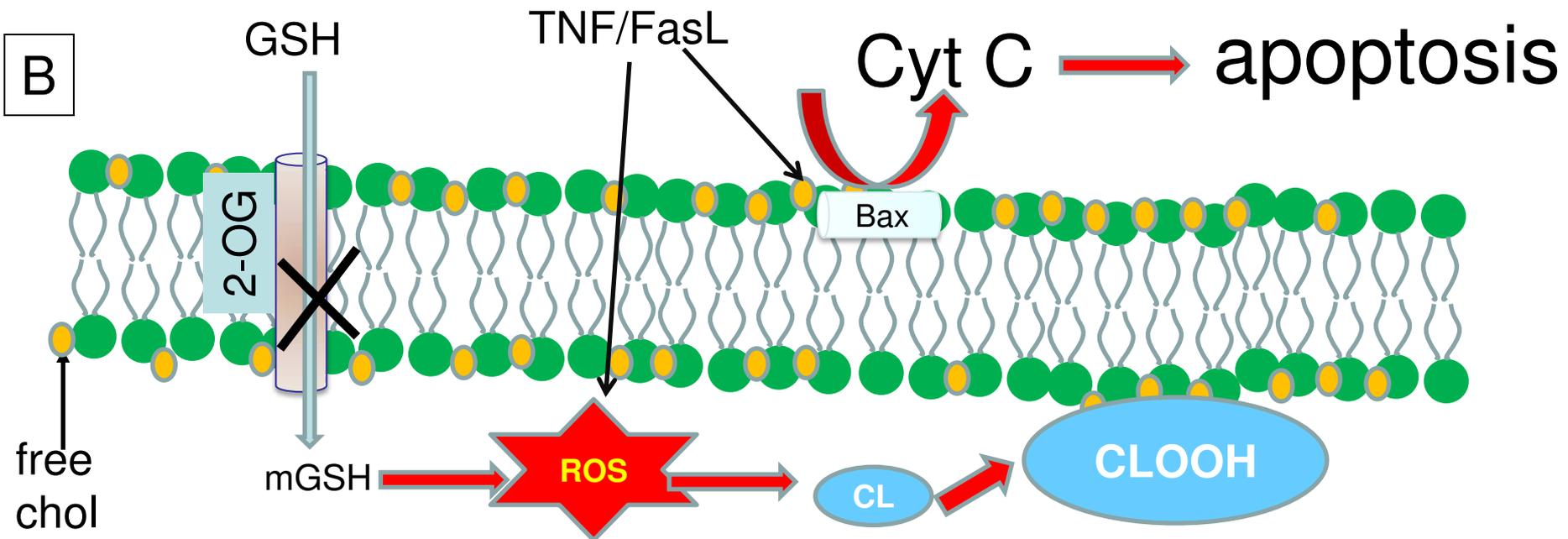
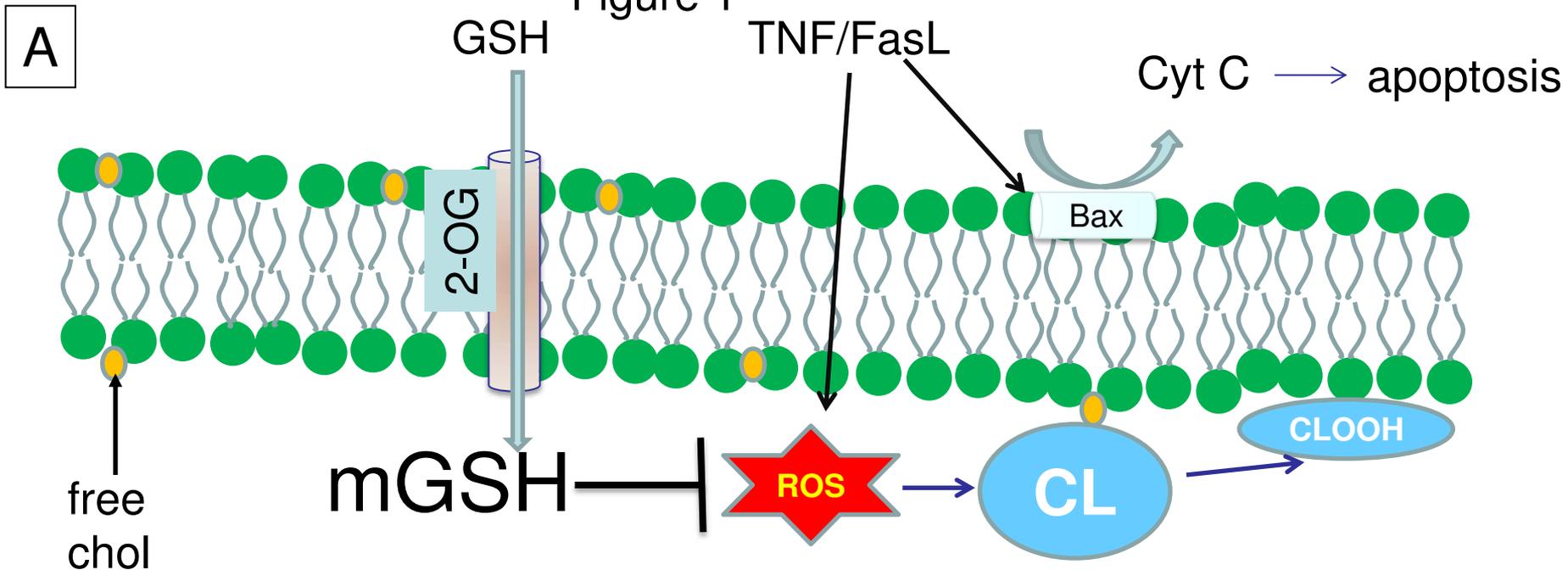


Figure 1

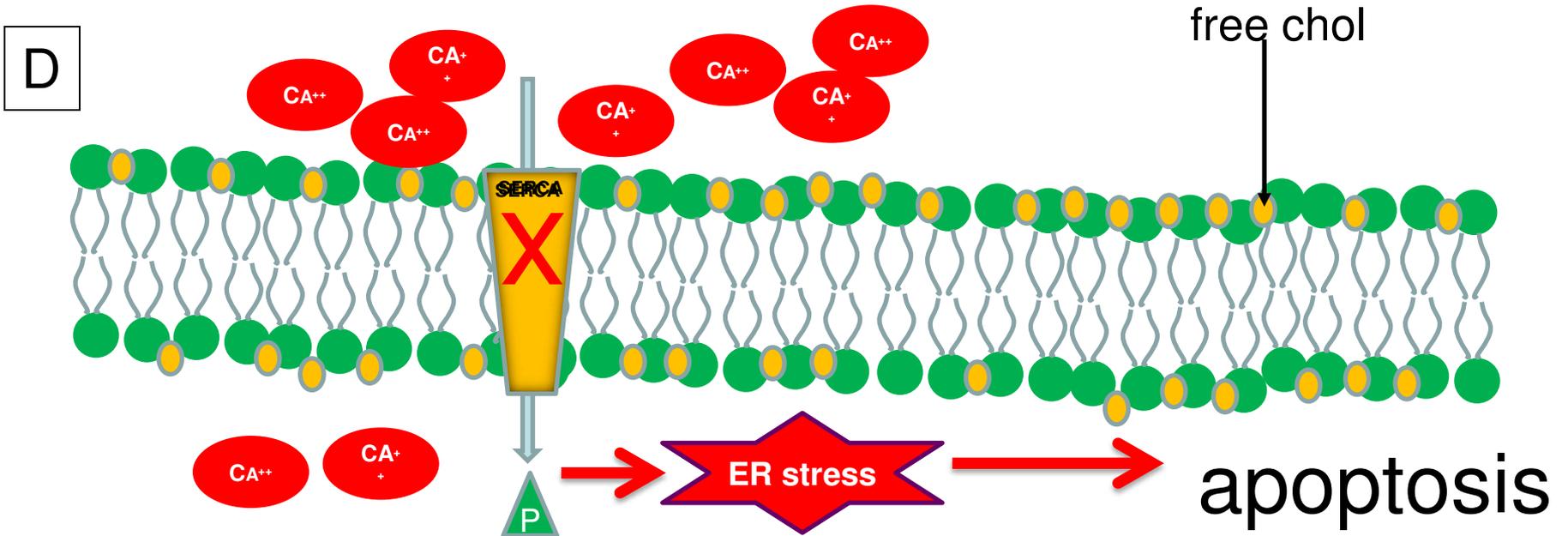
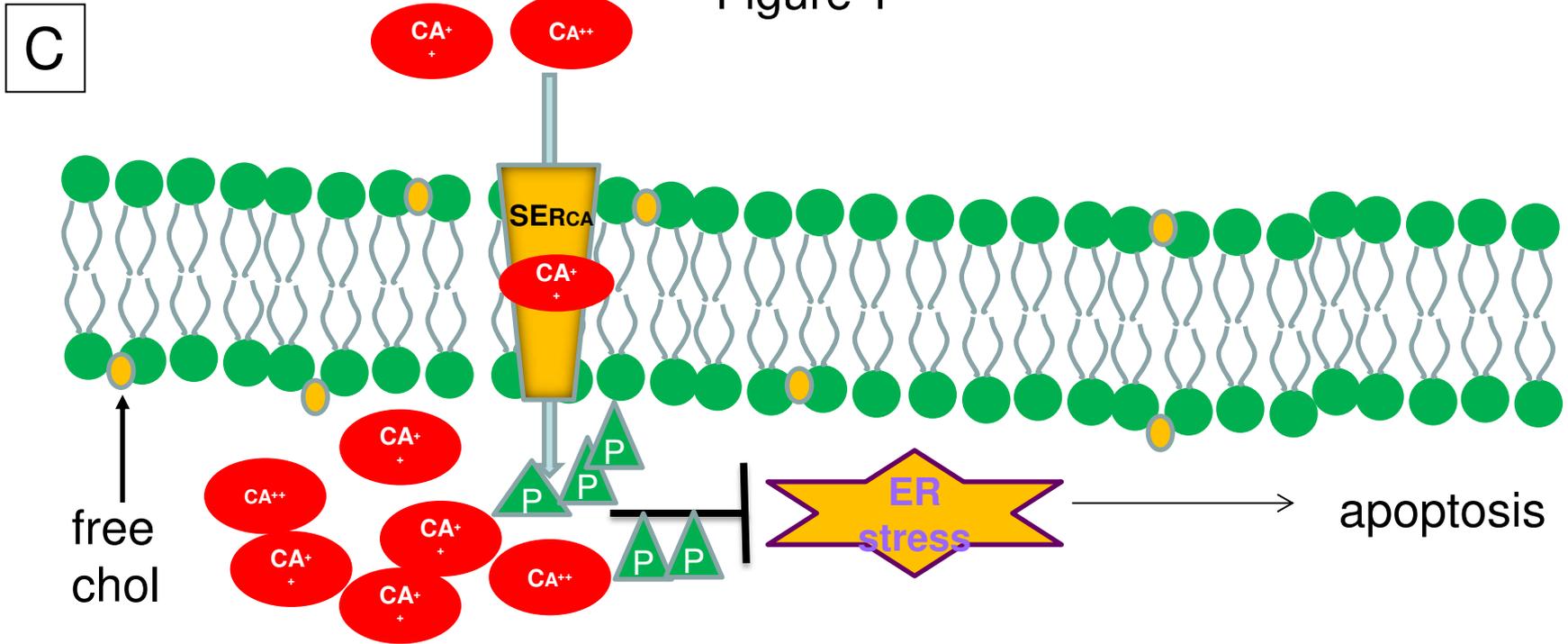
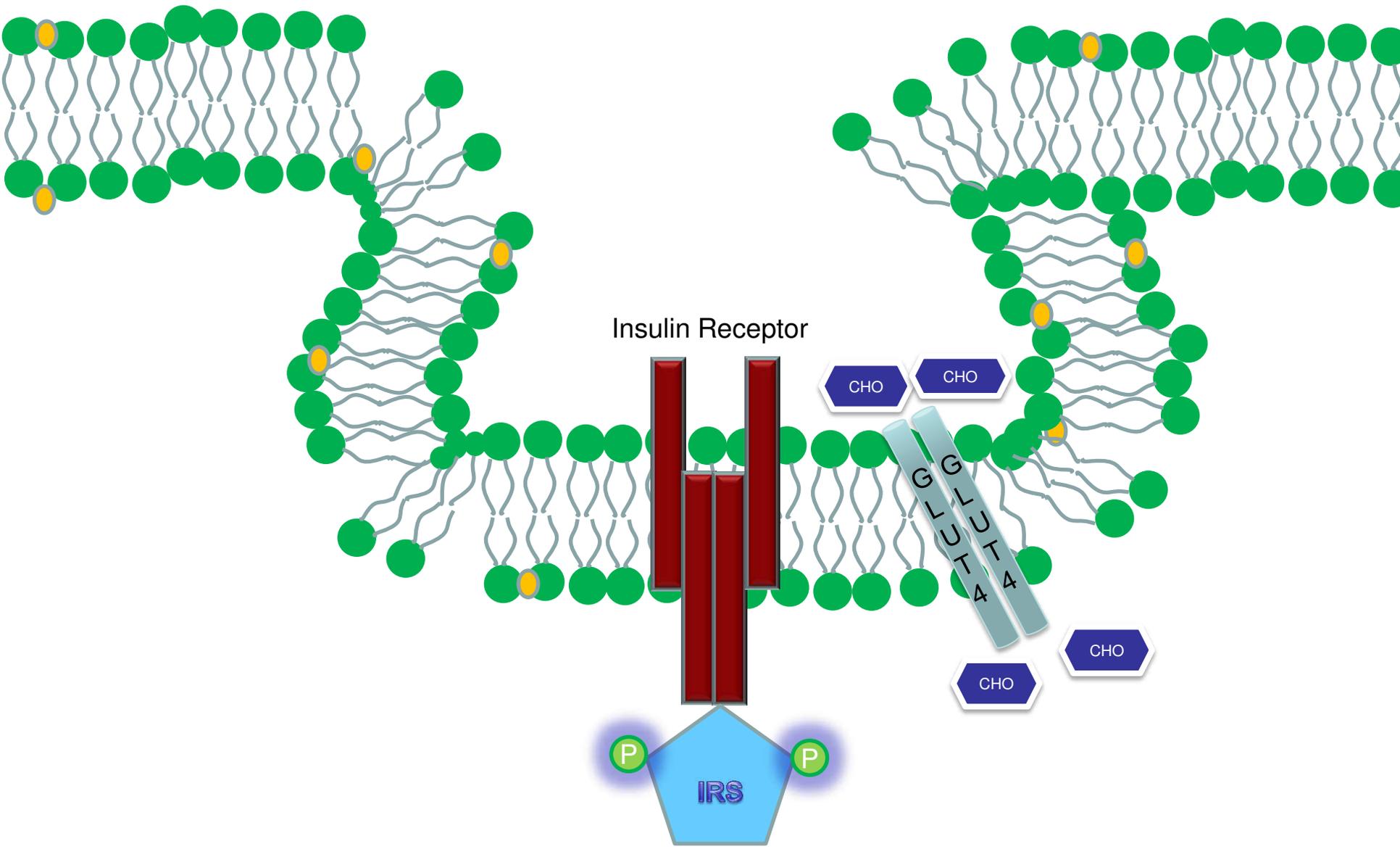
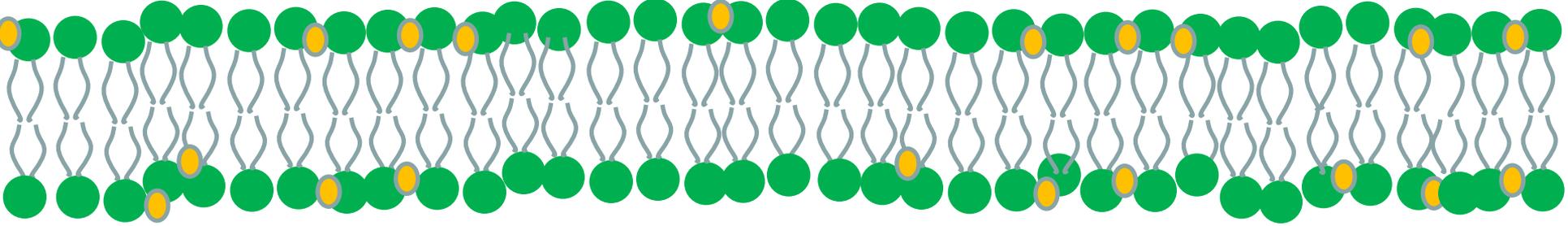


Figure 1

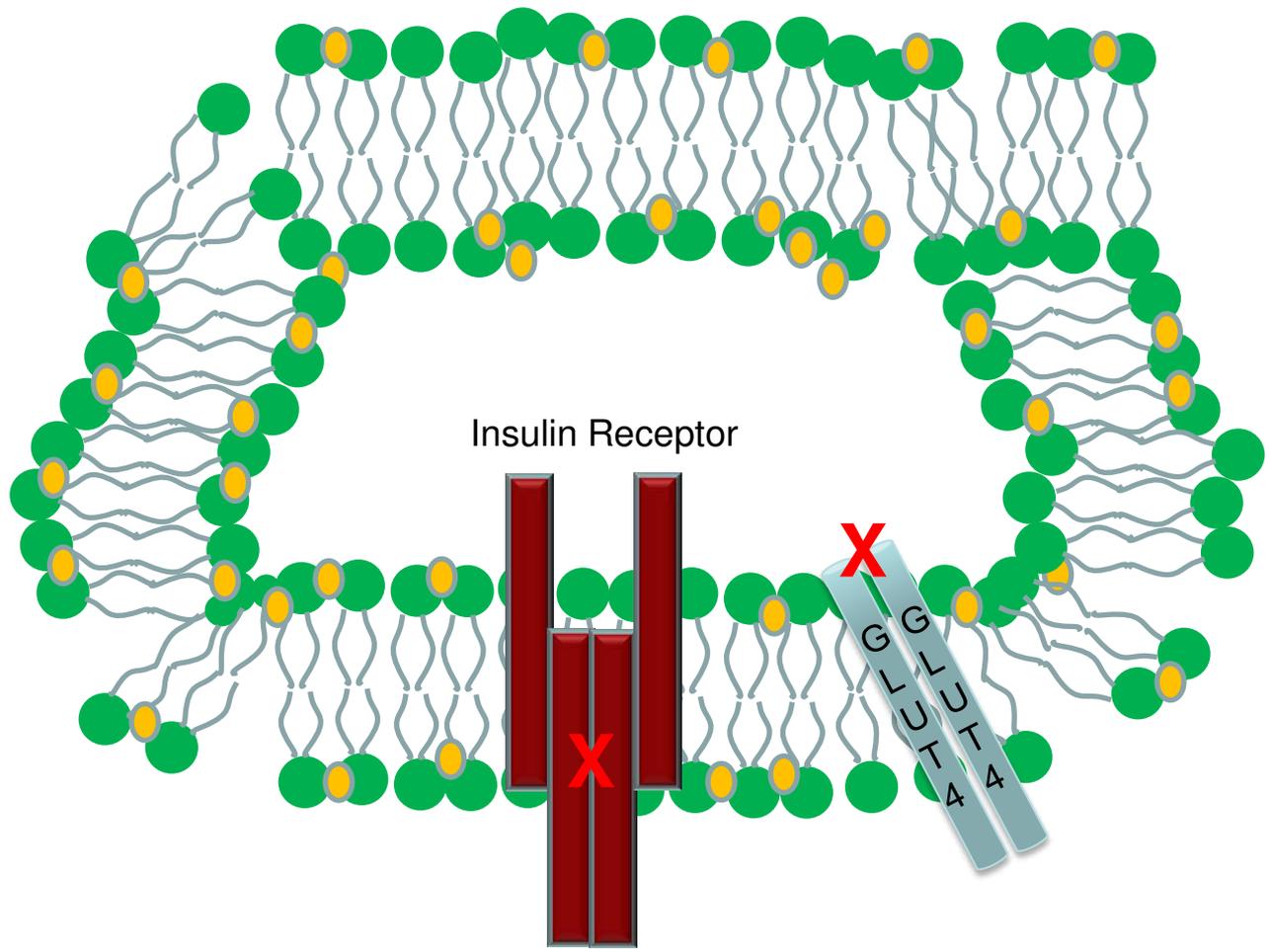
E





F

Figure 1



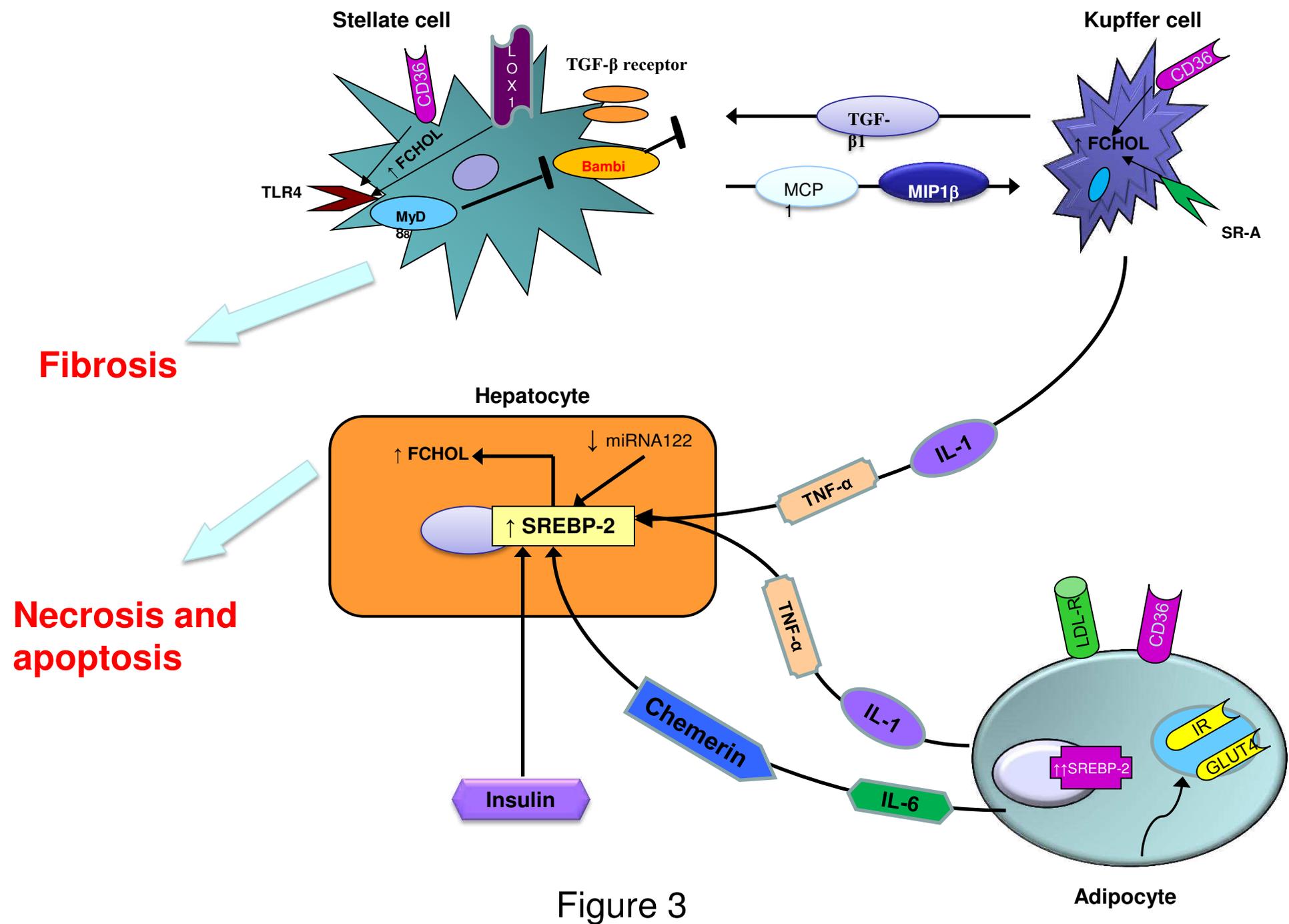


Figure 3

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

Cholesterol synthesis				
Factor	Localization	Regulators	Function	Biological effect
HMGCAR	Hepatocyte ER	SREBP-2 ↑ HMGCAR gene transcription miRNA34a: ↑ HMGCAR dephosphorylation and activation	