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Protocols for Mitochondria as the Target of Pharmacological Therapy in the Context of Nonalcoholic Fatty Liver Disease (NAFLD)

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Abstract

Nonalcoholic fatty liver disease (NAFLD) is one of the most frequent metabolic chronic liver diseases in developed countries and puts the populations at risk of progression to liver necro-inflammation, fibrosis, cirrhosis, and hepatocellular carcinoma. Mitochondrial dysfunction is involved in the onset of NAFLD and contributes to the progression from NAFLD to nonalcoholic steatohepatitis (NASH). Thus, liver mitochondria could become the target for treatments for improving liver function in NAFLD patients. This chapter describes the most important steps used for potential therapeutic interventions in NAFLD patients, discusses current options gathered from both experimental and clinical evidence, and presents some novel options for potentially improving mitochondrial function in NAFLD.

Keywords

Fatty liver; Mitochondria; Nitrosative stress; Nonalcoholic fatty liver disease; Oxidative Stress

1 Introduction

1.1 Nonalcoholic Fatty Liver Disease (NAFLD)

1. The term nonalcoholic fatty liver disease (NAFLD) refers to the presence of hepatic steatosis in the absence of other causes for secondary hepatic fat accumulation, e.g., heavy alcohol consumption, viruses, drugs, Wilson's disease, and starvation. The term NAFLD has been recently revisited and renamed as metabolic dysfunction-associated fatty liver disease (MAFLD) [1]. By an international panel, "inclusive" (not only exclusion) criteria rely on evidence of hepatic steatosis, in addition to one of the following three criteria, namely overweight/obesity, presence of type 2 diabetes mellitus, or evidence of metabolic dysregulation [1].
2. NAFLD is currently one of the most common liver disorders in Western industrialized countries, with a prevalence ranging from 10% to 46% in the United States [2–4] and a median of 20% worldwide with a documented rising trend with time [5]. This trend in North America and Europe is the consequence of the increasing prevalence of major risk factors for NAFLD, including obesity, sedentary lifestyles, type 2 diabetes mellitus, dyslipidemia, and metabolic syndrome [5–8]. However, lean subjects can develop NASH as well [7], which is frequent in Asia [9]. Overall, factors contributing to NAFLD include the environment, gut microbiome, disrupted glucolipid metabolic pathways, metabolic inflammation primarily mediated by innate immune signaling, comorbidities, and genetic risk factors [10, 11].
3. Patients with NAFLD develop hepatic steatosis due to excess accumulation (>5%) of triglycerides in the hepatocytes, with or without liver inflammation and fibrosis. Thus, the term NAFLD embraces a spectrum of conditions ranging from simple nonalcoholic fatty liver (NAFL) without evidence of significant inflammation to nonalcoholic steatohepatitis (NASH), where hepatic steatosis is associated with pericellular fibrosis, lobular inflammation and apoptosis, and a picture histologically indistinguishable from alcoholic steatohepatitis [12]. NASH, in turn, may progress to (cryptogenic) cirrhosis, and even hepatocellular carcinoma (HCC) [13–16]. Of note, HCC can also arise in NASH liver, before cirrhosis develops [17]. NASH affects 3% to 6% of the US population. Predisposing factors are metabolic disease and obesity. NASH progresses to cirrhosis in approximately 20% of cases. Early identification and targeted treatment of patients with NASH would prevent consequences related to progressive liver disease, growing economic burden due to health care for end-stage liver disease, need for liver transplantation, and care of patients with HCC. Moreover, development of NAFLD appears to be an additional risk factor for extrahepatic complications, which include cardiovascular disease and malignancy [18, 19].
4. Although most patients with NAFLD remain asymptomatic, some patients, especially those developing NASH, may complain of vague symptoms, which include fatigue, malaise, and right upper abdominal discomfort. However, several

NAFLD patients could ask for medical advice because of mildly elevated liver alanine aminotransferases or incidental increased echogenicity (“bright liver”) at ultrasound (or, eventually decreased hepatic attenuation by computed tomography, as well as increased fat signal by magnetic resonance imaging). NAFLD, however, may develop with normal aminotransferase levels.

5. The ultimate diagnosis of NAFLD requires the confirmation of steatosis by liver histology or imaging, no relevant alcohol consumption or other causes of liver steatosis (Table 1), and exclusion of a coexisting other chronic liver disease.
6. For alcohol, the consumption is more than three drinks/day (i.e., 21 drinks/week) in men or more than two drinks/day (i.e., 14 drinks/week) in women, according to the American Association for the Study of Liver Diseases (AASLD) [20], bearing in mind that a “standard” alcoholic drink contains about 14 g of pure alcohol [21], equal to 98 kcal.
7. Although NAFLD is prevalent and puts the populations at risk of evolution for chronic liver diseases, a systematic screening is still not advisable, according to the AASLD guidelines. In fact, there is no ultimate diagnostic test, except of the invasive liver biopsy which cannot be performed routinely. There is still no standard treatment for NAFLD, apart from healthy lifestyles [6, 7], and there is no agreement about the true cost-effectiveness of screening [20]. Further characterization of NAFLD populations requires specific research protocols [22].

