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Lipid oxidation products in the pathogenesis of non-alcoholic steatohepatitis

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ABSTRACT

Non-alcoholic fatty liver disease (NAFLD) is the major public health challenge for hepatologists in the twenty-first century. NAFLD comprises a histological spectrum ranging from simple steatosis or fatty liver, to steatohepatitis, fibrosis, and cirrhosis. It can be categorized into two principal phenotypes: (1) non-alcoholic fatty liver (NAFL), and (2) non-alcoholic steatohepatitis (NASH). The mechanisms of NAFLD progression consist of lipid homeostasis alterations, redox unbalance, insulin resistance, and inflammation in the liver. Even though several studies show an association between the levels of lipid oxidation products and disease state, experimental evidence suggests that compounds such as reactive aldehydes and cholesterol oxidation products, in addition to representing hallmarks of hepatic oxidative damage, may behave as active players in liver dysfunction and the development of NAFLD. This review summarizes the processes that contribute to the metabolic alterations occurring in fatty liver that produce fatty acid and cholesterol oxidation products in NAFLD, with a focus on inflammation, the control of insulin signalling, and the transcription factors involved in lipid metabolism.

1. Introduction

Primary non-alcoholic fatty liver disease (NAFLD) is the most common cause of chronic liver disease in Western countries. The term NAFLD refers to a lipid excess in the liver that does not result from alcohol consumption or secondary causes such as medications, viral infections, or endocrine conditions. The hallmark of NAFLD is the intracellular accumulation of lipids, resulting in the formation of lipid droplets within hepatocytes. This accumulation results from an imbalance between lipid synthesis and oxidation [1–3]. Starting from the benign condition of simple steatosis, NAFLD may evolve into non-alcoholic steatohepatitis (NASH), which is characterized by inflammation and necrosis, and may progress to hepatic fibrosis, cirrhosis, and hepatocellular carcinoma [4–6].

A burning problem constituting the focus of intense investigation is why most steatotic patients have a benign non-progressive disease, whereas others develop NASH and end-stage liver disease. The mechanisms of NAFLD progression are of significant biomedical and clinical importance, because the elucidation of these processes may help identify the subset of patients who will experience disease progression, and define novel therapeutic targets.

Oxidative stress is recognized as the main contributor to hepatocyte injury and disease progression in NAFLD [7–9]. In fact, hepatic lipid accumulation leads to a compensatory increased oxidation, which mainly involves mitochondria [10,11]. Even though reactive oxygen species (ROS) and reactive nitrogen species (RNS) are normally produced by the metabolism of normal cells, hepatic lipid accumulation causes an over-production of free radicals that is not counterbalanced

Abbreviations: NAFLD, non-alcoholic fatty liver disease; NAFL, non-alcoholic fatty liver; NASH, non-alcoholic steatohepatitis; ROS, reactive oxygen species; RNS, reactive nitrogen species; MDA, malondialdehyde; HNE, 4-hydroxynonenal; FAs, fatty acids; TAGs, triacylglycerols; PDC, pyruvate dehydrogenase complex; ACC, acetyl-CoA carboxylase; FAS, fatty acid synthase; SCFAs, short-chain fatty acids; MCFAs, medium-chain fatty acids; LCFAs, long chain fatty acids; VLCFAs, very long chain fatty acids; SFAs, saturated fatty acids; MUFAs, monounsaturated fatty acids; PUFAs, polyunsaturated fatty acids; SCD, stearoyl-CoA desaturase; ER, endoplasmic reticulum; CE, cholesteryl esters; D6D, delta-6 desaturase; D5D, delta-5 desaturase; mtGPAT, mitochondrial glycerol-3-phosphate-acyltransferase; DGAT, diacylglycerol-acyltransferase; HF/HC, high fat/high carbohydrate; ELOVL6, elongation of very long chain fatty acids 6; NEFAs, non esterified fatty acids; FATPs, fatty acids transport proteins; FAT/CD36, fatty acid translocase; VLDL, very low density lipoproteins; TCA, tricarboxylic acid cicle; CPT1, carnitine palmitoyl transferase 1; OxyPhos, oxidative phosphorylation system; PKA, cAMP/protein kinase A; LDL, low density lipoproteins; LRP, LDL receptor-related protein; HMG-CoA, hydroxymethylglutaryl-CoA; FC, free cholesterol; ACAT, acyl-coenzyme A:cholesterol acyltransferase; NPC, Niemann-Pick C protein; START, steroidogenic acute regulator (StAR)-related lipid transfer; CYP7A1, cholesterol 7α-hydroxlase; HDL, high density lipoproteins; NPC1L1, Niemann–Pick C1-like 1 protein; BA, bile acids; HETE, hydroxyeicosatetraenoic acid; UPR, unfolded protein response; GSH, reduced glutathione; TGFβ, transforming growth factor β; UCP-2, uncoupling protein-2; NRs, nuclear receptors; LXRs, liver-X receptors; SREBP, sterol regulatory element-binding protein; FXR, farnesoid-X receptor; PPARs, peroxisome proliferator-activated receptors; TLRs, toll-like receptors; NF-κB, nuclear factor-κB; JNK, c-Jun NH_2-terminal kinase

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by antioxidant defences, inducing liver injury [12]. At high concentrations, free radicals are dangerous for several cellular constituents. Thus, oxidative stress in NASH patients may result in a pro-oxidative environment with consequent formation of highly reactive molecules inducing lipid oxidation products [13]. The peroxidation of lipids is an attractive candidate mechanism for NASH development and progression because lipid peroxidation products, including malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE), potentially explain all of the histological features of steatohepatitis [14,15].

In the present review, we discuss recent findings on the role of lipid oxidation products, their newly identified targets, and the mechanistic studies emphasizing their importance in NAFLD, because these are topics of growing interest.

2. Lipid metabolism in NAFLD

The term "lipid" defines any of a group of organic compounds that are insoluble in water but soluble in organic solvents; these chemical features are found in a broad range of molecules such as fatty acids (FAs), sterols, phospholipids, sphingolipids, terpenes, and others [16]. The present discussion will pay special attention to FAs and cholesterol, owing to their important metabolic and nutritional functions [17].

The liver plays a central role in lipid metabolism, which involves the following inter-dependent and cross-regulated pathways:

- lipogenesis by conversion of excess carbohydrates;
- FAs uptake and oxidation to produce energy;
- cholesterol metabolism.

2.1. Lipogenesis modifications in NAFLD

The liver and adipose tissue are the main lipid producers, providing both an energy source and structural components for membrane building [18]. Hepatic lipogenesis starts with the *de novo* synthesis of FAs. Depending on the metabolic state, FAs are then either processed to triacylglycerols (TAGs) and stored, or rapidly metabolized through *β*-oxidation, the predominant source of energy during fasting. Thus, *de novo* synthesis of FAs is tightly controlled by nutritional as well as hormonal conditions: it is induced by excess carbohydrates, whereas it is inhibited by fasting or fat consumption as a result of insulin concentration and tissue insulin sensitivity [19]. Transcription factors such as liver X receptor (LXR), sterol regulatory element-binding protein-1c (SREBP-1c), and carbohydrate response element binding protein (ChREBP) exert significant control over the *de novo* synthesis of FAs [20].