catalyzes the conversion of HMGCAR to mevalonate, rate-limiting step in cholesterol biosynthesis	HMGCAR 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 HMGCAR 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 cells	miRNA143: ↓ ORP8 in obesity ⁸⁷	Cholesterol and oxysterol-binding proteins involved in nonvesicular cholesterol delivery from LE/LY, cholesterol transport between the ER and PM and intracellular 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 NPC1L1 ↓ NPC2 activity, promoting LE/LY cholesterol overload ⁹¹	NPC1L1 inhibition by ezetimibe reduces hepatic and intestinal free cholesterol absorption, reduces cellular 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-4 α , FXR: ↑ gene Transcription ^{110, 111, 116}	ABCG5 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	HNF1 α , 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 7 α hydroxylase (CYP7A1)	microsomal cytochrome P450 expressed only in hepatocytes	Bile acids, insulin, SHP: \downarrow transcription of CYP7A1 LXR α , FXR, HNF-4 α : \uparrow transcription of CYP7A1	CYP7A1 catalyzes the 7 α -hydroxylation of cholesterol to 7 α -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, : \downarrow transcription of CYP27A1 HNF-1 α : \uparrow transcription of CYP27A1	CYP27A1 catalyzes the “alternative” (or acidic) pathway of bile acids synthesis, leading to 27-hydroxycholesterol (27OHC) 27OHC : \downarrow SREBP-2-activation and \uparrow 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 NASH⁴⁴
Sterol 25-hydroxylase	microsomal diiron cofactor enzyme, not a cytochrome P450 family, with broad tissue distribution.	LPS/TLR-4 pathway \uparrow 25OHC synthesis	It catalyzes cholesterol 25-hydroxylation to 25-hydroxycholesterol (25OHC). 25OHC enhances lipogenesis via LXR α /SREBP-1c activation and inflammatory response via NF- κ B 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 \uparrow 