1.2 Mechanisms of Free Fatty Acid (FFA) Accumulation in the Liver in Health and Lipotoxicity During Insulin Resistance Status

1. In health, the three essential steps of FFA accumulation in the liver are influx of nonesterified fatty acids (NEFA), de novo lipogenesis (DNL) of FA, and influx of dietary FA (Fig. 1).
2. Influx of NEFA represents ~60% of total FFA in the liver and contributes to the enrichment of the FFA pool in hepatocytes. NEFA originate from lipolysis of triglycerides in adipocytes, a step controlled by insulin [23]; FFAs as fatty acyl-CoA can enter the mitochondria under the control of carnitine palmitoyltransferase (CPT)-1 and undergoes β -oxidation to form acetyl-CoA which ultimately enters the tricarboxylic acid cycle with production of ATP. Amounts of FFA are also used for esterification to triglyceride (TG) via the key enzymes diglyceride acyltransferase (DGAT)1 and DGAT2. This step is again under the control of insulin. Excess TG can be stored as lipid droplets (no more than 5% in the hepatocyte) or exported to blood as very-low-density lipoprotein (VLDL) [24]. TG can also be hydrolyzed under the actions of hydrolases such as the patatin-like phospholipase domain-containing protein 3 (PNPLA3), also known as adiponutrin, to enrich the FA pool [23, 25].
3. DNL of FA (~25%) originates from dietary sugars. With DNL, hepatocytes convert excess glucose and fructose to FAs. Insulin mediates the transport of absorbed dietary carbohydrates to its target tissues (skeletal muscle and liver for storage as glycogen). Glucose in hepatocytes is partly metabolized to pyruvic

acid via glycolysis and then to acetyl-CoA, to generate ATP in the tricarboxilic acid cycle and promote gluconeogenesis during hypoglycemia. The first step of DNL is the synthesis of malonyl-CoA from cytosolic acetyl-CoA by acetyl-CoA carboxylase (ACC). Malonyl-CoA serves as a substrate to form saturated FA, by FA synthase (FAS). Stearoyl CoA desaturase (SCD)1 is an endoplasmic reticulum (ER) enzyme that is responsible for the formation of monounsaturated FAs from saturated FAs [22].

4. Influx of FA from diet is ~15% of all amount of FFA in the liver [25]. Bile acids (BA) hydrolyze intestinal dietary TGs to form nascent chylomicrons. The FAs made from the hydrolysis of TGs are then taken up by adipose tissue and liver [26]. At the same time, BA act as potent metabolic regulators in the terminal ileum by activating the farnesoid X receptor (FXR) plus pregnane X receptor (PXR) and the G-protein-coupled bile acid receptor-1 (GPBAR-1) with effects in the liver and in the muscle, adipocytes, and brown adipose tissue for energy expenditure [27, 28].
5. Metabolic abnormalities, such as insulin resistance, expansion of visceral obesity, sedentary behaviors, and high-calorie diet can greatly influence all the abovementioned metabolic pathways of FFA. This ongoing inflammation induced by metabolic stress is associated with increases in lipolysis and influx of NEFA, de novo lipogenesis, influx of dietary FFA (especially fructose), TG deposition in droplets, as well as decreases in mitochondrial oxidation of FFA, or secretion/export of FFA or TG into VLDL [29] (Fig. 2a, b). Under conditions of insulin resistance, ingested glucose is redirected to the liver [24] where it is primarily processed to FA via DNL [23, 32]. When excessive FAs are synthesized, they are converted to fatty acyl-CoA, which is further esterified into TGs and stored in hepatocytes. DNL is also enhanced in NAFLD patients [24, 33].
6. At this stage, excess FFAs are initially stored as TG into lipid droplets, but thereafter FFAs in excess represent the substrate for lipotoxic species, which include lysophosphatidylcholine (LPC), diacylglycerols (DAG), and ceramides. Amounts of free cholesterol can contribute to accumulation. (Table 2 and Fig. 3).
7. Hallmarks of cellular events of damage involved in NAFLD/NASH are depicted in Table 3 [40]. When FFAs are oversupplied or their disposal is disrupted, excessive FFAs become the substrates to generate lipotoxic species, which provoke endoplasmic reticulum (ER) stress, produce reactive oxygen species (ROS), and lead to cell death and release of danger-associated molecular patterns (DAMPs).
8. Mechanisms of lipotoxicity (Fig. 2a, b) involve receptor/kinases-mediated interactions, the endoplasmic reticulum and other intracellular organelles, mitochondria [41], the nucleus, and signaling pathways. Further consequences are endoplasmic reticulum stress, inflammatory changes (metabolic inflammation or meta-inflammation) [42], production of reactive oxygen species, and cell death [43]. The innate immune system becomes part of this metabolic

inflammation, with the recruitment of Kupffer cells, dendritic cells, lymphocytes, as well as hepatocytes and endothelial cells [44, 45].

9. Ultimately, the activation of several transcription factors promotes the release of inflammatory cytokines and chemokines and stimulates hepatic stellate cells with propensity to collagen deposition, and further aggravation of the insulin resistance status, both potential harmful features in the progression of NASH [46].
10. Besides the liver, adipose tissue, skeletal muscle, the heart, the pancreatic islets, certain areas of the brain, and the intestinal microbiota represent additional organs potential targets of lipotoxicity.