When the *de novo* synthesis of FAs starts from glucose, the mitochondrial pyruvate dehydrogenase complex (PDC) is the gatekeeper for the generation of acetyl-CoA from glucose-derived pyruvate. It is worth noting that limiting the supply of PDC-generated acetyl-CoA from glucose negatively impacts the expression of key genes in the lipogenic pathway [21]. *De novo* synthesis of FAs occurs in the cytosol as the sequential addition of two-carbon units to acetyl-CoA as the starting molecule; the rate-limiting step in this pathway is catalyzed by acetyl-CoA carboxylase (ACC), which converts acetyl-CoA to malonyl-CoA [22]. The following steps in FA synthesis are catalyzed by fatty acid synthase (FAS), a key multifunctional enzyme whose activity regulates the rate of FA synthesis [23]. According to their chain length, FAs are referred to as short-chain FAs (SCFAs, C4–C10), medium-chain FAs (MCFAs, C12–C14), long-chain FAs (LCFAs, C16–C18), and very long-chain FAs (VLCFAs, C20 or more) [24]. FA elongation starts in the cytosol and leads primarily to hexadecanoic acid (palmitate, 16:0), and to a lesser extent tetradecanoic acid (myristate, 14:0) and octadecanoic acid (stearate, 18:0), as the end products. Microsomal FA elongation is considered the predominant pathway for elongating FAs of 12 carbons and longer, starting from FAs derived from endogenous pathways as well as exogenous FAs derived from the diet [25]. The last step for microsomal FA elongation involves seven enzymes called elongases (ELOVL1–7) [26,27].

The carbon atoms in FAs may be linked by single or double bonds, and the number of double bonds in the same molecule can range from one to six. Saturated FAs (SFAs) have no double bonds, monounsaturated FAs (MUFAs) have one double bond, and polyunsaturated FAs (PUFAs) have two to six double bonds [28]. Stearoyl-CoA desaturase (SCD) is an endoplasmic reticulum (ER) enzyme that catalyzes the critical committed step in the biosynthesis of MUFAs from SFAs. The preferred substrates are hexadecanoyl- (palmitoyl-CoA, 16:0) and octadecanoyl-CoA (stearoyl-CoA, 18:0); they are converted into (9*Z*)-hexadec-9-enoyl- (palmitoleoyl-CoA, 16:1) and (9*Z*)-octadec-9-enoyl-CoA (oleoyl-CoA, 18:1), respectively, which are the most abundant MUFAs in TAGs, phospholipids, and cholesteryl esters (CE) [29,30]. SCD-1 expression in the liver is regulated by adipose tissue-derived C16:1n7-palmitoleate; this compound is an example of a lipokine, and is used by adipose tissue to communicate with distant organs and regulate systemic metabolic homeostasis [31].

In mammalian cells, elongases and desaturases play critical roles in regulating the length and degree of unsaturation of FAs, which affect their functions and metabolic fates [32].

PUFAs are classified according to the number of carbon atoms, the number of double bonds, and the position of the double bond closest to the terminal methyl group. Of the four PUFA families, ω3 and ω6 are considered the most important. The precursor of the ω3 PUFAs, (9*Z*,12*Z*,15*Z*) − 9,12,15-octadecatrienoic acid (α-linolenic acid, 18:3), and that of the ω6 PUFAs, (9*Z*,12*Z*) − 9,12-octadecadienoic acid (linoleic acid, 18:2), are essential FAs that cannot be synthesized by mammals. Additional PUFAs are synthesized by the activity of a series of enzymes including delta-6 desaturase (D6D)—the first and rate-limiting enzyme in the ω3 and ω6 pathways—and delta-5 desaturase (D5D) (also active in the ω3 and ω6 pathways); these two desaturases, widely expressed in the mammalian liver and in other tissues, catalyze the insertion of additional double bonds within the PUFA carbon chain [33,34].

Because FAs and their metabolites are the major cause of lipotoxicity and promote the formation of ROS, they are stored for future use as TAGs, which are relatively inert and consist of three FAs esterified to a glycerol backbone [35]. TAG synthesis is catalyzed by the enzymes mitochondrial glycerol-3-phosphate-acyltransferase (mtGPAT) and diacylglycerol-acyltransferase (DGAT) [36]. TAGs are then either stored in lipid droplets within the hepatocyte or processed to very low density lipoprotein (VLDL). During NAFLD, an imbalance between uptake and synthesis that exceeds oxidation and removal occurs; in fact, patients affected by NAFLD show an increase in both uptake and synthesis [1,37], whereas experimental reduction of lipogenesis reverses hepatic steatosis [38,39]. In recent years, research has focused on the role of the altered activity of several lipogenic enzymes involved in the pathogenesis of NAFLD (Fig. 1). For most of these enzymes, animal models of their knockdown and/or knockout have been generated, providing a better understanding of important regulatory checkpoints in fat synthesis.

ACC has recently attracted significant attention because it catalyzes the synthesis of malonyl-CoA, the metabolic intermediate between lipogenesis and β-oxidation. In mammals, two ACC isoforms are described: ACC1 is highly expressed in the liver, is cytosolic, and participates in *de novo* lipogenesis; and ACC2 is mostly expressed in the muscular tissue, is mitochondrial, and negatively regulates mitochondrial β-oxidation by modulating local malonyl-CoA levels [40,41]. Whereas global inactivation of ACC1 leads to embryonic lethality, indicating that *de novo* lipogenesis is essential for embryonic development, *Acc2*$^{-/-}$ mice present with increased β-oxidation and do not develop high-fat/high-carbohydrate (HF/HC) diet-induced obesity and diabetes [42,43]. Liver-specific ACC1-knockout mice showed a reduction in *de novo* lipogenesis and TAG concentration but developed HF/HC diet-induced obesity and fatty liver [44]. The use of antisense

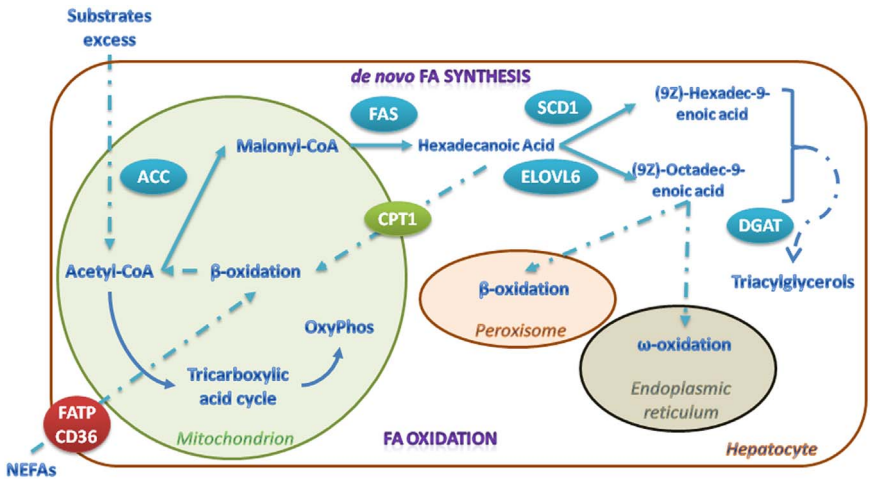

Fig. 1. Schematic representation of hepatic lipid synthesis and oxidation, with emphasis on the enzymes constituting important regulatory checkpoints in fat metabolism in non-alcoholic fatty liver disease (NAFLD), which are targets of genetic or pharmacological manipulation (please see the text for details). *De novo* synthesis of fatty acids (FAs) is started by acetyl-CoA carboxylase (ACC, the rate-limiting step), which converts acetyl-CoA into malonyl-CoA, and fatty acid synthase (FAS), which catalyzes all of the reaction steps in the conversion of malonyl-CoA to saturated FAs. The conversion of saturated to monounsaturated FAs is catalyzed by stearoyl-CoA desaturase-1 (SCD-1), whereas the formation of very long-chain FAs is mediated by elongases (ELOVL). The final step in triacylglycerol synthesis involves diacylglycerol acyltransferase (DGAT). Hepatic FA uptake is mediated by fatty acid transport proteins (FATPs) and fatty acid translocase (CD36). Oxidation of FAs occurs within mitochondria and peroxisomes (β-oxidation), or endoplasmic reticulum (ω-oxidation), and facilitates the degradation of activated FAs to acetyl-CoA. Mitochondria catalyze the β-oxidation of the bulk of short-, medium-, and long-chain FAs, and this pathway constitutes the major process by which FAs are oxidized to generate energy. Short- and medium-chain FAs freely enter mitochondria; in contrast, long-chain FAs are activated to acyl-CoA esters targeted to esterification or to mitochondrial β-oxidation. Transfer across the mitochondrial membrane is dependent on the activity of carnitine palmitoyl transferase 1 (CPT1). Peroxisomes are preferentially involved in β-oxidation chain shortening of very long-chain FAs, although the peroxisomal pathway is shorter. Very long-chain FAs are also metabolized by the cytochrome P450 CYP4A ω-oxidation system to dicarboxylic acids. The final product of FA oxidation, acetyl-CoA, is further processed through the tricarboxylic acid cycle, which provides reducing equivalents for oxidative phosphorylation (OxyPhos).

