



## Commentary

## Targeting mitochondria to oppose the progression of nonalcoholic fatty liver disease

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## ABSTRACT

Nonalcoholic fatty liver disease (NAFLD) is a condition characterized by the excessive accumulation of triglycerides in hepatocytes. NAFLD is the most frequent chronic liver disease in developed countries, and is often associated with metabolic disorders such as obesity and type 2 diabetes. NAFLD definition encompasses a spectrum of chronic liver abnormalities, ranging from simple steatosis (NAFL), to steatohepatitis (NASH), significant liver fibrosis, cirrhosis, and hepatocellular carcinoma. NAFLD, therefore, represents a global public health issue. Mitochondrial dysfunction occurs in NAFLD, and contributes to the progression to the necro-inflammatory and fibrotic form (NASH). Disrupted mitochondrial function is associated with a decrease in the energy levels and impaired redox balance, and negatively affects cell survival by altering overall metabolism and subcellular trafficking. Such events reduce the tolerance of hepatocytes towards damaging hits, and favour the injurious effects of extra-cellular factors. Here, we discuss the role of mitochondria in NAFLD and focus on potential therapeutic approaches aimed at preserving mitochondrial function.

## 1. Introduction

Nonalcoholic fatty liver disease (NAFLD) includes a spectrum of

disorders ranging from the simple liver steatosis to nonalcoholic steatohepatitis (NASH) with increasing inflammation and fibrosis (Fig. 1), to cirrhosis and its complications, including the potential evolution to

**Abbreviations:** ANG, angiotensin; ATP, adenosine triphosphate; BCAA, branched-chain amino acids; DAG, diacylglycerol; ER, endoplasmic reticulum; Fe/S, iron-sulfur; FFA, free fatty acid; GPD2, glycerol phosphatase dehydrogenase-2; GFP, green-fluorescence protein; GSH, glutathione; HCC, hepatocellular carcinoma; iNOS, inducible nitric oxide synthase; KGDH,  $\alpha$ -ketoglutarate dehydrogenase; LCFA, long chain fatty acids; MAM, mitochondria-associated membranes; MPC, mitochondrial pyruvate carrier; mtDNA, mitochondrial DNA; MPTP, mitochondrial permeability transition pore; NAFL, nonalcoholic fatty liver; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; NO, nitric oxide; NOX4, NADPH oxidase-4; PDH, pyruvate dehydrogenase; PSSG, protein mixed disulphides; PSH, protein sulfhydryl; ROS, reactive oxygen species; SAME, S-adenosyl-L-methionine; SOD, superoxide dismutase; TCA, tricarboxylic acids; TFAM, transcription factor A; TG, triglycerides; TNF- $\alpha$ , tumor necrosis factor-alpha

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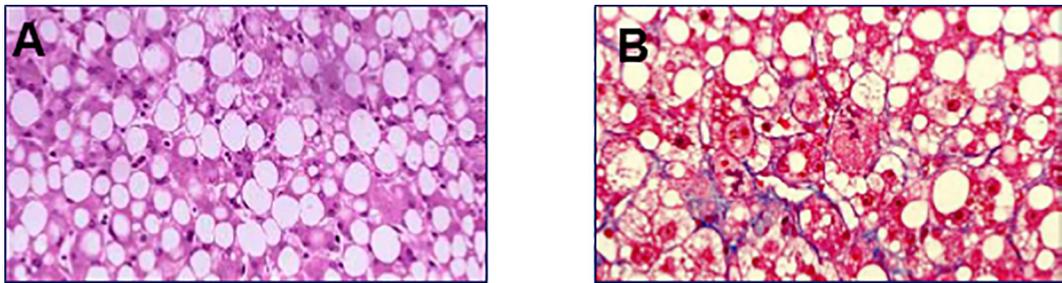
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**Fig. 1.** Light micrographs of human liver stained with hematoxylin-eosin. (A) simple steatosis showing intracellular fat deposition organized as droplets confluent to form vacuoles; (B) steatohepatitis showing fatty infiltration of hepatocytes, inflammation and fibrotic septa determining significant changes of the liver architecture. Magnification: 200 $\times$ . Courtesy of Maria L. Caruso, MD, Dept of Pathology, National Institute of Gastroenterology “S de Bellis”, Castellana Grotte, Bari, Italy.

the hepatocellular carcinoma. NAFLD develops in the absence of other known causes of secondary accumulation of fat (i.e. relevant alcohol consumption, viruses). The prevalence of NAFLD is growing alarmingly worldwide with a clear association with metabolic diseases such as obesity, insulin resistance, type 2 diabetes, and dyslipidemia [1,2]. The existence of a bi-directional relationship between NAFLD and various components of the metabolic syndrome points to a strong association between NAFLD and cardiovascular disease (CVD) morbidity and mortality. In fact, NAFLD may strongly contribute to the development of hypertension, diabetes and cardiovascular disease [3].

The processes leading to NAFLD are multifactorial. Overnutrition and fat accumulation in hepatocytes in the presence of insulin resistance in genetically predisposed individuals are major factors leading to the onset and progression to NASH. Among the genetic factors, polymorphisms in genes like *PNPLA3* (which encodes patatin-like phospholipase domain-containing protein 3) and *TM6SF2* (which encodes transmembrane 6 superfamily member 2) could govern the severity of steatosis/steatohepatitis [4,5] even in lean subjects [6]. Hepatocytes are cells intensively specialized in a variety of metabolic and detoxification processes. Therefore, mitochondria in hepatocytes are not only particularly prone to the effects of altered substrate influx but also trigger signalling pathways which may lead to cell injury (e.g. cytochrome c release) or cell protection (e.g. increased ATP generation). Mitochondrial dysfunction has an inevitably negative impact on energy generation as well as on the maintenance of ionic and redox gradients, thus being determinant for the progression of NAFLD toward NASH [7,8]. Furthermore, morphological alterations in mitochondria (crystalline inclusions, disruption of cristae) are associated with their functional impairment [9,10]. This review discusses the critical role of mitochondria in the development and progression of NAFLD and points out to potential therapeutic interventions.

## 2. Mechanisms of hepatocyte toxic fat accumulation in NAFLD

The mitochondrial damage that occurs in NAFLD is the consequence of a complex and multifactorial cascade of events involving long-chain fatty acids (LCFA) and triglycerides (TGs). The steps modulate the onset, and progression of liver steatosis. TGs form by the combination of one molecule of glycerol connected to three molecules of LCFA. LCFA are essential components of diets and are involved in a number of vital processes including energy production, energy storage, synthesis of plasma membrane, and anchorage of proteins. Hormone-like properties of LCFA include nuclear receptor-mediated (HNF4, PPAR) gene expression, activation of the G-protein-coupled receptor (GPCR) GPR40 and insulin release by  $\beta$ -cells. In addition, LCFA activate the innate immune response by modulating cellular toll-like receptor (TLR) signalling, and suppress food intake by acting in a subpopulation of hypothalamic neurons (with inhibition of the release of neuropeptide Y (NPY) and Agouti-related protein (AgRP)).