1.3 Focus on Mitochondrial Function in the Liver

1. In the hepatocyte, the mitochondrial activities encompass both metabolic pathways and signaling networks [47] (Fig. 4). The viability of mitochondria depends on several factors which include the integrity of mitochondrial DNA (mtDNA), membrane constituents, lipoprotein trafficking, pro- and antioxidant balance, and metabolic demand and supply [31].
2. Mitochondria are the most important sites for β -oxidation of long-chain FFA, and this process is a major source of cell energy, ultimately leading to ATP production.
3. The long-chain fatty acyl-CoA is transferred from cytosol into matrix, prior to undergoing β -oxidation in the mitochondrial matrix. This important function is achieved by the carnitine palmitoyltransferase (CPT-1), located on the outer mitochondrial membrane.
4. CPT-1 catalyzes the formation of long-chain fatty acylcarnitine from long-chain fatty acyl-CoA and carnitine. The acylcarnitine complexes are subsequently transported across the inner mitochondrial membrane [49–51].
5. Mitochondrial dysfunction is detrimental to cells and organs. This step is mediated by the lack of energy supply and excessive ROS generation. In addition, mitochondria are a definitive target of lipotoxicity. Conditions like type 2 diabetes mellitus and NAFLD change hepatic mitochondrial metabolism [32, 52], induce remodeling of mitochondrial lipids [53] while increasing mitochondrial mass and respiratory capacity [54]. Associated conditions include altered acetyl-CoA metabolism [55] and excessive turnover of the tricarboxylic acid cycle [32].
6. Because of the progression of NAFLD to its more deleterious necro-inflammatory form, NASH appears as a type of mitochondrial disease [56, 57] due to the coexistence of both structural and functional abnormalities [58] contributing to oxidative stress and apoptosis [29]. Events in mitochondria might occur at an early stage of disease, as also shown in humans undergoing the stable-isotope ^{13}C -breath test [59–61] with a substrate targeting mitochondria, i.e., ^{13}C -ketoisocaproic acid [62].

7. Mitochondrial defects stimulate excessive production of reactive oxygen species (ROS), and oxidative stress ultimately leads to impaired FFA oxidation processes [63].
8. Excess lipids toward hepatocytes leads to deleterious effects that include dephosphorylation of a voltage-dependent anion channel located in the mitochondria, inner membrane permeabilization, mitochondrial depolarization, and disruption of the capacity to synthesize ATP, in addition to the loss of antioxidant capacity [64–66]. The ultimate event is cell death [67].
9. An additional mechanism of damage includes the increased synthesis of mitochondrial free cholesterol (Table 2), which originates from (SREBP)-2-mediated upregulation of β -hydroxy β -methylglutaryl-CoA (HMG-CoA) reductase, increased synthesis of free cholesterol followed by apoptosis and JNK-dependent pro-inflammatory pathways [39, 68].
10. Other pathways of mitochondrial damage include decreased nicotinamide adenine dinucleotide (NAD⁺) levels and involvement of the histone deacetylases sirtuin-1 and -3, modulating an adaptive response to increased hepatic levels of FFA [69].
11. Excessive production of ROS in mitochondria can be disclosed by morphological changes, which include cristae swelling [70], decreased ATP synthesis, and production of lipid peroxidation products malondialdehyde (MDA) and hydroxynonenal (HNE) [71]. Oxidant 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 liver [72].
12. Disruption of intracellular homeostatic processes and mitochondrial function activate both apoptotic signaling and necroptotic events [73]. 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 proapoptotic proteins from mitochondrial intermembrane space [74]. The final MPTP structure is still a matter of research. More recently, research has focused on the involvement of ATP synthase in pore formation [75–78].
13. Beyond the release of cytochrome c and other proapoptotic 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 [79, 80]. Necroptosis appears to be a key event in NASH [81]. In this scenario, excessive production of ROS is responsible for the oxidative stress, lipid and protein oxidation, and damage to mtDNA [82].
14. 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 [80]. NO exerts a controller activity on mitochondrial respiration and biogenesis [83]. Both ROS and NO may disrupt mitochondrial function by post-translational changes of the mitochondrial proteome. This step results 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 [84]. A progressive deterioration of mitochondrial function, with decreased respiratory rates and ATP synthesis, occurs in NAFLD.

15. Mitochondria play a key role in the development of NAFLD and its progression to NASH [85]. Robust evidence has suggested 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 results in a chronic metabolic overload from BCAA. These amino acids are essential to mediate efficient channeling of carbon substrates for oxidation through the mitochondrial TCA cycle. Therefore, impairment of BCAA-mediated upregulation of the TCA cycle may significantly contribute to mitochondrial dysfunction in NAFLD [86]. Indeed, the link between insulin resistance and mitochondrial abnormalities is widely documented [87].
16. 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 [88].

2 Therapeutic Strategies in NAFLD

2.1 General Measures

1. There is no ultimate established therapy for NAFLD, due to the complexity of pathogenic mechanisms involved, short duration of available and future trials, need for translational value of the studies, and likely need for combination of treatments.
2. There are general measures that apply to all patients with NAFLD, irrespective of their ultimate effects on liver mitochondria (see below). As first step, lifestyle modification to reach or maintain the ideal body weight plays a paramount role [89] (Table 4).
3. There is a remarkable list of upcoming drugs and clinical trials available, in the attempt to achieve several targets, which include oxidant stress, apoptosis, metabolism, innate immunity, bile acid metabolism, nuclear receptors, fibrosis (i.e., fibrogenesis plus fibrinolysis), gut microbiota, and intestinal permeability [19, 113–115] (Fig. 5 and Table 5).
4. By contrast, a recent Cochrane Review focusing on 77 available trials on antioxidants, bile acids, and thiazolidinediones vs. no intervention concluded that “*Due to the very low quality evidence, we are very uncertain about the*

effectiveness of pharmacological treatments for people with NAFLD including those with steatohepatitis. Further well-designed randomized clinical trials with sufficiently large sample sizes are necessary” [177].

5. Notably, a single therapy leads benefits in no more than 40% of patients, and trials are usually too short to be recommended lifelong. Combination therapies might represent an alternative approach and increase the success rate of agents for NASH.