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	<u>Activators:</u> IL-1b, IL-6, SAA ¹⁵ , Insulin ¹¹¹ , PM cholesterol depletion ⁶⁰ , <u>Inhibitors:</u> miRNA122 ⁶⁹ , 7-OHC, 27-OHC ¹⁰⁹	↑ LDL receptor	↑ extracellular cholesterol uptake
		↑ HMG-CoAR, ↑ squalene synthase ⁶⁰	↑ 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 inflammation 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 β subunit (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	<u>Activators:</u> Natural bile acids (chenodeoxycholic acid), semisynthetic bile acids (obeticholic acid), PUFA (arachidonic, linolenic, or docosahexaenoic), synthetic agonists(GW4064, 6 α -ECDA, fexaramine)	↑ CYP7A1, ↑ ABCG5/G8 ¹¹⁵	↑ bile acid synthesis and cholesterol excretion into bile
		↓ SREBP-1c-mediated lipogenesis (SHP-dependent and -independent mechanisms) ¹¹⁵	↓ liver triglyceride
		↑ PPAR- α -mediated activation of fatty acid β -oxidation ¹¹⁵	↓ secretion and ↑ clearance of VLDL, thereby reducing plasma triglycerides
		↓ ApoC-III and apoA-1 synthesis ↑ ApoC-II synthesis and VLDL-receptor expression ↓ hepatic lipase activity ¹¹⁵	↓ synthesis and uptake of HDL-C, with a net reduction in plasma HDL-C levels
		↓ hepatic expression of gluconeogenic 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- β 1 genes ¹¹⁷	↓ hepatic fibrogenesis ^{117, 118}

Liver X receptor(LXR)- α

Cells	modulators	Molecular targets	Biological effect
Hepatocytes, enterocytes,	<u>Activators:</u> glucose ¹¹⁹	↑ transcription 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	↓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
	<u>Inhibitors:</u> natural oxysterol 25OHC3S , Synthetic oxysterol 22(S)OHC 40-42	↑ 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 lipoprotein 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 7 α 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; PEPCCK: 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