TGs are physiologically stored within the adipocytes in the post-prandial period and released intermittently during lipolysis, when

needed. Small lipid droplets are found normally in hepatocytes [11,12] and cardiomyocytes [13] but with the ongoing expansion of visceral adiposity, insulin resistance, de-repression of adipocyte lipases, and lipolysis (“obesogenic scenario”), a massive release of LCFA occurs into the splanchnic circulation towards the liver, striated muscle, cardiomyocytes, and pancreas. In these tissues, both the excess of LCFA and TG contribute to lipotoxicity, leading to clinical consequences such as NAFLD, and obesity cardiomyopathy [14,15] (Fig. 2). Mathematical algorithms model LCFA uptake according to protein-mediated transport of LCFA anions (saturable transport  $\approx$ 95%) and passive transmembrane diffusion of protonated LCFA (unsaturable transport) [16,17]. In the obesogenic scenario, the uptake of LCFA is upregulated in the liver [12], cardiomyocytes [18], and adipose tissues [19]. Uptake of LCFA is up-regulated before weight gain [20], while it is down-regulated after weight loss [21]. In addition,  $V_{max}$  of LCFA intake progressively increases from non-obese to obese to super-obese patients [17] in both omental [17,22] and subcutaneous [19] fat. We could speculate that events governing LCFA intake, work simultaneously at different tissue levels.

As depicted in Fig. 3A, increased visceral adiposity is associated with local and inflammatory changes due to macrophagic-mediated inflammation, upregulated adipocytokines and change in the levels of adipocyte-derived hormones. In the liver, LCFA influx is regulated by protein transporters: fatty acid translocase (FAT) or class B scavenger receptor (CD36) [23], and fatty acid transporting polypeptide (FATP) family [24–28], as well as others [29,30], including plasma membrane fatty acid binding protein (FABPpm) [31–34], caveolin-1 (CAV1) [30,35]. Dietary fat contributes to the hepatic uptake of LCFA and of triglycerides via low-density lipoprotein (LDL) receptor. Dietary and/or serum sugars enhances *de novo* lipogenesis [36] while insulin and glucose induce up-regulation of the carbohydrate-responsive element-binding protein (ChREBP) and sterol regulatory element-binding protein 1 (SREBP1) which will further increase intrahepatic lipid supply by about 25% [37,38]. Insulin-dependent pathways might involve endoplasmic reticulum stress to activate SREBP1 [39]. The intestinal microbiota can also contribute to NAFLD (i.e. dysbiosis [40], over-extraction of dietary nutrients [41], increased intestinal permeability [42], intestinal degradation of choline with negative effect on lipid homeostasis [36,43–48], and bacteria and/or bacterial product translocation of endotoxins and endogenous ethanol via the portal circulation [49]). Fat accumulation is facilitated upon the ongoing decrease of very low-density lipoprotein (VLDL) assembly and secretion, decreased mobilization of triglycerides, diminished  $\beta$ -oxidation of LCFA, and decreased synthesis of ApoB100 [15]. Under such conditions, intrahepatic lipids are synthesized by expanded *de novo* lipogenesis plus dietary fat influx (40%) and adipose tissue lipolysis (60%) [50]. LCFA conversion to triglycerides might initially limit the ongoing damage leading to hepatic cytotoxicity and steatohepatitis.

Indeed, simple steatosis is an early and largely reversible form of NAFLD, while NASH represents the manifestation of hepatocyte injury/death and the resultant wound-healing process. The histologic picture

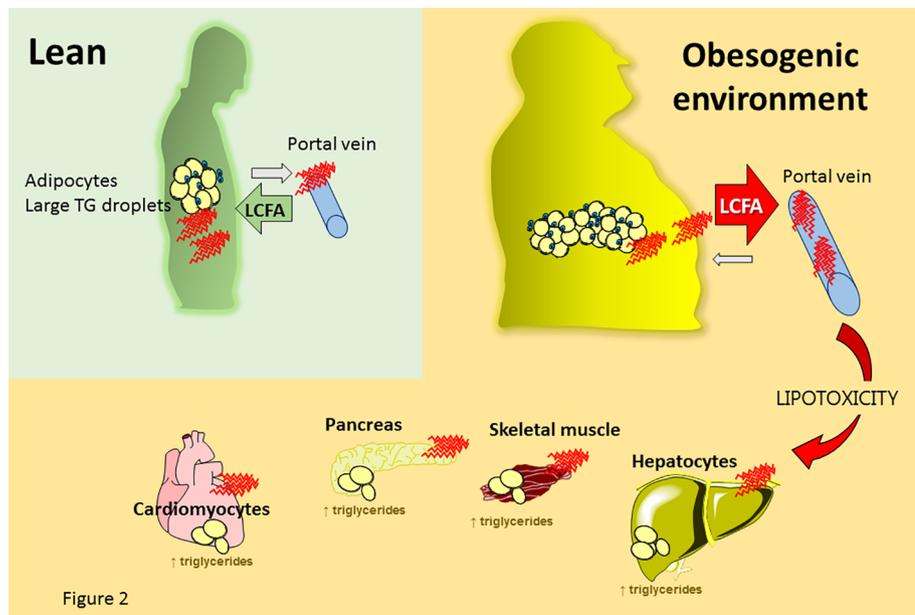


Figure 2

resembles that of alcoholic hepatitis and evolution towards more severe forms of fibrosis, cryptogenic cirrhosis and hepatocellular carcinoma is possible [50]. Fig. 3B depicts the steps involving hepatic lipid-induced insulin resistance. Few key events occur in the context of overnutrition, increased expression of hepatic suppressor of cytokine signaling-3 (SOCS3) and increased insulin resistance [51]. The increased portal flow of intra-abdominal fat-derived LCFA is associated with increased hepatic uptake and intracellular accumulation of LCFA, increase of lipotoxic substances such as ceramides, diacylglycerols (DAG) [52], and activation of the epsilon isoform of protein kinase C (PKC $\epsilon$ ) [53]. Hepatocellular DAG increased if the rates of DAG synthesis due to re-esterification of fatty acids and *de novo* lipogenesis are greater than the rates of mitochondrial long-chain CoA (fat) oxidation and rates of incorporation of DAG into triacylglycerides, or both combined events. Activated PKC $\epsilon$  binds and inhibits the insulin receptor (IR-1) tyrosine kinase with decreased phosphorylation of the glycogen synthase kinase 3 (GSK3) ultimately leading to decreased glycogen synthesis. The decreased phosphorylation of forkhead box subgroup O (FOXO) will drive the FOXO translocation to the nucleus, and increased gene transcription of the gluconeogenic enzymes phosphoenolpyruvate carboxykinase (PEP-CK) and glucose-6-phosphate (G6P) resulting in decreased insulin-mediated suppression of hepatic gluconeogenesis. The increase of IRS-1 and the downstream targets PI3K/Akt and sterol regulatory element-binding protein 1c (SREBP-1c) will further contribute to lipid accumulation and steatohepatitis [54]. Impaired mitochondrial and extra-mitochondrial oxidation [15] and production of reactive oxygen species (ROS) metabolites [55–57] also contribute to hepatocellular injury.