3 Potential Strategies Effective also on Mitochondria in NAFLD

3.1 Rationale for Targeting Mitochondrial Function

1. Few therapeutic strategies have emerged in this field so far (Table 6). Targets are different and are depicted in Fig. 7. Oxidative stress plays a central role in the development and progression of NAFLD. Mitochondrial and peroxisomal oxidation of fatty acids generates ROS. Mitochondrial (*)O₂(-)/H₂O₂ has deleterious effects on the development of metabolic diseases, including NAFLD [48]. Mitochondrial abnormalities are early detected also by breath testing using specific substrates targeting mitochondria [60–62, 213–217]. Thus, antioxidants targeting mitochondrial (*)O₂(-)/H₂O₂, for example, represent one attractive strategy to counteract liver inflammation in NASH [218, 219]. More evidence is required before definitive approaches enter the clinical trials on a regular base.

3.2 Physical Exercise

1. Physical exercise induces mitochondrial biogenesis in the striated muscle and also in the liver [220]. Physical activity can, therefore, play a role in the prevention and the treatment of NAFLD through the modulation of function and structure of mitochondria [221].
2. The systemic challenge of a single acute bout of physical exercise has effects on hepatic metabolic and redox state, with uncertain effects on mitochondrial membrane permeability to protons, state 4 respiration, increased state 3 respiration, and stress response to mitochondrial permeability transition [222, 223].
3. Long-term physical activity as endurance training (or voluntary running) appears to ameliorate markers of liver mitochondrial integrity and function and might facilitate hepatic features typical of a phenotype more resistant to stress [223].
4. Otsuka Long-Evans Tokushima Fatty (OLETF) rats develop obesity and type 2 diabetes with multiple stigmata of the metabolic syndrome. After 16 or 36 weeks of daily voluntary wheel running, OLETF rats develop increased hepatic mitochondrial fatty acid oxidation, enhanced oxidative enzyme function, and protein content. In addition, rats display suppressed levels of proteins related to hepatic de novo lipogenesis [224, 225]. Changes parallel several markers of mitochondrial oxidative phosphorylation apparatus, i.e., increased palmitate oxidation, increased citrate synthase, β -hydroxyacyl-CoA dehydrogenase and

carnitine, palmitoyl-CoA transferase 1 activities, and cytochrome c and ETC complex IV. Additional effects of physical exercise include increased phosphorylated form of acetyl-CoA carboxylase (ACC) and a decrease in ACC, fatty acid synthase, and stearoyl-CoA desaturase (SCD) activities (markers of inhibition of de novo hepatic lipogenesis) [226].

5. The exercise could increase hepatic mitochondrial oxidative capacity and, in turn, improve the insulin resistance driving liver steatosis. The mechanism includes increased fatty acid oxidation and decreased fatty acid-derived ceramide and diacylglycerols synthesis associated with liver insulin resistance [227, 228].
6. Comparison of effects of physical activity with those of sedentary behavior is shown in Fig. 8.

3.3 Antidiabetic Drugs

1. Peroxisome proliferator-activated receptors (PPARs) are a member of the superfamily of nuclear receptors, which bind a wide range of fatty acids and fatty acid derivatives, and transcriptionally regulate intracellular metabolic processes [230]. Three types of PPARs exist with differences in ligand selectivity and tissue distribution, i.e., PPAR- α , PPAR- δ (also named PPAR- β), and PPAR- γ .
2. PPAR- α (expressed in the liver, adipose tissue, heart, skeletal muscle, and kidney) regulates gluconeogenesis, lipid transport, and the hormone fibroblast growth factor (FGF)-21. Activation of PPAR- α shifts hepatic metabolism toward fatty acid transport and β -oxidation [115], improving plasma lipids by decreasing TG and increasing high-density lipoprotein (HDL) cholesterol [231]. One additional effect might include the inhibition of inflammatory genes via nuclear factor-kB, decreased expression of acute-phase response genes [231]. Although PPAR- α deletion in the animal model parallels the worsening of liver steatosis, receptor activation by typical PPAR- α agonists such as fibrates, while lowering serum TG, has not shown a consistent effect on NAFLD [232].
3. PPAR- δ (expressed in the liver, skeletal muscle, and macrophages) contributes to improved insulin sensitivity, decreased hepatic glucose production [233], increased fatty acid oxidation, and decreased macrophage and Kupffer cell activation [234]. In the liver, PPAR- δ has anti-inflammatory activities in macrophages and Kupffer cells [234]. Activation of PPAR- δ decreases steatosis but increases concerns about safety [235].
4. Elafibrinor acting as a PPAR α / δ agonist displays a distinct effect, i.e., increases fatty acid β -oxidation (PPAR α activity), but also improves insulin resistance and inflammation (see above) [233, 234]. Exact and potential effects on liver mitochondria deserve further investigations.
5. PPAR- γ exists mainly in adipose tissue and regulates lipogenesis, glucose metabolism, and adipose tissue differentiation. Thiazolidinediones (TZDs) are PPAR γ agonists and act as insulin sensitizers and antidiabetic drugs (*also see* Table 4), and this class of drugs might also play a role in NAFLD patients

[116, 122, 236]. Pioglitazone improves NASH [116, 121–124]. Rosiglitazone is also effective [127–129]. Of note, a mitochondrial target of thiazolidinediones (mTOT) may exist, acting as a mitochondrial membrane complex involved with pyruvate transport. TZDs, therefore, appear to modulate pyruvate entry into the TCA cycle [237]. Liver steatosis appears to be associated with increased activity of TCA cycle with decreased availability of acetyl-CoA [55]. Pioglitazone seems to partly correct this situation and decrease hepatic TCA cycle flux in the mouse model of NASH [238]. There is evidence suggesting that the mechanism might involve inhibition of mitochondrial pyruvate fluxes [239, 240].