oligonucleotide inhibitors to independently or synergistically knock down ACC1 and ACC2 expression helped resolve the respective roles of these enzymes in the control of *de novo* lipogenesis, revealing that targeting ACC is beneficial in both hepatic steatosis and insulin resistance [39].

FAS determines the rate of *de novo* hepatic lipogenesis; the global deletion of its gene results in embryonic lethality, but liver-specific FAS knockout does not protect against the development of fatty liver [45,46].

Elongation of very long-chain fatty acids protein 6 (ELOVL6) is a microsomal enzyme that regulates the elongation of C12–C16 SFAs and MUFAs. Results from three independent mouse models with loss or gain of function of Elovl6 show that this enzyme is crucial for the development of hepatosteatosis and liver injury, suggesting that the hepatic long-chain FA composition is a determinant in NASH [47].

Global deletion of the stearoyl-CoA desaturase-1 gene (*SCD1*) reduces lipogenic gene expression and increases β-oxidation, protecting against HC/HF diet-induced obesity and insulin resistance [48,49]. Antisense nucleotide inhibitors against hepatic SCD1 also prevent HF/HC diet-induced steatosis [50,51]. Mice with liver-specific knockout of SCD1 are protected against HF/HC diet-induced obesity and hepatic steatosis, and exhibit reduced rates of FA synthesis because the expression of key genes for the lipogenic pathway is reduced [52].

Inactivation of the hepatic diacylglycerol O-acyltransferase 2 (DGAT2) isoform in obese mice results in a significant reduction of TAGs but an increase in oxidative stress and hepatocellular apoptosis, and a worsening of hepatic inflammatory activity and fibrosis; this suggests a protective role of TAGs against the development of hepatic inflammation [53].

2.2. Alterations of fatty acid uptake and oxidation in NAFLD

Non-esterified FAs (NEFAs) are the most abundant circulating energy source under fasting conditions. NEFAs can be produced by the hydrolysis of complex lipids by lipases, or the hydrolysis of fatty acid-CoA by thioesterases. Hepatic NEFA uptake is mediated by diffusion or transporters [fatty acid transport proteins (FATPs) and fatty acid translocase (FAT), CD36], but it is unregulated [18]. Thus, when circulating levels are increased—during post-prandial conditions or during insulin resistance—a relentless hepatic uptake of NEFAs occurs [54]. If energy demand is low, hepatic NEFAs are esterified as TAGs and stored in the cytosol or secreted in the plasma as VLDLs. However, under conditions of energy deficiency, NEFAs are utilized as follows:

- by mitochondria and peroxisomes through β-oxidation;
- by the ER through the ω-oxidation [11].

Mitochondrial β-oxidation is primarily involved in the oxidation of SCFAs, MCFAs, and LCFAs; this process progressively shortens FAs into acetyl-CoA subunits, which may enter the tricarboxylic acid (TCA) cycle for further oxidation to water and carbon dioxide, or may condense in ketone bodies as oxidizable energy substrates for extra hepatic tissues. LCFAs, activated to acyl-CoA esters, cannot directly cross the mitochondrial inner membrane, and the mitochondrial gate is a major factor in the control and regulation of β-oxidation [55]. Transfer across the mitochondrial membrane involves three different steps: (1) transference of the acyl group from CoA to carnitine, catalyzed by carnitine palmitoyl transferase 1 (CPT1); (2) intermembrane transport, catalyzed by acylcarnitine translocase; and (3) reconversion to acyl-CoA ester in the inner face of the mitochondrial inner membrane by CPT2 (Fig. 1). CPT1, being the mitochondrial gateway for FAs, is the main controller of the hepatic β-oxidation flux [56]; its activity is inhibited by malonyl-CoA, the product of the first step of FAs synthesis. β-oxidation involves four individual reactions that generate reducing equivalents (NADH or FADH2), which can then be oxidized to water by the mitochondrial respiratory chain. The energy produced is harnessed in the form of ATP by the oxidative phosphorylation system (OxyPhos), coupled with the transfer of electrons along the respiratory chain.

Peroxisomes are preferentially involved in the β-oxidation chain shortening of VLCFAs [57]. Nevertheless, the peroxisomal pathway is quantitatively minor [58].

Saturated and unsaturated FAs may undergo microsomal ω-oxidation by CYP4A enzymes. This pathway starts with a hydroxylation

reaction in the ER; the resulting ω-hydroxy fatty acids are then dehydrogenated in the cytosol to dicarboxylic acids, which are converted to dicarboxylyl-CoAs. The latter are oxidized by the classical β-oxidation pathway. Although ω-oxidation is a minor pathway of FA metabolism, significant amounts of dicarboxylic acids can be formed when the liver is overloaded with FAs, for example, when the subject is obese or suffers from diabetes, or when the mitochondrial oxidation system is incapable of metabolizing FAs [59].

Many recent studies have investigated the activity of several enzymes involved in FA uptake and oxidation in NAFLD (Fig. 1). Hepatic FA uptake contributes to the steady balance of TAGs in NAFLD [60]. FATPs and CD36 are key contributors to the transmembrane process. Six FATP isoforms have been identified in mammals; examples include FATP2, which is expressed in the liver and kidney, and FATP5, which is a liver-specific isoform [61,62]. In mouse hepatocytes, adenovirus-mediated knockdown of FATP2 or targeted genetic deletion of FATP5 decreases FA uptake and hepatosteatosis [63,64]. CD36 expression is low in hepatocytes [65]. Global deletion of CD36 reduces muscular but increases hepatic triglyceride content [66]. However, NASH patients show CD36 upregulation in the liver, suggesting that this transporter may contribute to hepatic lipid accumulation [67]. Of note, the polyphenol curcumin may activate the transcription of CD36 via the cAMP/protein kinase A (PKA)/CREB pathway, increasing lipolysis and FA β-oxidation [68].

CPT1 inhibition by malonyl-CoA tightly regulates mitochondrial β-oxidation in the liver. An oxidative impairment of CPT1 occurs in animal models of NASH [69]. The expression of a malonyl-CoA-insensitive CPT1 causes a metabolic switch allowing β-oxidation of *de novo*-synthesized LCFAs, demonstrating that control of CPT1A activity by malonyl-CoA is an essential driving force for the metabolic fate of hepatic LCFAs [70].