Further events leading to hepatocyte death, liver fibrosis, and NASH are depicted in Fig. 4 [15,58]. Excessive influx of LCFA in the liver upregulates several death receptors, i.e. DR5, FAS, TRAIL-R2, TNFRSF1A (see legend) [59–61]. Excess intracellular FAs can result in intrinsic endoplasmic reticulum stress, and mitochondrial dysfunction. Generation of ROS, activation of JNK [62], caspase-2 pathway [63–65], and increased  $\beta$ -oxidation represent the principal toxic pathways. Hepatocyte death follows the release of extracellular DAMPs and is predisposing to inflammation and fibrosis upon accumulation of LCFA, intestinal endotoxins and effect of DAMP on the TLR [66] of recruited macrophages (involving activation of inflammasomes, pro-inflammatory cytokines, chemokines and inflammation [67,68]). Activated stellate cells are sensitive to the ongoing oxidative stress and cholesterol accumulation [66], and will promote myofibroblasts, collagen production and fibrosis [69–71]. T cells might also be involved in

Fig. 2. Distinct regulatory functions of adipocytes in lean and obese subjects. In lean subjects, up regulated uptake of long-chain fatty acids (LCFA) by adipocytes from the portal vein occurs i.e. adipocytes import LCFA intermittently post-prandially; LCFA accumulate into large droplets mainly of triglycerides (TGs). Lipolysis releases LCFA according to the metabolic needs. In the obesogenic scenario, subjects accumulate expanded visceral adiposity, without preventing the effect of lipases; this condition causes excessive flux of LCFA into portal vein with subsequent lipotoxic deposition into non-adipose cells (cardiac myocytes, hepatocytes, pancreatic  $\beta$ -cells and striated muscle cells).

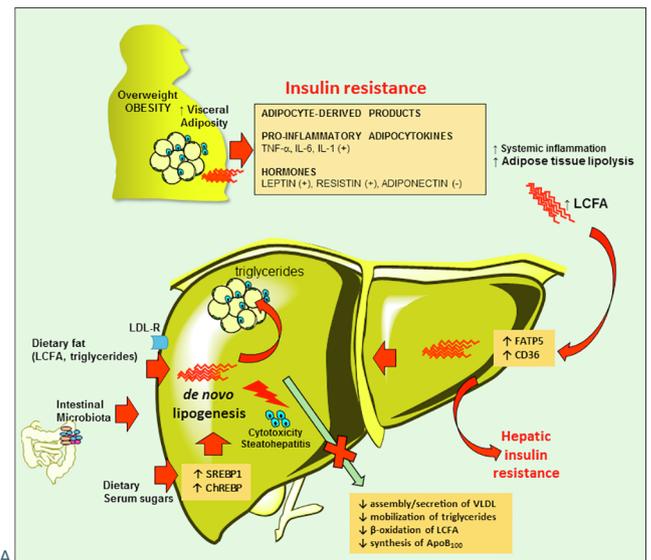


Fig. 3. Lipid accumulation in NAFLD. (A) In a condition of expanded visceral adiposity and insulin resistance, events include increased flow and uptake of long-chain fatty acids (LCFA) in the liver, increased uptake of dietary fats and sugars, intestinal dysbiosis, and decreased export of VLDL. (B) Events contributing to hepatic lipid-induced insulin resistance in the liver. The hepatocyte is overloaded with LCFA leading to activation of diacylglyceride (DAG) and epsilon isoform of protein kinase C (PKC $\epsilon$ ). Further steps include defective mitochondrial function and decreased rates of incorporation of DAG into triacylglycerides, defective insulin receptor (IR-1), decreased glycogen synthesis, along with increased hepatic gluconeogenesis. Abbreviations: ApoB, apolipoprotein B; ChREBP, carbohydrate-responsive element-binding protein; DNL, *de novo* lipogenesis; LCFA, long-chain fatty acids; IL, interleukin; FATP5, fatty acid transport protein 5 (bile acyl-CoA synthetase); PKC $\epsilon$ , protein kinase C $\epsilon$ ; SREBP, sterol regulatory element-binding protein 1; TNF, tumor necrosis factor; VLDL, very low density lipoprotein. Symbols:  $\uparrow$ , increased,  $\downarrow$ , decreased. Adapted from Portincasa P, Wang DQH. Nonalcoholic fatty liver and gallstone disease. In: Wang DQH, Portincasa P, eds. Gallstones. Recent advances in epidemiology, pathogenesis, diagnosis and management. New York: Nova Science Publisher Inc., 2017:387–414 [163] and Grattagliano I, De Bari O, Di Palo D, Montecucco F, Carbone F, Oliveira P, Wang DQH, Portincasa P. Mitochondria in liver diseases. In: Oliveira P, ed. Mitochondrial Biology and Experimental Therapeutics. Cham, Switzerland: Springer Nature, 2018:91–126 [82].

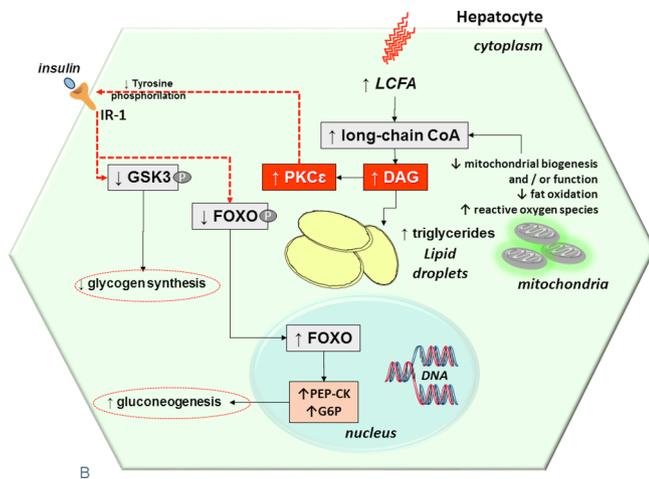


Fig. 3. (continued)

the progression of liver disease [50]. Endotoxins can originate from gut microbiota and bacterial products of Gram-species [72].