6. Some novel antidiabetic drugs, such as liraglutide (glucagon-like peptide 1 analogue and GLP-1 receptor agonist) [135] and sitagliptin (dipeptidyl peptidase-4 inhibitor) [241, 242], might be effective in treating NAFLD, but more evidence from control studies is required.
7. In nephrectomized rats, pioglitazone prevents the leakage of cytochrome c from the mitochondria, stabilizes the mitochondrial transmembrane potential, inhibits ROS generation, and activates the electron transport chain complexes I and III. At least in this model of disease, pioglitazone has renoprotective effects through modulating mitochondrial electron transport chain and mitochondrial dynamics, while protecting against fibrosis [180]. Further studies should extend such promising results under other conditions characterized by mitochondrial dysfunction, i.e., NAFLD. More evidence is required to establish the role of novel drugs in treating NAFLD, as selective modulators of PPAR α (pemafibrate, K-877), and PPAR γ (INT-131), PPAR δ (HPP-593), and PPAR α/γ (DSP-8658) agonists [115].
8. Metformin (dimethylbiguanide) improves hepatic and peripheral tissue sensitivity to insulin. In cultured HepG2 cells loaded with oleic acid to induce steatosis, metformin decreases steatosis and improves hepatocyte function. Mechanisms include reduction of oxidative stress injury, regulation of protein expression related to mitochondrial apoptosis pathway, and the inhibition of cell apoptosis [243]. Metformin also activates AMPK to stimulate mitochondrial biogenesis and fatty acid β -oxidation [181]
9. Liraglutide is an acylated glucagon-like peptide-1 (GLP-1) agonist. In the cultured liver HepG2 cells, it ameliorates NASH by inhibiting the nucleotide-binding oligomerization domain, leucine-rich repeat-containing receptor-containing pyrin domain 3 (NLRP3) inflammasome and pyroptosis activation via mitophagy [244]. In addition, liraglutide ameliorated NAFLD in HFD-fed mice by enhancing mitochondrial architecture, attenuating ROS production, and promoting autophagy through the SIRT1/SIRT3 pathway [182]

3.4 Bile Acids

1. Bile acids (BA) are soluble amphiphilic molecules, which represent the major lipid components of bile, together with phospholipids and cholesterol. Primary BA are synthesized from cholesterol in the liver and conjugated to glycine or

taurine to increase their solubility in bile. Upon hepatic secretion into bile, BA are concentrated in the gallbladder during fasting, expelled into the intestine in response to dietary fat and bio-transformed to secondary BA by the colonic gut microbiota. Both primary and secondary BA undergo reabsorption in the ileum and the colon, respectively, and then return to the liver, with minimal loss in the faces. Besides 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 farnesoid X receptor (FXR) and G-protein-coupled bile acid receptor-1 (GPBAR-1) in the liver, intestine, muscle, and brown adipose tissue [27]. In the liver, the BA-FXR interaction negatively regulates BA synthesis and acts transcriptionally to decrease hepatic lipogenesis and steatosis [245], whereas decreasing hepatic gluconeogenesis and decreasing peripheral insulin resistance [246].

2. The mechanism of action of OCA might imply the activation of FXR, with decreased hepatic lipogenesis because of downregulation of the transcription factor SREBP1c and increased SIRT1 expression [247, 248]. Also, FXR activation leads to intestinal production of fibroblast growth factor 19 (FGF19) which promotes mitochondrial fatty acid β -oxidation and hepatic glycogen synthesis after binding to FGF receptor (FGFR)4 in the hepatocytes [27, 249].
3. Among BA, the naturally occurring “tertiary” dihydroxy ursodeoxycholic acid (UDCA), the epimer of CDCA, shows multiple hepatoprotective effects and improves liver condition in patients suffering from several chronic liver diseases [250]. Based on previous results, UDCA is also tested in patients with NASH. In a previous open-label pilot study, UDCA showed some beneficial effects on liver enzymes and degree of steatosis (at biopsy) in patients with NASH [251]. However, in a subsequent randomized trial, the same research group randomized 166 patients with liver biopsy-proven NASH. Patients received 13–15 mg/kg/day of UDCA or placebo for 2 years. After 2 years, 126 patients completed the study, with 107 liver biopsies available. Although UDCA was safe, there was no advantage over placebo with regard to serum liver biochemistries, degree of steatosis, necroinflammation, or fibrosis. Likely, UDCA with its hydrophilic properties has poor affinity for FXR or might even antagonize FXR activity [252]. There is no information about analogues of UDCA, i.e., tauroursodeoxycholic acid (TUDCA) or nor-UDCA. At a mitochondrial level, lipophilic BA, like deoxycholic acid (DCA), chenodeoxycholic acid (CDCA), and lithocholic acid (LCA), inhibit the electron transport chain. The effects are unspecific on the inner mitochondrial membrane of intact mitochondria at high concentrations (100 μ mol/L), and specific (impairment of complex I and complex III) in broken mitochondria or in intact mitochondria incubated with low BA concentrations (10 μ mol/L) [253]. Under conditions of liver disease, i.e., cholestasis with excess retention of BA, mitochondrial antioxidative capacity is reduced [254]. In addition, impaired mitochondrial function is clinically relevant during cholestasis. At toxicologically relevant concentrations, most, but not all, BA alter mitochondrial bioenergetics [255]. UDCA displays antioxidant