In NAFLD both lipid oxidation and the TCA cycle are enhanced, whereas ketogenesis is not modified, suggesting that hepatocytes try to counteract excess lipid by increasing oxidation [71]. The higher production of reducing equivalents by lipid oxidation causes an overflow of electrons through the mitochondrial respiratory chain, resulting in higher free radical generation [72,73]. This may lead in turn to mitochondrial dysfunction, with consequent progression of liver pathology [72,74]. Recent research has focused on several proteins that modulate FA mitochondrial oxidation as potential therapeutic targets. Retinol-binding protein 4, a specific vitamin A transporter that exhibits elevated circulating levels in NAFLD patients, is linked to hepatic mitochondrial dysfunction and lipid accumulation [75]. Furthermore, FA oxidation may be promoted by fibronectin type III domain-containing 5 protein, deficiency in which causes liver steatosis by enhancing lipogenesis via the AMPK/mTOR pathway [76]. Leucine-rich pentatricopeptide repeat-containing protein may drive mitochondrial oxidative phosphorylation, promoting FA uptake and oxidation in hepatocytes, and reducing both hepatic and circulating triglyceride and cholesterol levels [77]. Peroxisomal β-oxidation generates hydrogen peroxide and is not coupled with phosphorylating systems [78]. Microsomal oxidation also participates in the adaptive response induced by lipid accumulation and in redox unbalance; in particular, cytochromes P4502E1 and P4504A (CYP2E1 and CYP4A, respectively) are the major microsomal sources of oxidative stress in NAFLD [79]. Thus, increased oxidation of fat by microsomes and peroxisomes occurs in NAFLD, and contributes to oxidative stress [80–82]; malnutrition causes a secondary form of NAFLD. It is noteworthy that hepatic peroxisomal and mitochondrial dysfunction were found in a recent experimental model of malnutrition (comprising rats fed on a low-protein diet) characterized by hypoalbuminemia and hepatic steatosis, consistent with the human phenotype [83].

2.3. Change in cholesterol metabolism during NAFLD progression

Cholesterol uptake, synthesis, esterification, intracellular transport, and excretion in hepatocytes are modulated through a coordinated network involving cholesterol sensors and nuclear transcription factors [84].

Uptake from plasma lipoproteins and *de novo* synthesis represent the main sources of cellular cholesterol. Dietary cholesterol is transported from the intestine to the liver through chylomicron remnants after hydrolysis of chylomicron triglyceride by the lipoprotein lipase in muscles and adipose tissue. The uptake of chylomicron remnants is mediated by low-density lipoprotein (LDL) receptors or by LDL receptor-related protein (LRP), or they may be sequestered in the space of Disse by the binding of apolipoprotein E (apoE) to heparan sulphate proteoglycans and/or the binding of apoB to hepatic lipase [85].

The pathway of cholesterol synthesis is quite elaborate: it starts with acetyl-CoA, which is exported from the mitochondria to the cytosol to synthesize hydroxymethylglutaryl-CoA (HMG-CoA). The subsequent steps occur in the smooth ER, where HMG-CoA reductase reduces HMG-CoA to (3*R*) − 3,5-dihydroxy-3-methylpentanoic acid (mevalonate); this enzyme is the major target of regulation in the entire pathway [86].

Free cholesterol (FC) may be promptly esterified after its synthesis, and a balance between the availability of free and esterified cholesterol is critically important for hepatocellular function. Two different acyl-coenzyme A:cholesterol acyltransferase (ACAT) enzymes responsible for the intracellular esterification of cholesterol have been described, of which ACAT2 is mostly present in the liver [87,88].

Intracellular trafficking involving several transporters plays a major role in the proper disposition of internalized cholesterol and in the regulation of cholesterol efflux. Caveolin-1, the main structural protein of caveolae, exhibits cholesterol-binding and transport activity [89]. Niemann–Pick C1 and C2 proteins (NPC1 and NPC2) are involved in the trafficking of endocytosed lipoprotein cholesterol from the endolysosomal compartment to the rest of the cell [90]. Finally, a family of proteins containing steroidogenic acute regulatory protein (StAR)-related lipid transfer (START) domains involved in cholesterol trafficking is particularly relevant to cholesterol trafficking from intracellular stores to mitochondria [91].

A hepatocyte is a unique type of cell that is capable of degrading cholesterol and removing it in large quantities via biliary elimination. Hepatocytes are able to do this because they express high levels of the enzyme cholesterol 7α-hydroxylase (CYP7A1), which initiates and rate-limits this multi-step conversion process [92]. Sterol efflux from cells is mediated by ABC transporters, a family of integral membrane proteins that actively transport a variety of small molecules across cell membranes; at least four ABC-transporters have been identified in mammals: ABCA1, ABCG1, ABCG4, and ABCG5/ABCG8 [93].

Regardless of its origin, hepatic cholesterol undergoes several alternative metabolic pathways: (1) excretion or efflux into the blood in the form of VLDL or through ABCA1 to nascent high-density lipoprotein (HDL) particles; (2) excretion and uptake through bile via ABCG5/G8 and Niemann–Pick C1-like 1 protein (NPC1L1), respectively; (3) deposition as CE; and (4) the hepatic cholesterol becomes the substrate for bile acid (BA) synthesis [94].

The role of cholesterol accumulation in NAFLD is an emerging topic, because it can further contribute to the alteration of the cellular redox status. Several studies have focused on altered cholesterol metabolism during NAFLD (Fig. 2). High plasma cholesterol levels, a major risk factor for atherosclerosis, can be reduced by inhibiting lipoprotein production; this is associated with hepatic steatosis, even though it has been recently demonstrated that the lowering cholesterol effect induced by microRNA-30c limits steatosis by diminishing lipid synthesis [95]. The authors of several studies have reported that FC (but not CE) accumulates in cases of human NAFLD, and is strongly associated with the progression and severity of liver damage [96,97]. This accumulation arises from high cholesterol synthesis rather than elevated intestinal absorption [98,99]. Interestingly, dietary fat and cholesterol may promote steatosis, the accumulation of cholesterol crystals, and

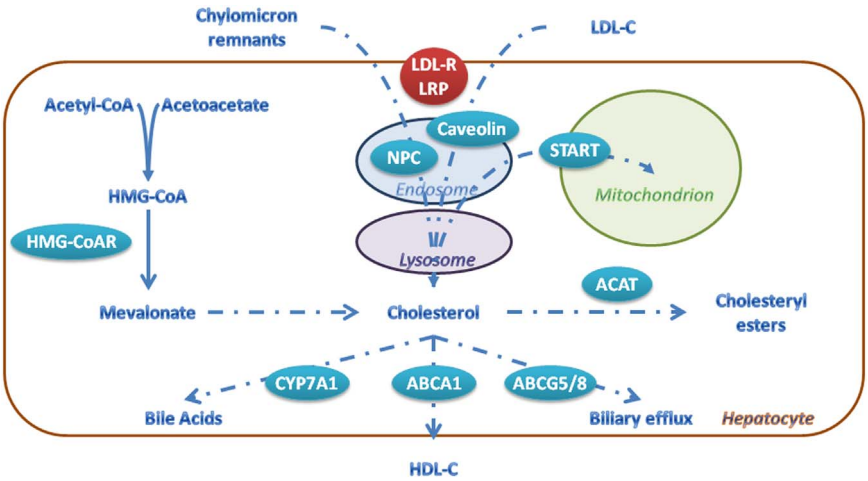

Fig. 2. General overview of hepatic cholesterol metabolism, particularly focusing on altered pathways in non-alcoholic fatty liver disease (NAFLD) (please see the text for details). Dietary cholesterol is transported from the intestine through chylomicron remnants, whereas endogenous cholesterol is transported from peripheral tissues by a low-density lipoprotein (LDL-C); once in the liver, the uptake of both is mediated by LDL receptors or by the LDL receptor-related protein (LRP). Cholesterol synthesis starts when acetyl-CoA synthesizes hydroxymethylglutaryl-CoA (HMG-CoA), then HMG-CoA reductase reduces HMG-CoA to mevalonate; HMG-CoA reductase is the major regulator of the entire pathway. Free cholesterol may be promptly esterified after its synthesis through acyl-coenzyme A:cholesterol acyltransferase (ACAT) enzymes. Intracellular trafficking involves transporters such as caveolin-1, the main structural protein of caveolae, as well as Niemann–Pick C1 and C2 proteins (NPC1 and NPC2), which account for the trafficking of endocytosed lipoprotein cholesterol from the endolysosomal compartment to the rest of the cell. Moreover, a family of proteins containing steroidogenic acute regulatory protein (StAR)-related lipid transfer (START) domains is relevant to facilitating cholesterol trafficking from intracellular stores to mitochondria. Cholesterol 7α-hydroxylase (CYP7A1) initiates and rate-limits the conversion of cholesterol to bile acids; sterol efflux from cells is also mediated by ABC transporters: ABCA1 and ABCG5/8 modulate cholesterol efflux to nascent high-density lipoprotein (HDL) particles and to bile, respectively.

inflammasome activation through distinct but complementary pathways [100].