Under such conditions, the balance between the extent of injurious events and the capacity to repair is highly impaired [73]. T-cells might also be involved in the progression of liver disease [50]. The involvement of the innate immune system suggests a role for toll-like receptors (TLRs) and the nucleotide-binding domain (NOD)-like receptor protein 3 (NLRP3) inflammasome, a cytosolic receptor. TLR4, in turn, leads to the activation of NF-κB, macrophage-derived chemokines, and activation of Kupffer cells. Injured hepatocytes release micro particles containing mtDNA, which will activate TLR9 pathways, due to their circular, prokaryote nature [74–76]. The recruitment of monocytes and macrophages to the liver will initiate the inflammatory cascades. The NLRP3 inflammasome responds to various noxious signals [DAMPs and pathogen-activated molecular patterns (PAMPs)] with recruitment of an apoptosis-associated speck-like protein (ASC) and activation of pro-caspase 1. The ongoing mitochondrial dysfunction, lysosomal damage, and oxidative stress occurring during NASH could also activate the

NLRP3 inflammasome. In addition, NF-κB regulates NLRP3 and pro-interleukin-1β [54]. Fibrosis is the consequence of the ongoing liver damage, inflammatory cell death and reparative effort which involves activated transforming growth factor β (TGF-β)-mediated activation of hepatic stellate cells with production of fibrous matrix [77]. Liver cancer is the ultimate consequence of such intense regenerative efforts.

The nuclear liver X receptor (LXR) also contributes to fat accumulation as transcriptional activator for lipogenesis via modulation of SREBP-1c and expression of fatty acid synthase, carbohydrate-responsive element-binding protein (ChREBP) and stearoyl-CoA desaturase-1 (SCD-1), all components of *de-novo* fatty acid biosynthesis. The intestinal farnesoid X nuclear receptor (FXR) upregulates SREBP-1c by enhancing ceramide synthesis, increasing *de-novo* lipogenesis and promoting the appearance of NAFLD [78]. Increased apoptosis occurs in NAFLD [54] while impaired autophagy contributes to accumulation of damaged cellular products in hepatocytes, inflammation and endoplasmic reticulum stress [79]. Finally, the prolonged disruption of physiological circadian rhythm in mice with fatty livers leads to the development of NASH by dysregulating the cross-talk between two nuclear hormone receptors, FXR and constitutive androstane receptor (CAR), resulting in suppression of FXR, accumulation of bile acids, bile acid-induced overactivation of CAR, liver injury, fibrosis, and even cancer [80].

Lipotoxicity encompasses the dysregulation of the intracellular lipid composition with accumulation of harmful lipids resulting in sub-cellular organelle dysfunction, including mitochondria, and leading to cell injury and even death. Generation of ceramides increases with proinflammatory cytokines, including IL-1 and IL-6, and contributes to inflammation via interaction with TNFα. In NASH, the increased expression of SREBP-2 leads to upregulation of HMG-CoA reductase, the rate limiting step of cholesterol synthesis, with a final accumulation of free cholesterol in mitochondria. Indeed, mitochondria are a well-established target of lipotoxicity and participate in the process of apoptosis and in generation of oxidative stress-related products. In fact, in NASH hepatocytes, signals from death receptors converge on mitochondrial pathways of cell death, whereby caspase 8 cleaves Bid, resulting in release of cytochrome c. In steatotic cells, FXR activation

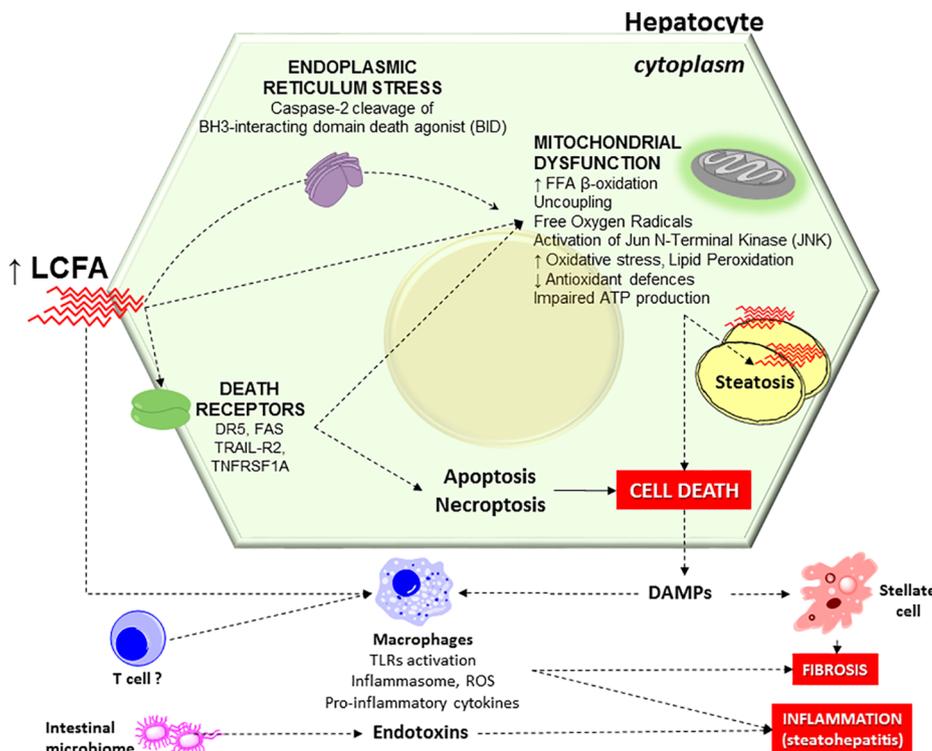


Fig. 4. Pathways leading to hepatocyte injury, death, inflammation and fibrosis during NAFLD and NASH. Increased accumulation of LCFA activate endoplasmic reticulum stress, mitochondrial dysfunction, death receptors, macrophagic and stellate cell activation. The role of microbiota-derived endotoxins might contribute to the inflammatory changes. Abbreviations: DAMPs, damage-associated molecular patterns; DR5, death receptor 5; LCFA, long-chain fatty acids; ROS, reactive oxygen species; TLRs, Toll-like receptors; TNFRSF1A, tumor necroptosis factor receptor superfamily member 1A. Adapted from Portincasa P, Wang DQH. Nonalcoholic fatty liver and gallstone disease. In: Wang DQH, Portincasa P, eds. Gallstones. Recent advances in epidemiology, pathogenesis, diagnosis and management. New York: Nova Science Publisher Inc., 2017:387–414 [50] and Grattagliano I, De Bari O, Di Palo D, Montecucco F, Carbone F, Oliveira P, Wang DQH, Portincasa P. Mitochondria in liver diseases. In: Oliveira P, ed. Mitochondrial Biology and Experimental Therapeutics. Cham, Switzerland: Springer Nature, 2018:91–126 [82].

induced by bile acids alters different pathways involved in fatty acid metabolism: activation of peroxisome proliferator-activated receptor alpha (PPAR $\alpha$ ) with increased fatty acids oxidation, SREBP-1c inhibition and modulation of VLDL production.