and anti-inflammatory properties and prevents mitochondrial dysfunction in the progression of obesity-associated diseases. The effect of UDCA and TUDCA as well as the toxicity of lipophilic BA (CDCA and LCA) on the function of the electron transport chain is investigated in isolated rat liver mitochondria [256]. CDCA and LCA reduce state 3 oxidation rates and respiratory control ratios of L-glutamate, succinate, and duroquinol at a concentration of 30 $\mu\text{mol/L}$, without affecting ADP/O ratios of these substrates and oxidative metabolism of ascorbate. UDCA up to 100 $\mu\text{mol/L}$ does not interfere with mitochondrial oxidative metabolism, while at 300 $\mu\text{mol/L}$ has effects similar to CDCA and LCA. TUDCA has no significant inhibitory effect up to 300 $\mu\text{mol/L}$. The toxic effects of CDCA and LCA on mitochondrial oxidative metabolism are partially reversed with UDCA at 30 $\mu\text{mol/L}$ or 100 $\mu\text{mol/L}$, whereas UDCA at 300 $\mu\text{mol/L}$ plus CDCA or LCA produces greater toxicity compared with individual BA. TUDCA does not reduce the toxic effects of CDCA and LCA on mitochondrial metabolism. These studies show BA produce distinct effects on mitochondrial oxidative metabolism. UDCA, but not UDCA, decreases the toxicity of lipophilic BA on the function of the electron transport chain up to a concentration of 100 $\mu\text{mol/L}$ but increases BA-induced mitochondrial toxicity at higher concentrations. A possible mechanism is the decreased incorporation of BA into mitochondrial membranes. The protective effects of UDCA might go beyond the simple action on mitochondria and involve several mechanisms of metabolic damage. In fact, UDCA shows features of multi-targeted therapies and modulates glucose and lipid biosynthesis fluxes, inflammatory response, angiogenesis, and macrophage differentiation. In ob/ob mice, UDCA significantly reduces lipid droplets, as well as FFA and TG, improves mitochondrial function, and enhances white adipose tissue browning. UDCA increases hepatic energy expenditure, mitochondria biogenesis, and incorporation of BA metabolism (Abca1 and Abcg1 mRNA as well as BSEP, FGFR4, and TGR5 proteins). In addition, UDCA downregulates NF-kappa B and STAT3 phosphorylation through a negative regulation of the expression of SOCS1 and SOCS3 signaling. These changes occur together with decreased angiogenesis through downregulation of VEGF, VCAM, and TGF-betaRII expression. UDCA also reduces whole body adiposity, while decreasing adipose tissue expression of macrophage infiltration (CD11b, CD163, and CD206), as well as lipogenic capacity markers (lipofuscin, SREBP-1, and CD36). UDCA also upregulates adipose browning in association with upregulation of SIRT-1-PGC1-alpha signaling in epididymis adipose tissue (EWAT) [183].

4. Aramchol (Arachidyl-amido cholanoic) has some beneficial effects on liver steatosis in humans but does not improve liver enzymes, glucose metabolism, and insulin sensitivity [147, 148] (see above). In the animal model, Aramchol treatment improves steatohepatitis and fibrosis by decreasing stearoyl-coenzyme A desaturase 1 (SCD1) and increasing the flux through the transsulfuration pathway maintaining cellular redox homeostasis [147]. SCD1 deficiency in mice reduces lipid synthesis and increases mitochondrial FA β -oxidation (mitochondria) and insulin sensitivity in various tissues, including the liver. In

this context, SCD1 deficiency has been demonstrated to prevent liver steatosis in several mouse models of NAFLD, like mice fed the high-carbohydrate and high-fat diet.

3.5 Antioxidant Agents

1. Except for high doses of Vitamin E (*see* Table 4), the ultimate role of antioxidants in NAFLD is not established, due to several confounding factors [257].
2. Problems include nonspecific or insufficient efficacy of proposed agents or multifactorial origin of ROS in such a complex disease such as NAFLD. Moreover, available agents show poor cell membrane permeability and selectivity.
3. Effective therapeutic strategies should reduce the oxidative stress without severely disturbing general redox homeostasis.
4. Determining the tissue and cell specificity of antioxidative therapy is essential, together with identification of reliable biomarkers of liver damage [258].
5. Tempol is a nitroxide (4-Hydroxy-2,2,6,6-tetramethylpiperidine-*N*-oxyl) which undergoes 1 electron reduction reaction to hydroxylamines or 2 electron reduction reaction to oxammonium cations. This transformation provides redox metabolic actions. A study tested the efficacy of Tempol in mitigating NASH. Tempol acts by selectively modulating the gut microbiota composition and metabolism under pro-steatotic conditions. Following the change in gut microbiota, liver histology shows a marked reduction in lipid droplets. Tempol also decreases liver weight and liver/body mass ratios in mice fed a steatogenic diet by interfering with the intestine-specific disruption of FXR [259]. 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 [184].
6. Resveratrol, a polyphenol extract from red grapes and berries, exerts mitochondrial protectant properties [185]. Decrease of high-fat diet (HFD)-induced steatosis involves regulation of AMPK and SIRT1 [186–188]. Data obtained from clinical trials are still vague [260]. Although promising, the possible health benefits of resveratrol are only demonstrated by an attenuation of liver fibrosis via the AKT and NF- κ B pathway, but without effects on liver fat accumulation.
7. Other antioxidant agents target mitochondria, but their definitive roles in clinical field need further proofs. Mitoquinone (Mito-Q) and Mitovitamin E (MitoVit-E) are promising novel class of antioxidants. Both molecules have a covalently

attached lipophilic triphenylphosphonium (TPP) cationic moiety. This feature makes them able to transport and concentrate antioxidant molecules within mitochondria [189–191]. Mito-Q improves metabolic syndrome in rats fed a high-fat diet for eight weeks [261], and the effect is associated with increased expression of cardiolipin synthase and cardiolipin levels in liver mitochondria [262]. Mito-Q prevents hypercholesterolemia, hypertriglyceridemia, mtDNA oxidative damage, hyperglycemia, and hepatic steatosis in experimental models of atherosclerosis and metabolic syndrome [263]. Low doses of Mito-Q and MitoVit-E protect cells against peroxide-induced oxidative damage 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 reduce ROS-induced transferrin receptor-mediated iron uptake in mitochondria, lipid peroxidation, lipid peroxide-induced inactivation of complex I, and aconitase [264]. A phase II study in patients with chronic hepatitis C demonstrates that Mito-Q decreases circulating aminotransferase levels; this effect may suggest reduced hepatic inflammation and necrosis in these patients [265].