Alterations in cholesterol uptake may also be involved in the pathogenesis of NASH, because the expression of LDL receptor is reduced in NAFLD patients, and mutant mice for LRP6 exhibit both steatohepatitis and steatofibrosis [101,102].

Changes related to proteins involved in intracellular cholesterol transport are also reported for NAFLD and NASH. Caveolin-1 expression and distribution are altered in lipid droplets and mitochondria from dietary rodent models of NAFLD [103,104]. NPC1 or NPC2 deficiency causes FC accumulation in most tissues, including the liver [90]. Decreased NPC1 in mice is associated with NAFLD and hyperinsulinemia [105]. Compared with lean subjects, hepatic StAR protein expression is 7- and 15-fold higher in patients with steatosis and NASH, respectively [97].

With regard to the catabolism of cholesterol to BA, patients with NAFLD and NASH present with a decreased expression of CYP7A1; thus, decreased cholesterol biotransformation contributes to intrahepatic accumulation [101]. Moreover, cholesterol overload in NASH is caused by impaired efflux, as suggested by reduced hepatocyte ABCA1 activity [106,107] as well as decreased ABCG5/G8 expression [106,107]; interestingly, ABCA1 overexpression improves experimental liver disease [108]. ABCG5/G8 knockout mice present with NAFLD, hepatocyte apoptosis, ER stress, and hepatic insulin resistance, accelerating high-fat, diet-induced NASH [109]. It is worth noting that the excretion of biliary cholesterol by liver-specific ABCG5/G8 overexpression is counteracted by its re-absorption through intestinal NPC1L1; in this model, the inhibition of this latter transporter by ezetimibe is ultimately able to induce hepatic cholesterol loss [110].

3. Lipidomics contribution to the study of NAFLD

Lipidomics is a subcategory of metabolomics that facilitates the identification and quantification of the diverse lipid species found in biological samples using pioneering methodologies such as mass spectrometric approaches [111]. Data processing and interpretation are major challenges in this field because of the origins, cellular localization, and metabolic pathways underlying lipid alterations. Nevertheless, innovative lipidomic technologies may be useful for investigating the pathophysiology of NAFLD and, most importantly, for the identification of non-invasive diagnostic biomarkers of pathology progression [111–113].

Although the majority of hepatic lipids in NAFLD subjects accumulate in the form of TAGs, various NEFAs, FC, CE, ceramide, and phospholipids are stored in the liver [114]. Experimental evidence suggests that the quality, as opposed to the quantity, of stored lipids may play a determinant role in the progression from steatosis to steatohepatitis [115].

Studies using various dietary animal models have attempted to characterize the specific lipid signature of NASH [116,117]. Our recent study, performed using nutritional models of high FA, high cholesterol, or high fat and high cholesterol diets, has generated some interesting hypotheses on the role of the interaction of lipid and cholesterol metabolites in liver injury during progression from simple steatosis to steatohepatitis [118].

The authors of a human study profiled the NEFA, DAG, TAG, FC, CE, and phospholipid contents in normal livers, and compared them with those in NAFL and NASH, evaluating the distribution of FAs within these classes. Despite increased levels of DAGs and TAGs in NAFLD relative to a normal liver, hepatic NEFAs were unchanged (even though the SFA palmitic acid and the MUFA oleic acid were elevated in NAFLD), but the accumulation of hepatic FC progressively increased with the evolution of liver injury [96]. Thus, since hepatic NEFAs were not consistently elevated, we can conclude that their efficient esterification to form TAGs is a protective mechanism against NEFA-induced lipotoxicity [119]. The authors of further human studies described that, even in the absence of any difference in dietary FA composition, biologically active long-chain PUFAs (5Z,8Z,11Z,14Z,17Z) −5,8,11,14,17-icosapentaenoic acid (eicosapentaenoic acid), (4Z,7Z,10Z,13Z,16Z,19Z)-docosa-4,7,10,13,16,19-hexaenoic acid (docosahexaenoic acid), and (5Z,8Z,11Z,14Z) −5,8,11,14-eicosatetraenoic acid (arachidonic acid) were lower in NASH than in simple steatosis [120,121].

A study on the plasma lipidome of NAFLD patients showed that the circulating lipid pool explained the large hepatic availability of several FAs (such as oleic acid), but also suggested that impaired peroxisomal

PUFA metabolism and non-enzymatic oxidation are associated with progression to NASH. In particular, this study found significant increases in the plasma levels of palmitoleic and oleic MUFAs, and all-*cis*−6,9,12-octadecatrienoic acid (γ-linolenic acid, 18:3n-6) and *cis*,*cis*,*cis*−8,11,14-eicosatrienoic acid (dihomo-γ-linolenic acid, 20:3n-6) PUFAs, but decreased linoleic acid content in both NAFL and NASH [112]. Differences in the plasma lipid content between NAFLD patients with simple steatosis or steatohepatitis are actively investigated to diagnose NASH non-invasively. Interestingly, an increase in the plasma levels of the lipoxygenase metabolites 5(S)-hydroxyeicosatetraenoic acid (5-HETE), 8-HETE, and 15-HETE may characterize NAFLD progression, and 11-HETE levels are significantly increased in NASH [112]. In another study, a panel of 20 plasma metabolites including glycerophospholipids, sphingolipids, sterols, and various aqueous small molecular weight components involved in cellular metabolic pathways, facilitated differentiation between NASH and steatosis [122].

To date, free radical-mediated linoleic acid oxidation products and specific PUFA metabolites have been identified as novel lipidomic biomarkers of human NASH [113,123]. In particular, Feldstein et al. reported that levels of 9- and 13-hydroxyoctadecadenoic acids (HODE), and 9- and 13-oxo-octadecadenoic acids (oxoODE) were significantly elevated in patients with NASH, compared with patients with steatosis; furthermore, there was a strong correlation between these oxidation products and liver histopathology [113]. Loomba et al. demonstrated that profiling of plasma levels of eicosanoid and other PUFA metabolites can be used to differentiate between steatosis and steatohepatitis, and suggested 11,12-dihydroxy-eicosatrienoic acid (11,12-diHETrE), 13,14-dihydro-15-keto prostaglandin D2 (dhk PGD2), and 20-carboxy-arachidonic acid (20-COOH AA) as biomarkers for the non-invasive diagnosis of NASH [123].

Thus, oxidized lipid metabolites may be promising markers for the non-invasive diagnosis of NASH and, once well-defined, they may be used to monitor therapeutic effects in huge patient cohorts.

4. Role of lipotoxic products in NAFLD development

The progression from simple steatosis to NASH may be the result of hepatic lipotoxicity. This term was coined to describe the toxic effects of excessive NEFAs on pancreatic beta cell survival [124], and it is currently used to refer to cellular injury and death caused by several lipid classes [2]. The identification of lipotoxic species is challenging because a wide variety of metabolites can be derived from FAs of varying chain length, degrees of desaturation, and location [125].