### 3. Mitochondrial dysfunction in NAFLD/NASH

Mitochondrial activities in hepatocytes encompass both metabolic pathways and signalling networks [81] with a viability depending on several factors including the integrity of mitochondrial DNA (mtDNA), membrane constituents, lipoprotein trafficking, pro- and anti-oxidant balance and metabolic demand and supply [82]. Mitochondria are the most important sites for  $\beta$ -oxidation of fatty acids (FAs), whose main regulation depends on carnitine palmitoyltransferase 1 (CPT-1), the enzyme required for intra-mitochondrial transport of long-chain FAs [83]. The progression of NAFLD to its more deleterious NASH form has been indicated as a type of mitochondrial disease [13,84], a possibility occurring at an early stage in humans [85]. Mitochondrial defects stimulate ROS production, oxidative stress, and lead to impaired FA oxidation processes [86]. Activation of intracellular stress cascades or death receptor pathways in liver diseases can result in mitochondrial damage, by means of inner membrane permeabilization. Loss of impermeability of the inner mitochondrial membrane can lead to mitochondrial depolarization and disruption of the capacity to synthesize ATP, besides leading to the loss of antioxidant capacity [87–89].

On the other hand, an excessive production of ROS in mitochondria can be manifested by morphological changes, including cristae swelling [90], decreased ATP synthesis, and production of lipid peroxidation products malondialdehyde (MDA) and hydroxynonenal (HNE) [48]. Oxidative stress and inflammation are closely associated with elevated oxidative metabolism in obese individuals and this link may be exaggerated by increased work through anabolic pathways in fatty livers [91]. Disruptions of intracellular homeostatic processes and mitochondrial function activate both apoptotic signalling and necroptotic events [92]. Apoptosis is associated with changes in mitochondrial cardiolipin and phosphatidylcholine redox state and distribution. Apoptosis is also associated with increased probability of mitochondrial permeability transition pore (MPTP) opening as well as release of pro-apoptotic proteins from mitochondrial intermembrane space [93]. The final MPTP structure is still a matter of research. More recently, research has focused on the involvement of ATP synthase in pore formation [94–97]. Beyond the release of cytochrome *c* and other pro-apoptotic factors into the cytosolic compartment, lysosomal damage, oxidative stress and MPTP opening seem to be involved in the activation of NLRP3 inflammasome and of the executioner caspase 3 which interacts with pro-caspases 6, 7 and 2 [98,99]. Necroptosis appears to be a key event in NASH [100]. In this scenario, excessive production of ROS is responsible for the oxidative stress, lipid and protein oxidation and damage to mtDNA [101].

Nitrosative stress also acts as an additional mechanism for cell damage in NAFLD. Locally produced nitric oxide (NO) derivatives bind to proteins and thiols, resulting in enzyme inactivation and conformational changes in different membrane transporters [99]. NO exerts a controller activity on mitochondrial respiration and biogenesis [102]. Both ROS and NO may disrupt mitochondrial function by post-translational changes of the mitochondrial proteome which result in mitochondrial dysfunction. Indeed, mitochondrial proteomics points to defects in the assembly of multi-protein complexes, and resolution of highly hydrophobic proteins of the inner mitochondrial membrane [103]. A progressive deterioration of mitochondrial function, with decreased respiratory rates and ATP synthesis occurs in NAFLD. Simoes et al. [104] have reviewed the role of mitochondria in the development of NAFLD and its progression to NASH. Moreover, robust evidence suggests the existence of a relationships between human plasma branched-chain amino acids (BCAA) and insulin resistance, which is in line with the hypothesis that the mitochondrial modulation of the

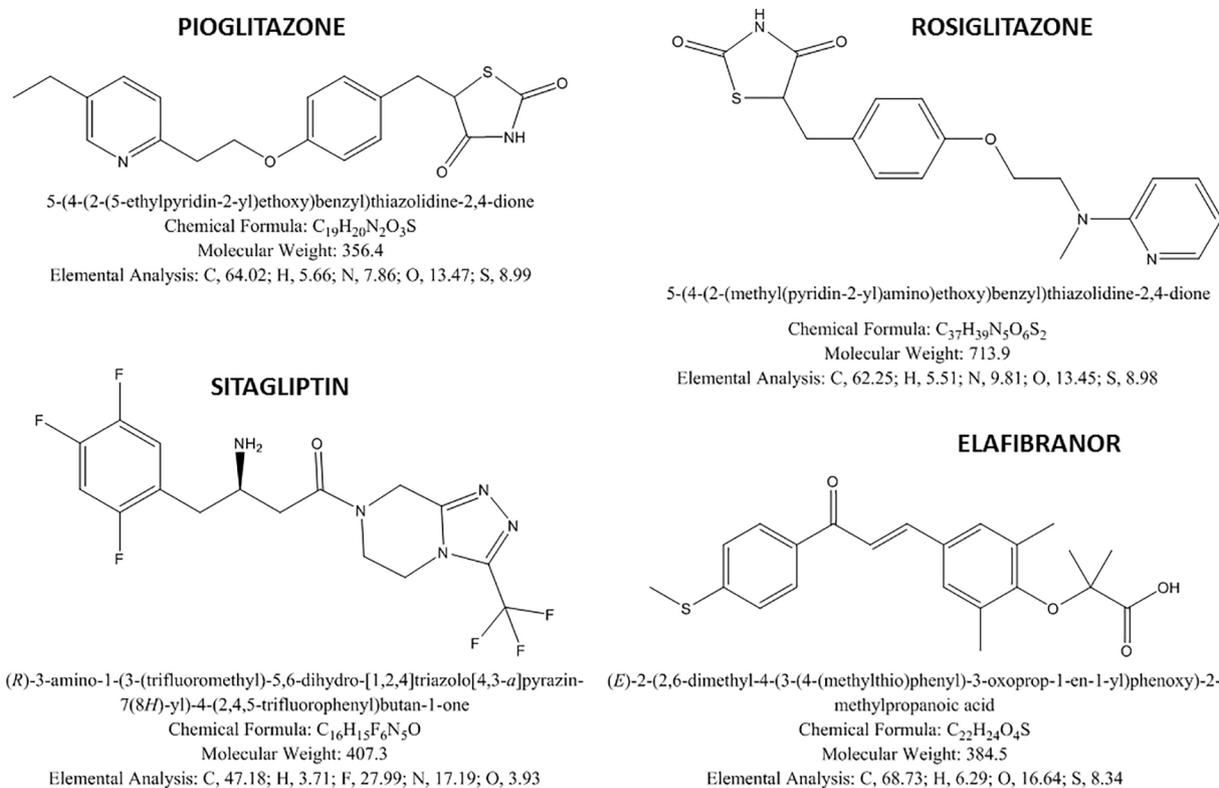
metabolism by BCAA result in a chronic metabolic overload from BCAA. These amino acids are essential to mediate efficient channeling of carbon substrates for oxidation through the mitochondrial tricarboxylic acid (TCA) cycle. Therefore, impairment of BCAA-mediated upregulation of the TCA cycle may significantly contribute to mitochondrial dysfunction in NAFLD [105]. Indeed, the link between insulin resistance and mitochondrial abnormalities has been widely documented [106].

Finally, mitochondrial genetics also seem to play a major role in NASH by active modulation of oxidative stress and controlling the efficiency of oxidative phosphorylation [107].