8. Pentoxifylline improves histological features of NASH compared to placebo in a randomized and controlled trial [202, 203]. Among the other effects, pentoxifylline appears to upregulate mitochondrial biogenesis and FA β -oxidation via increased expressions of PGC1 α and its downstream or parallel PPAR α [201].
9. Medicinal plants are often used as dietary supplements [266], and silymarin is extracted from milk thistle (*Silybum marianum*) with silybin as its major active compound. Silybin displays some hepatoprotective effects [267] and might improve insulin resistance, liver injury, and liver enzymes in patients with NAFLD [268, 269]. The silybin–phospholipid complex containing vitamin E improves liver steatosis in NAFLD patients [270] and significantly lowers 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 attenuates changes in mitochondrial respiratory complexes, with a major protective effect on complex II subunit CII-30 [192]. Recently, additional protective effects of silybin on rat hepatoma FaO cells are shown to mimic a progressive model of liver steatosis [193]. Of note, silybin reduces excessive TG accumulation and changes the expression of transcription factors (e.g., PPARs, enzymes for mitochondrial, peroxisomal, and ER oxidations of FFA). Silybin also rescues the FFA-induced mitochondrial dysfunction (size and function) and the apoptotic signals and oxidative stress, which resemble a condition of steatohepatitis [70].
10. Corilagin is a polyphenols tannic acid compound found in many ethnopharmacological plants and shows antioxidant properties [194]. In the animal model, corilagin reduces lipid deposition of diet-induced NAFLD via diminishing oxidative stress, restoring autophagic flux, as well as improving

mitochondrial function (decreased mitochondrial DNA oxidative damage and increased mitochondrial biogenesis-related transcription factors expression, mitochondrial DNA content and oxygen consumption rate).

11. Anthocyanins, plant flavonoids contained in the berries of bilberry and black currant, promote activation of AMPK and its downstream PGC-1 α . In mice, beneficial effects are described for restored mitochondrial content, biogenesis, OXPHOS, and FA β -oxidation, all pathways involved in oxidative stress, steatosis, inflammation, and fibrosis [195, 196]
12. Dihydromyricetin is a type of flavonoid found in the *Ampelopsis species like japonica, megalophylla, and grossedentata, Cercidi-phyllum japonicum, Hovenia dulcis, Rhododendron cinnabarinum*, some Pinus species; some Cedrus species; and in *Salix sachalinensis*. This compound ameliorates NAFLD in HFD-fed mice and in fatty hepatocytes loaded with palmitic acid. The mechanism involves SIRT3 leading to improved mitochondrial respiratory capacity and redox homeostasis in hepatocytes, less hepatic lipid accumulation, and decreased oxidative stress [197].
13. Berberine (isoquinoline alkaloid) improves OXPHOS in the liver of HFD-fed rats while increasing mitochondrial SIRT3 activity [198].
14. As antioxidant strategy, prevention of mitochondrial ROS production through uncoupling might be a valid alternative to removal of ROS by using antioxidants. The artificial uncoupler, 2,4-dinitrophenol (DNP), has toxic effects [271]. Further studies need to assess the ultimate role of this therapeutic strategy especially in NAFLD [272]. Controlled-release mitochondrial protonophore (CRMP) is a controlled-release oral formulation of DNP that produces mild hepatic mitochondrial uncoupling. In rat models, CRMP reduces hypertriglyceridemia, insulin resistance, hepatic steatosis, and diabetes. CRMP also normalizes plasma transaminase concentrations, ameliorates liver fibrosis, and improves hepatic protein synthetic function in a methionine/choline-deficient rat model of NASH. There was no systemic toxicity [273] .
15. The antioxidant hydroxytyrosol (HT) shows some beneficial effects also on mitochondrial function in HFD-fed mice when coadministered with n-3 LCPUFA eicosapentaenoic acid (EPA) [173].
16. Cysteamine (an aminothioliol) acts as a scavenger of ROS. This step parallels the enrichment of glutathione stores, with potential benefit for NAFLD. Liver enzymes are improved in children with biopsy-proven NAFLD with cysteamine bitartrate, but liver histology as well NASH does not improve [199, 200].
17. Avocado oil might improve mitochondrial dynamics acting on the respiratory chain in the animal model [205, 206], but the ultimate efficacy is controversial [204].

3.6 Mitotherapy

1. The term mitotherapy defines a procedure characterized by the exogenous intravenous injection of functional mitochondria from hepatoma cells. This promising procedure might successfully improve the phenotype of liver steatosis induced by high-fat diet. The mechanism includes decreased lipid content and improved cellular redox balance.
2. According to the protocol, exogenous mitochondria are tagged with green-fluorescence protein (GFP) and retrieved in mouse liver, lungs, brain, muscle, and kidneys [207, 208]. This strategy leads to attenuation of lipid deposits, prevention of cell injury, and 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 [56]. Further research is evolving in this direction, especially that concerning the nature of the administrated mitochondria with potential metabolic and proteomic differences vs. mitochondrial isolated from normal, nontumor-derived hepatocytes.