A considerable body of evidence indicates that NEFAs—and not their esterified products—mediate lipotoxicity.

NEFAs may exert lipotoxic effects in the liver by triggering apoptosis, mitochondrial–lysosomal dysfunction, and ER stress [119]. The experimental diversion of palmitic acid to TAG formation—achieved by co-treatment with oleic acid or by overexpression of SCD1—prevents SFA toxicity [126,127]. An excess of NEFAs may induce excessive ROS production and consequent cellular dysfunction and death through apoptosis and/or necrosis [2,128]. Hepatocytes overwhelmed by NEFAs (in particular saturated NEFAs) activate a variety of intracellular responses resulting in lipotoxic stress in both mitochondria and the ER [129]. Because mitochondrial FA oxidation is still functional in NAFLD [130], an increased rate of substrate oxidation during the early stages of liver injury may be followed by an impairment of mitochondrial function and increased oxidative stress, despite the adaptive uncoupling mechanism, as the disease progresses [72,73]. Moreover, the enhanced mitochondrial oxidative metabolism in humans with high levels of intrahepatic TAGs provides a potential link with oxidative stress and liver damage [71]. Studies performed on liver cells demonstrated that excess SFAs enhanced TCA cycle fluxes with a consequent increase in mitochondrial ROS production and apoptosis [131], whereas exposure to a lipid emulsion triggered an excess of mitochondrial ROS leading to necrosis but not apoptosis [132]. An excess of SFAs in hepatic cell lines impairs TAG synthesis in the ER, probably because of the formation of lipid intermediates, which induce ER stress with the accumulation of unfolded or misfolded proteins [133]. This causes the unfolded protein response (UPR), activating an intracellular signalling cascade that leads to an increased transcription of ER-resident chaperones and an overall decrease in protein synthesis [128]. A human study demonstrated that the UPR is activated to varying degrees in the livers of NAFLD sufferers [134]. The UPR signalling pathway is linked to lipid and membrane biosynthesis, the action of insulin, inflammation, and apoptosis [135]. Under normal conditions, the redox environment in the ER lumen is different from that in other cell organelles, and favours disulfide formation for protein folding and assembly, indicating that ROS formation and oxidative stress are integral components of UPR [136]. Oxidative stress during ER disruption may be the result of an enzymatic mechanism or reduced glutathione (GSH) depletion [137]. Moreover, accumulation of unfolded proteins in the ER may generate mitochondrial ROS production through Ca^{2+} release and depolarization of the inner membrane [138]. Thus, ER stress-induced oxidative stress and UPR triggered by altered lipid metabolism are closely related in the progression of NAFLD, and may represent other interesting therapeutic targets.

Free cholesterol accumulation also plays an important role in liver injury within the context of NAFLD. Although the precise mechanisms of FC lipotoxicity in NASH have still not been completely delineated, current evidence indicates that FC accumulation may activate both Kupffer and stellate cells, and induce UPR, mitochondrial dysfunction, and ER stress [139]. FC can be the direct cause of apoptosis and necrosis in hepatocytes [140]. Accumulation of cholesterol crystals in Kupffer cells and increased inflammasome activation were observed in a rodent model of NASH [141]. Moreover, FC accumulation in hepatic stellate cells increases levels of toll-like receptor 4 protein (TLR4) and sensitizes them to transforming growth factor β (TGFβ), resulting in the progression of liver fibrosis [142]. FC accumulation induces mitochondrial oxidative stress through the depletion of GSH, sensitizing hepatocytes to pro-inflammatory cytokines [143].

Oxidants such as free radicals or non-radical species may attack NEFAs containing double bonds (particularly PUFAs), producing lipid peroxy radicals and hydroperoxides [144]. Glycolipids, phospholipids, and non-esterified cholesterol are also well-known targets of potentially lethal peroxidative modification [145]. NEFAs and FC can be oxidized by lipoxygenases, cycloxygenases, and cytochrome P450 [144]. Lipid hydroperoxides are the primary products of lipid peroxidation. Among the different aldehydes that can be formed as secondary products during lipid peroxidation, MDA and 4-HNE are the most extensively studied: MDA appears to be the most mutagenic product of lipid peroxidation, whereas 4-HNE is the most toxic [146,147]. Human NAFLD HNE adducts—major aldehydic metabolites of lipid peroxidation—are widely detected in the cytoplasm of hepatocytes and sinusoidal cells in zone 3, where histological damage is often observed. Moreover, the amount of HNE adducts significantly correlates with the grade of necro-inflammation and the stage of fibrosis [148]. We have also reported a significant increase in mitochondrial HNE–protein adducts during NASH development [72]. The toxicity of HNE may be exerted by the addition of reactive carbonyl functional groups on proteins, a process generically termed “protein carbonylation” [149]. Protein carbonylation can be the direct result of several reactions, or it may be derived from an indirect mechanism involving the hydroxyl radical-mediated oxidation of lipids. Recent studies have suggested the higher prevalence of protein carbonylation from lipid-derived aldehydes rather than via direct amino acid side chain oxidation [150]. Post-translational protein oxidation frequently leads to enzyme inactivation or targeted degradation, but it can also cause a functional gain for certain metabolic signalling pathways [149]. Some proteins involved in FA metabolism may be post-translationally modified. In particular, specific adducts between HNE and CPT-1 were observed in a model of NAFLD, with consequent decreased expression and activity

of the protein [69]. Similarly, adducts between 4-HNE and uncoupling protein 2 (UCP-2) are also associated with increased uncoupling in NASH [72].

Free cholesterol is also susceptible to enzymatic and non-enzymatic oxidation, leading to the formation of oxysterols. These compounds participate in the regulation of cellular cholesterol homeostasis, but they are also suspected to play key roles in several pathologies, including cardiovascular and inflammatory disease, cancer, neurodegeneration, and NAFLD [151]. Serum oxysterol levels increase in patients with biopsy-proven NAFLD. However, despite indications that oxysterols are pro-apoptotic, pro-inflammatory, and pro-fibrogenic, there is no experimental evidence that they change cell viability in hepatocytes [152,153]. To date, there has been little research into the role of oxysterols in NASH pathogenesis.

5. Lipid oxidation products induce insulin resistance in NAFLD

Insulin resistance is one of the pathophysiological hallmarks of NAFLD [154,155]. Under normal conditions, postprandial insulin secretion: (1) induces hepatic glucose uptake by stimulating the translocation of glucose transporter 2 (GLUT2) from intracellular vesicles to the plasma membrane; (2) inhibits gluconeogenesis; and (3) stimulates glycogen synthesis [19]. When not redirected to glycogen pools, excess glucose is used in *de novo* lipogenesis. Moreover, insulin triggers lipogenesis, suppresses peripheral lipolysis by inhibiting hormone-sensitive lipase, and indirectly antagonizes mitochondrial FA oxidation by increasing malonyl-CoA concentration [156,157]. In clinical practice, insulin resistance is defined as a state in which a given concentration of insulin is associated with a subnormal glucose response [158].

Hepatic lipid accumulation is strongly dependent on insulin sensitivity; however, this causal relationship has not yet been completely defined, because it is not clear whether hepatic steatosis increases insulin resistance or, conversely, NAFLD is the final effect of insulin resistance. It is conceivable that a common underlying molecular pathophysiology may account for both [159]. Some experimental evidence suggests that a redox imbalance induced by excess lipid oxidation may interfere with the normal insulin signalling pathways, inducing insulin resistance [160,161]. Furthermore, according to their oxidative properties, different lipid classes may exert diverse effects on insulin sensitivity. Dietary supplementation of n-3 long-chain polyunsaturated FAs reduces hepatic lipid content, with concurrent antioxidant and anti-inflammatory responses, and an improvement in insulin sensitivity [162,163]. Several studies suggest that HNE is able to induce insulin resistance in adipose tissue, skeletal muscle, and liver cells by modulating the insulin-dependent signalling pathway at different levels [164–167]. However, data related to the effects of reactive aldehydes on insulin signalling in NAFLD are lacking, and further investigation is warranted.