### 4. Therapeutic interventions on mitochondrial metabolic pathways and function

The presence of obesity and insulin resistance, and the degree of hepatic steatosis contribute to the severity of liver inflammation and fibrosis in NAFLD; by counteracting such participating factors, both liver histology and mitochondrial function can be improved [108,109]. The most efficient treatments include weight loss and exercise [110], but adjunctive therapies are actively being investigated [58,111]. Indeed, initial lifestyle interventions contribute to modulate the expression of the disease. Measures include reduction of body weight by dietary restriction or, in selected cases, by bariatric surgery (five to ten percent or even more of body weight, at a rate of 0.5–1.0 Kg weekly, depending on the presence of overweight, obesity, biopsy-proven NASH and increased serum alanine aminotransferase, ALT). The program should also include regular physical activity (i.e. 30 min moderate exercise five times weekly or increase daily footsteps to 10,000 daily), since aerobic exercise improves skeletal muscle insulin sensitivity, a factor contributing to lower insulin resistance and liver steatosis [109,112,113]. The consumption of fructose-enriched beverages [114–116] and alcohol [117] must be limited. Intake of three to five cups of caffeinated coffee daily is recommended [118] since regular coffee consumption can significantly reduce the risk for hepatic fibrosis and cirrhosis [119] among NASH patients [120]. Because the fatty liver-associated lipotoxicity drives hepatocyte death and promotes NASH, interventions aiming at preventing or treating NASH should firstly counteract the ongoing lipotoxicity. Ideally, the therapeutic agents should improve NAFLD/NASH by acting on intracellular metabolic pathways and the ongoing process of mitochondrial disruption [121].

Few agents may act also by targeting mitochondrial function. Peroxisome proliferator-activated receptors (PPARs), which are ligand-activated transcription factors, regulate intracellular metabolic processes and shift hepatic metabolism toward lipid oxidation [122]. Thiazolidinediones are PPAR $\gamma$  agonists and act as antidiabetic drug, and might play a role in this respect: pioglitazone improves NASH [123–127], but one of its side effects is weight gain [128]. Rosiglitazone is also effective in this regard [129–131]. Pioglitazone prevents the leakage of cytochrome *c* from mitochondria, stabilizes the mitochondrial transmembrane potential, inhibits ROS generation and activates the electron transport chain complexes I and III [132]. The European Association for the Study of the Liver (EASL) and the American Association for the Study of Liver Diseases (AASLD) [133,134] advise the use of pioglitazone to treat subjects with and without type 2 diabetes mellitus with biopsy-demonstrated NASH. Pioglitazone, in fact, improved liver histology in these patients [125]. However, it has no indication to treat NAFLD without biopsy-based proof of NASH [133,134]. Other antidiabetic drugs, such as liraglutide (glucagon-like peptide 1 analogue) [135] and sitagliptin (dipeptidyl peptidase-4 inhibitor) [136,137] may also be effective in NAFLD. In patients treated with elafibranor (an agonist of peroxisome proliferator-activated receptors  $\alpha$  and  $\delta$ , involved in mechanisms improving insulin sensitivity, glucose homeostasis, lipid metabolism and inflammation), NASH resolved without worsening of fibrosis. Liver enzymes, glucose and lipid



**Fig. 5.** Structures of agents targeting the steatotic liver: peroxisome proliferator-activated receptors (PPARs) pioglitazone and rosiglitazone; sitagliptin (dipeptidyl peptidase-4 inhibitor). Elafibranor is an agonist of peroxisome proliferator-activated receptors  $\alpha$  and  $\delta$ , involved in mechanisms improving insulin sensitivity, glucose homeostasis, lipid metabolism and inflammation). In particular, pioglitazone possesses protective mitochondrial effects, preventing the leakage of cytochrome c from mitochondria, stabilizing the mitochondrial transmembrane potential, inhibiting ROS generation and activating the electron transport chain complexes III and I.

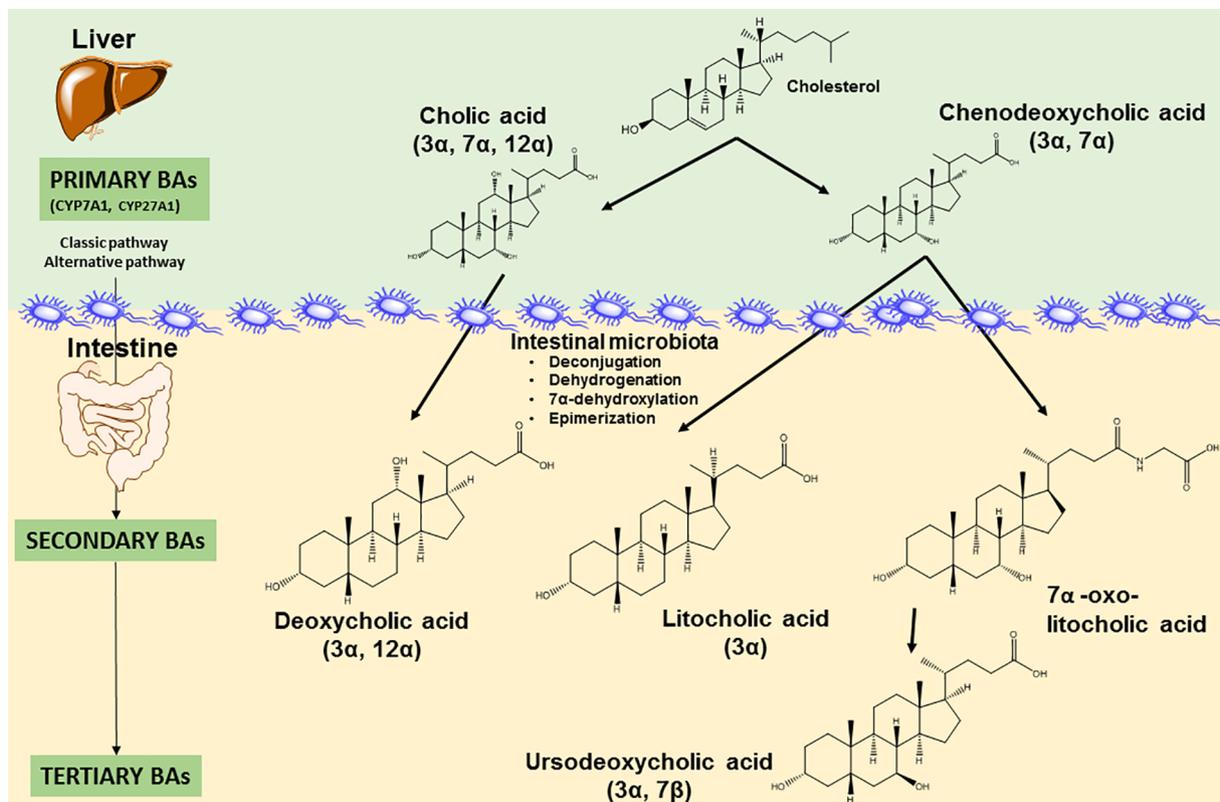
profiles, and systemic inflammatory markers also improved [138] (Fig. 5).

Bile acids (BA) are soluble amphiphilic molecules, which represent the major lipid components of bile. Primary BA are synthesized from cholesterol in the liver and conjugated to glycine or taurine to increase their solubility. Upon secretion into bile, BA are concentrated in the gallbladder during fasting, expelled in the intestine in response to dietary fat and bio-transformed to secondary BAs by the colonic gut microbiota (Fig. 6). Both primary and secondary BA undergo re-absorption in the ileum and the colon, respectively, and then return to the liver, with minimal loss in the feces. Beside their digestive function, more lipophilic BA also play a role as signaling molecules in modulating epithelial cell proliferation, gene expression, and lipid and glucose metabolism by activation of the FXR and G-protein-coupled bile acid receptor-1 (GPBAR-1) in the liver, intestine, muscle and brown adipose tissue [139]. BA promote insulin sensitivity and decrease gluconeogenesis and circulating triglycerides [140]. In a randomized, multi-center double-blind placebo-controlled, parallel group clinical trial in USA, the synthetic variant of the natural primary BA chenodeoxycholic acid and potent FXR activator 6-ethylchenodeoxycholic acid (obeticholic acid, OCA, Intercept Pharmaceuticals) 25 mg/day orally for 72 weeks improved liver histology of NASH without worsening of fibrosis (Fig. 7). In addition, serum alanine aminotransferase and aspartate aminotransferase concentrations decreased during OCA treatment [141]. The mechanism of action of OCA might imply the activation of FXR, with decreased hepatic lipogenesis because of down-regulation of the transcription factor SREBP1c and increased SIRT1 [142,143].

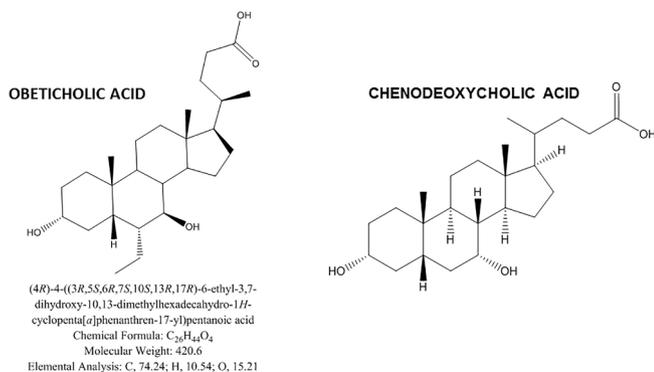
Since mitochondrial and peroxisomal oxidation of fatty acids generate ROS, the use of antioxidant therapy represents one of the major strategies to counteract liver inflammation in NASH [144] (Fig. 8). One recent study tested the efficacy of the antioxidant Tempol in mitigating

liver injury associated to NASH. Tempol acts by selectively modulating the gut microbiota composition and metabolism under pro-steatotic conditions. Following the change in gut microbiota, liver histology showed a marked reduction in lipid droplets. Tempol also decreased liver weight and liver/body mass ratios in mice fed a steatogenic diet by interfering with the intestine-specific disruption of FXR [78]. In fact, intestinal FXR influences the ceramide/SREBP1C/CIDEA pathway and administration of ceramide attenuates the effects of the steatogenic diet that induces steatohepatitis. Therefore, inhibition of intestinal FXR is crucial for gut microbiome-mediated progression of NAFLD. The inhibition of intestinal FXR signaling also results in an improvement in mitochondrial function, repression of ceramide synthesis and reduction of its serum levels and, ultimately, in decreased hepatic steatosis [145]. Another antioxidant molecule, resveratrol, a polyphenol extract from red grapes and berries, exerts mitochondrial protectant properties [146]. However, although promising, the possible health benefits of resveratrol were only demonstrated by an attenuation of liver fibrosis via the AKT and NF- $\kappa$ B pathway, but without effects on liver fat accumulation.

Other agents target antioxidant compounds into mitochondria. Mitoquinone (Mito-Q) and Mitovitamin E (MitoVit-E) represent a promising novel class of antioxidants. Both molecules contain a covalently attached lipophilic triphenylphosphonium (TPP) cationic moiety able to transport and concentrate antioxidant molecules within mitochondria [147–149]. Mito-Q improves metabolic syndrome in rats fed a high-fat diet for eight weeks [150] and the effect is associated with increased expression of cardioprotective synthase and cardioprotective levels in liver mitochondria [151]. In another study, Mito-Q prevented hypercholesterolemia, hypertriglyceridemia, mtDNA oxidative damage, hyperglycemia, and hepatic steatosis in experimental models of atherosclerosis and metabolic syndrome [152]. Low doses of Mito-Q and MitoVit-E protected cells against peroxide-induced oxidative damage



**Fig. 6.** Bile acid (BA) biosynthesis in humans. The primary BAs are synthesized in the liver starting from cholesterol as trihydroxy cholic acid (CA) and dihydroxy chenodeoxycholic acid (CDCA). The biosynthetic pathways include the “classical pathway” where a cytochrome P450 oxidase, the cholesterol 7 $\alpha$ -hydroxylase (CYP7A1), induces the 7 $\alpha$ -hydroxylation of cholesterol. The “alternative” pathway occurs with another a cytochrome P450 oxidase, the sterol-27-hydroxylase (CYP27A1), and produces mainly CA. The two primary BAs account for more than 75% of total BA production. In the intestine (mainly colon), the microbiota operates by 7 $\alpha$ -dehydroxylation on CA and CDCA to synthesize the «secondary» dihydroxy deoxycholic acid (DCA) and the monohydroxy lithocholic acid (LCA). CDCA is also 7 $\alpha$ -dehydrogenated to dihydroxy 7 $\alpha$ -oxo-LCA further metabolized to its 7 $\beta$ -epimer and “tertiary” bile acid t. e dihydroxy ursodeoxycholic acid (UDCA) [139].



**Fig. 7.** Structure of the potent farnesoid X receptor (FXR) activator 6-ethylchenodeoxycholic acid (obeticholic acid, OCA). The structure of chenodeoxycholic acid appears for comparison.

and apoptosis, in contrast to what occurs with low doses of the untargeted antioxidants (e.g. Vit-E and ubiquinone). The protective effects of Mito-Q and MitoVit-E are likely mediated through the inhibition of cytochrome c release and caspase-3 activation. Furthermore, Mito-Q and MitoVit-E reduced ROS-induced transferrin receptor-mediated iron uptake in mitochondria, lipid peroxidation, lipid peroxide-induced inactivation of complex I, and aconitase [153]. A phase II study in patients with chronic hepatitis C demonstrated that Mito-Q decreased circulating aminotransferase levels; this effect may suggest reduced hepatic inflammation and necrosis in these patients [154].