Hepatic cholesterol accumulation *per se* does not induce insulin resistance but increases hepatic steatosis [168,169]. Several experimental models have clarified that both high fat levels and cholesterol are needed to produce hepatic steatosis and insulin resistance [168,170]. Because free cholesterol accumulation in mitochondria leads to glutathione depletion, lipid peroxidation [143], and the formation of oxysterols [153], it is conceivable that it could impair the redox status and alter insulin-dependent signalling. Serum concentrations of the oxysterols 7-ketocholesterol and 7β-hydroxycholesterol are consistently associated with insulin resistance [171,172]. Nevertheless, to date no studies designed to clarify the impact of specific oxysterols on insulin signalling during NAFLD have been carried out.

Taken together, the current evidence suggests that the lipids that accumulate in cases of liver steatosis are bioactive substances that interfere with the ability of hepatocytes to respond to changes in the level of insulin. However, to date, studies have failed to identify a specific lipid that can determine hepatic insulin resistance [159]. Thus, excess bioactive lipids do not act as contributing factors but rather as part of a signal in which radical molecules generate reactive intermediates and exert signalling functions.

6. Lipid oxidation in the cross-talk between metabolism and inflammation during NAFLD

Both lipid metabolism and inflammation are simultaneously and coordinately regulated in experimental NAFLD [173,174]. Control of lipid metabolism is mediated by a network of nuclear receptors (NRs) that respond to several ligands to tightly regulate and coordinate the expression of the enzymes involved in hepatic lipogenesis or lipoxidation [175]. Several endogenous and exogenous lipids such as cholesterol and FAs act as physiological NR ligands. Thus, NRs may be viewed as "lipostats" because their activation frequently promotes the metabolism/catabolism of their respective ligands and/or provides negative feedback for self-termination of their synthesis [176]. Interestingly, NEFA- and cholesterol-derived oxidative products may act as NR ligands, may alter their DNA binding, or may impair their nuclear import [177]. As with lipid metabolism, a crucial factor in inflammation—the critical response to tissue damage or infection in which secreted mediators (such as cytokines, chemokines, and eicosanoids) coordinate cellular defences and tissue repair—is the level of gene transcription [178,179].

By signalling to the nucleus, the end-products of NEFA and cholesterol peroxidation, such as HNE and oxysterols, play a primary role in the modulation of both lipid metabolism and inflammation in NAFLD (Fig. 3).

Oxysterols are natural ligands of liver-X receptors (LXRs) alpha (NR1H3) and beta (NR1H2), which are NRs and are critical for the control of lipid homeostasis [180]. In addition to their function in lipid metabolism, LXRs modulate immune and inflammatory responses in macrophages, and this property makes them particularly attractive for intervention in human metabolic diseases [181,182]. LXR expression is four times higher in NAFLD than in healthy subjects [183]. A significant increase in the serum levels of 4β-hydroxycholesterol, 25-hydroxycholesterol, and 27-hydroxycholesterol, which are LXRα ligands, has been reported in NAFLD patients [153]. When LXRα is activated by these oxysterols, the FA biosynthetic pathway is stimulated through the upregulation of sterol regulatory element-binding protein 1c (SREBP-1c) [184]. SREBP-1c controls hepatic *de novo* lipogenesis, primarily by regulating the expression of key genes involved in FA homeostasis and glucose metabolism [185–187]. SREBP-1c expression is enhanced in NAFLD patients, leading to the increased synthesis of FAs in hepatocytes [188–190]. Liver-specific expression of transcriptionally active SREBP-1c is associated with hepatic steatosis [191]. Hepatic expression of LXRα and its related lipogenic and inflammatory genes is abnormally increased in NAFLD patients, and LXRα expression correlates with the degree of hepatic fat deposition as well as hepatic inflammation and fibrosis [192,193]. However, additional studies are needed to clarify the mechanism by which oxysterols modulate the LXR-dependent signalling pathway in NAFLD.

Two key nuclear receptors, sterol regulatory element binding protein-2 (SREBP-2) and farnesoid X receptor (FXR), are involved in the regulation of hepatic cholesterol/oxysterol homeostasis. Hepatic SREBP-2 upregulation parallels the severity of liver disease in NAFLD [97,101,107]. FXR is an NR and is functionally related to LXR signalling [194] because it regulates a wide variety of target genes that are critically involved in the control of bile acids, lipid and glucose homeostasis, and the regulation of immune responses [195]. FXR-deficient mice display elevated serum levels of TAGs and cholesterol, demonstrating the crucial role of FXR in lipid metabolism [196]. The decreased expression of hepatic FXR is associated with an increased expression of LXRs, SREBP-1c, and hepatic TAG synthesis in NAFLD patients [197]. Consistent with this, activation of FXR by bile acids or

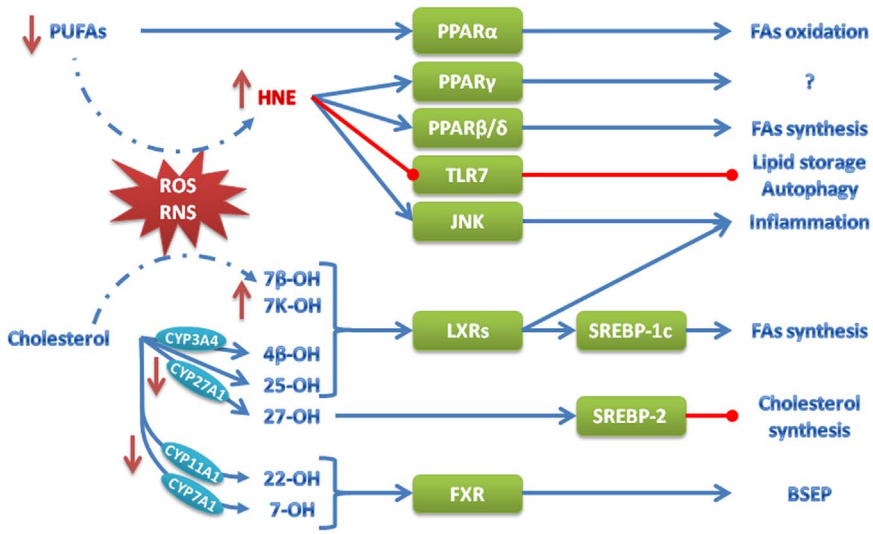

Fig. 3. Lipid metabolism and inflammation pathway regulation by lipid oxidation products in non-alcoholic fatty liver disease (NAFLD) (please see the text for details). Polyunsaturated fatty acids activate peroxisome proliferator-activated receptor α (PPARα), which induces genes involved in fatty acid (FA) oxidation; PPARα expression is reduced in NAFLD, and a defect in PPARα-inducible FA oxidation accounts for severe hepatic lipid overload. The main role of PPARγ in the liver is related to the regulation of glucose and lipid metabolism; hydroxynonenal (HNE) may activate PPARγ, but this field requires further investigation in the liver. HNE activates PPARβ/δ receptor, which controls genes involved in glucose homeostasis and FA synthesis/storage. HNE may inhibit the toll-like receptor 7 (TLR7) signalling pathway, which in turn reduces lipid accumulation and autophagy in hepatocytes, preventing the progression of NAFLD. Finally, HNE activates the JNK signalling pathway in hepatocytes, being an active mediator of hepatocellular injury. Oxysterols are the natural ligands of the liver-X receptors (LXRs), which are critical for the control of both FA synthesis and inflammation. There are significantly increased serum levels of 4β-hydroxycholesterol (4β-OH), 25-hydroxycholesterol (25-OH), and 27-hydroxycholesterol (27-OH) in NAFLD patients. When LXRα is activated by these oxysterols, the FA biosynthetic pathway is stimulated through the upregulation of sterol regulatory element-binding protein 1c (SREBP-1c). Hepatic expression of CYP27A1, which synthesizes 27-OH, is reduced in non-alcoholic steatohepatitis (NASH); because 27-OH may prevent SREBP-2 activation, this reduction may enhance SREBP-2-mediated cholesterol synthesis, further aggravating hepatic cholesterol overload. The oxysterols 22-hydroxycholesterol (22-OH) and 7-hydroxycholesterol (7-OH) activate farnesoid X receptor (FXR), inducing the expression of the bile salt export pump (BSEP), and accounting for the secretion of bile acids from the liver. However, the hepatic expression of both CYP11A1 and CYP7A1, which convert cholesterol to 22-OH and 7-OH, respectively, is reduced in NASH.