Medicinal plants can be used as dietary supplements [155] and

silymarin is extracted from milk thistle (*Silybum marianum*) with silybin as its major active compound. Silybin displays some hepatoprotective effects [156] and might improve insulin resistance, liver injury and liver enzymes in patients with NAFLD [157,158]. The silybin-phospholipid complex containing vitamin E improved liver steatosis in NAFLD patients [159], and significantly lowered fat infiltration in the liver of rats fed a high fat diet, likely by modulating thioredoxin changes and the production of nitric oxide (NO) derivatives and by significantly lowering lipid peroxidation. Silybin also attenuated changes in mitochondrial respiratory complexes, with a major protective effect on Complex II subunit CII-30 [46]. Recently, we showed additional protective effects of silybin on rat hepatoma FaO cells mimicking a progressive model of liver steatosis [160]. Of note, silybin reduced excessive TG accumulation, changed the expression of transcription factors (PPARs, enzymes for mitochondrial, peroxisomal, and ER oxidations of FAs). Silybin also rescued the FA-induced mitochondrial dysfunction (size and function) and the apoptotic signals and oxidative stress, which resemble a condition of steatohepatitis [90].

Finally, the “mitotherapy”, i.e., the exogenous intravenous injection of functional mitochondria from hepatoma cells has recently found to improve successfully the phenotype of liver steatosis induced by high-fat diet, by decreasing lipid content and restoring cellular redox balance. In brief, exogenous mitochondria were tagged with green-fluorescence protein (GFP) and were found in mouse liver, lungs, brain, muscle, and kidneys [161,162]. This disruptive strategy results in attenuation of lipid deposits, prevention of cell injury, improved energy production with restored hepatocyte function. However, it is not clear how intact mitochondria enter the different cells and restore the cell metabolic activity [13]. Further research is evolving in this direction,

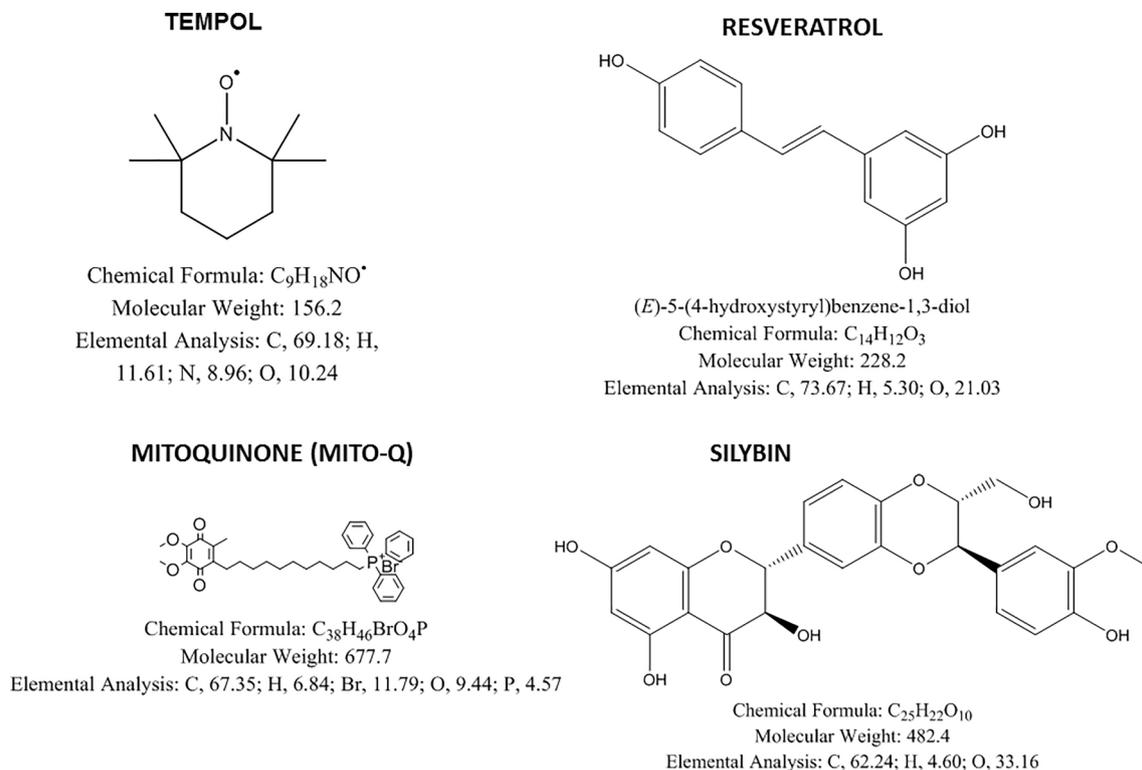


Fig. 8. Structures of antioxidant compounds used for the treatment of liver steatosis, with results showing improvement of mitochondrial function.

especially concerning the nature of the administrated mitochondria with potential metabolic and proteomic differences vs. mitochondrial isolated from normal, non-tumour-derived hepatocytes.

Finally, the role of several other agents is currently being investigated, i.e. obeticholic acid (Ocaliva/Intercept Pharmaceuticals), Mgl-3196 (Madrigal Pharmaceuticals), GS-9674 (Gilead Sciences), NGM282 (NGM Bio), arachidyl amido cholanoic acid (Aramchol, Galmed Pharmaceuticals), and tropifexor (Novartis). However, the ultimate effect of such molecules on mitochondrial function is not defined and deserves further and specific investigations.

## 5. Summary

Impairment of several intracellular metabolic pathways can have a role in the pathogenesis of NASH. Lipotoxicity associated with glucotoxicity represent the most recent pathogenic view. Several new drugs and molecular targets appear promising; however, the results of recent clinical trials indicate that the complex pathogenesis of NASH is still the limiting step to reach an optimal pharmacological approach. Treatments may need to target some subcellular organelles that are fundamental in the pathogenic process, other than acting on genetic risk variants or on metabolic/environmental stressors. Ideally, disease-modifying factors may act both on mitochondrial function and energy supply and on regulators of lipid metabolism such as peroxisomes. In this view, the future research should focus its attention also by considering the potential role of microbiota and intestinal nuclear receptors.

## 6. What is already known about this subject

- Nonalcoholic fatty liver disease (NAFLD) is the most frequent chronic liver disease in developed countries and puts the populations at risk of evolution towards liver fibrosis, cirrhosis and hepatocellular carcinoma
- Mitochondrial dysfunction is involved in the onset of nonalcoholic fatty liver disease (NAFLD) and contributes to the progression from

NAFLD to nonalcoholic steatohepatitis (NASH)

- Mitochondria could be a target to improve liver function in NAFLD

## 7. What this study adds

- A description of complex mechanisms involved in mitochondrial dysfunction in NAFLD and steatohepatitis
- Identification of potential therapeutic interventions, either in use or experimental, able to improve mitochondrial function in NAFLD

## Notes

Compliance with ethical standards.

## Conflicts of interest

The authors declare that they have no conflicts of interest.

## Statement of human and animal rights

Not applicable.

## Informed consent

Not applicable.

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