synthetic FXR agonists lowers the level of plasma TAGs by repressing hepatic SREBP-1c expression and increasing hepatic FA oxidation [198,199]. The oxysterol 22-hydroxycholesterol activates FXR (but not LXRs), inducing the expression of the bile salt export pump (BSEP), which accounts for bile acid secretion from the liver [200]. This finding suggests that oxysterols may act as dual ligands that regulate both LXRs and FXR target genes in NAFLD, in a complementary pathway addressed to remove excess cholesterol. Hepatic expression of CYP7A1, the liver-specific microsomal cytochrome P450 that converts cholesterol to 7-hydroxycholesterol during bile acid formation, was reduced in an experimental model of NASH, resulting in an impaired activation of FXR with deleterious metabolic and proinflammatory effects [107]. Hepatic expression of CYP27A1, the mitochondrial cytochrome P450 that synthesizes 27-hydroxycholesterol, is reduced in NASH [201]. 27-hydroxycholesterol may prevent SREBP-2 activation and up-regulate ABC transporter expression via LXRα, inhibiting cholesterol uptake and synthesis, and promoting cholesterol excretion [202]. Thus, the reduction of CYP27A1 expression observed in NASH may enhance SREBP-2-mediated cholesterol synthesis and uptake, and may reduce cholesterol excretion by ABCA1 transporter, further aggravating hepatic cholesterol overload [107].

HNE may affect lipid metabolism via signal transduction and gene expression, mostly by the regulation of peroxisome proliferator-activated receptors (PPARs). These NRs are activated by peroxisome proliferators, a miscellaneous group of rodent hepatocarcinogens that include hypolipidemic drugs, plasticizers, and herbicides; in vertebrates, four PPAR isoforms termed α, γ, and β/δ are described [203]. PPARα induces genes involved in mitochondrial, peroxisomal, and microsomal FA oxidation [204–206]. The PUFAs eicosapentaenoic acid (EPA, C20:5n-3) and docosahexaenoic acid (DHA, C22:6n-3), and LCFA-CoA bind and activate PPARα increasing FA oxidation, gluconeogenesis, and ketogenesis [207]. The expression of PPARα was significantly reduced in a rodent model of NAFLD; however, PPARα agonists improve steatosis [208,209]. A defect in PPARα-inducible FA oxidation accounts for severe hepatic lipid overload [206,210,211]. The expression of PPARα is reduced in NAFLD patients, and PPARα gene polymorphism distribution may influence the pathogenesis of human hepatic steatosis [189,212]. PPARγ plays a crucial role in adipogenesis and insulin sensitization; the main role of PPARγ in the liver is related to the regulation of glucose and lipid metabolism [213,214]. The use of PPARγ ligands such rosiglitazone or pioglitazone for the treatment of NAFLD patients is controversial. Some studies have demonstrated an improvement in glucose and lipid metabolism, as well as hepatic inflammation and fat storage [215–217]. However, pioglitazone was not effective in improving the histological features of NASH in another human trial [218]. It is worth noting that the treatment response of PPARγ ligands is extremely variable and difficult to predict, owing to single nucleotide polymorphisms in several target genes [219]. HNE is effective in the modulation of PPARγ activity through the pathways that regulate leukemic cell growth and differentiation [220]. However, there are no data on the free radical biology of PPARγ in hepatocytes, and this field merits further investigation.

PPARβ/δ is expressed in most metabolically active tissues, and controls an array of genes involved in glucose homeostasis and FA synthesis/storage, mobilization, and catabolism in a tissue-specific manner [221–223]. HNE activates the PPARβ/δ receptor in transient transfection reporter assays [224]. Moreover, the activation of PPARβ/δ by 4-HNE increases the expression of alcohol dehydrogenase and glutathione-S-transferases, providing evidence of a feedback regulation of gene expression that reduces liver toxicity, and revealing cross-talk between PPARβ/δ and energy homeostasis during inflammation [224].

HNE is described as one of the most potent physiological regulators of transcription factors that mediate inflammation and cellular immune response [225]. Toll-like receptors (TLRs), a family that comprises approximately 13 pattern recognition receptors, are the most important innate immune response factors involved in host defence against foreign pathogens [226]. TLRs may play a crucial role in the pathogenesis of NAFLD [227–229]. The activation of the TLR7 signalling

pathway reduces lipid accumulation and autophagy in hepatocytes, preventing the progression of NAFLD; however, both HNE and MDA are able to inhibit this pathway, exacerbating NAFLD [230]. HNE may activate other TLRs, such as TLR4, inducing cellular apoptosis, but this has not yet been evidenced in hepatocytes [231,232]. It is interesting to note that HNE, together with 27-hydroxycholesterol, may activate TLR4 and its nuclear factor-κB (NF-κB) downstream signalling, contributing to atherosclerotic plaque instability and rupture [232]. This latter evidence suggests that these oxidized lipids could contribute to inflammatory signalling in NAFLD. NF-κB and activator protein 1 (AP-1) are downstream signalling molecules that couple receptor ligation to the activities of several classes of signal-dependent transcription factors, such as c-Jun NH_2-terminal kinase (JNK) [226]. HNE sensitizes hepatocytes to tumor necrosis factor killing by suppressing NF-κB transactivity in alcoholic liver disease [233]. Moreover, HNE can cause synergistic overactivation of the JNK signalling pathway in hepatocytes, strengthening the idea that this compound is not just a passive biomarker of hepatic oxidant stress but rather an active mediator of hepatocellular injury [234]. These data support the notion that intracellular HNE levels can be modulated as a potential therapeutic option for NAFLD, but further investigation is required.

7. Concluding remarks

The development and progression of NAFLD is characterized by hepatocellular redox imbalance, which may depend on, but also contribute to, the impaired regulation of lipid metabolism. Oxidative stress and lipid peroxidation products, such as reactive aldehydes and oxysterols, are generated during the transition from simple steatosis to steatohepatitis. The mechanisms by which such compounds contribute to the progression of NAFLD are complex and not fully understood. Oxidized lipids such as HNE and oxysterols are not only markers of oxidative stress but play a significant role in the modulation of cell metabolism and in the control of inflammatory pathways. Accordingly, antioxidants may limit NAFLD progression by reducing the formation of lipoperoxides. However, the encouraging results from preclinical experiments have not been confirmed in clinical trials, probably because better definitions of biologically relevant reactive species, oxidation mechanisms, and targets of cellular modification are required to design more efficient interventions. All these data suggest a scientific rationale for targeting liver lipid oxidation with novel therapeutic strategies, supporting the assumption that a selective control of cellular redox in hepatocytes may have a favourable impact on organ dysfunction, lipid homeostasis, and inflammation. The modulation of lipid oxidation products might impact the signalling pathways that lead to the development and progression of NAFLD.

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