3.7 Miscellanea of Novel Agents

1. Research is evolving and testing the potential beneficial effects of other agents on mitochondria.
2. Aramchol also acts on mitochondrial function in improving NAFLD/NASH (see above). SCD1 deficiency in mice is associated with reduced lipid synthesis and increased mitochondrial FA β -oxidation and insulin sensitivity in various tissues, including the liver [274, 275]. Aramchol improves steatohepatitis and fibrosis in mice by decreasing SCD1 and increasing the flux through the transsulfuration pathway. These steps maintain cellular redox homeostasis [147].
3. Baicalin is the flavonoid component of the herbal medicine *Scutellaria baicalensis*, and the agent binds and activates CPT-1, the rate-limiting enzyme of FA β -oxidation (Fig. 3). As a consequence, FA degradation is increased and some aspects of liver steatosis is also improved [209].
4. Nitro-oleic acid treatment improves the function of hepatic mitochondrial complexes I, IV, and V and decreases oxidative stress, with protection in mice from diet-induced hepatic steatosis [210].
5. Carboxyatractyloside acts as a specific inhibitor of ANT. Systemic administration of low-dose carboxyatractyloside appears to protect mice against fatty liver [211].
6. Genistein, an isoflavone, does not inhibit hepatic steatosis, but attenuates steatohepatitis induced in methionine-choline-deficient (MCD) diet-fed mice. The mechanism includes AMPK inactivation and inhibition of inflammation [212].
7. GS-0976 is a potent acetyl-CoA carboxylase (ACC) inhibitor also effective on mitochondrial ACC2 (see above for detailed discussion). If ACC is inhibited,

mitochondrial FA β -oxidation is increased, and this effect might reduce hepatic steatosis and fibrosis [139].

8. The ultimate effect of such molecules on mitochondrial function is not defined and deserves further and specific investigations.

4 Summary

NAFLD is now one of the most common chronic liver diseases worldwide, putting the populations at increasing risks of end-stage liver disease, HCC, and extrahepatic diseases such as CVD. Both prevention and treatment of NAFLD at any possible stage are therefore of paramount importance. A number of 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. Combination therapies might be considered in the near future. Ideally, disease-modifying factors may act both on mitochondrial function and energy supply other than on intracellular regulators of lipid metabolism. Adequate duration and power are needed in order to evaluate the long-term efficacy and safety of each potential therapeutic option.

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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	α -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 disulfides
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-α	tumor necrosis factor-alpha

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Fig. 1.

Mechanisms of lipid accumulation in the liver. In health, free fatty acids (FFA) originate from lipolysis of triglycerides (TG) in adipose tissue leading to circulating nonesterified fatty acids, (NEFA, ~60%), from dietary fatty acids (~15%), and from dietary sugars undergoing de novo lipogenesis (DNL, ~25%) mediated by several proteins (sterol regulatory element-binding protein 1, stearoyl CoA-desaturase, and fatty acid synthase). FA in hepatocytes undergo mitochondrial β -oxidation or reesterification with glycerol to form TG. TG can be stored in lipid droplets or exported to blood as very-low-density lipoproteins (VLDL). NAFLD is the consequence of excess flow of FFA and accumulation of intrahepatic TG due to several abnormalities depicted in the boxes, and associated with increased influx of FFA, increased DNL, decreased β -oxidation, and decreased secretion/export of TG as very-low-density lipoproteins (VLDL). Abbreviations: DNL, de novo lipogenesis; FFA, free fatty acids; NEFA, nonesterified fatty acids; TG, triglycerides; VLDL, very-low-density lipoproteins.

Fig. 2.

(a) Mechanisms of lipotoxicity in the liver. Excessive accumulation of free fatty acids (FFA) is the result of increased influx, increased synthesis, decreased mitochondrial oxidation of FFA, or decreased secretion/export of FFA. Adiponutrin (also known as patatin-like phospholipase domain-containing protein 3, PNPLA3) governs the lipolysis of lipid droplets with FA flowing back to the hepatocyte FFA pool. With excess FFA, their disposal through beta-oxidation or formation of triglyceride is insufficient and lipotoxic species form (lysophosphatidylcholine, LPC; diacylglycerol, DAG; ceramides) that mediate

endoplasmic reticulum (ER) stress, oxidant stress, and activation of the inflammasome (the multiprotein cytoplasmic complex that responds to damage-associated molecular patterns (DAMPs), as part of the innate immunity response). External factors, poorly understood so far, may include dysregulation of cytokines and adipokines, ATP depletion, toxic uric acid, sleep apnea leading to periodic hypoxia, and products of the gut microbiome (tumor necrosis factor (TNF- α), endogenous ethanol, endotoxins like lipopolysaccharides (LPS)). Such events promote the phenotype of NASH, hepatocellular injury, inflammation, stellate cell activation, and progressive accumulation of excess extracellular matrix. Further target structures of damage are other intracellular organelles, the nucleus, receptors, and signaling pathways. *See also* [19, 22, 30, 31]. **(b)** Detailed picture representing the ultimate consequence of circulating damage-associated molecular patterns (DAMPs) on hepatocytes. DAMs represent the continuous stimulus for at least three intracellular events in the hepatocyte: (1) response of pattern-recognition receptors (PRRs) such as Toll-like receptors (TLRs) and NOD-like receptors (NLRs); (2) activation of intracellular cascades, kinases representing the downstream signaling pathways, such as the apoptosis signal-regulating kinase 1 (ASK1) and TGF- β -activated kinase 1 (TAK1). Both kinases are activated through posttranscriptional modification (PTM) and activate other key kinases (C-Jun N-terminal kinase, JNK), AMP-activated kinase, AMPK, and I κ B; (3) activation of transcription factors such as interferon regulatory factors (IRFs), nuclear factor (NF)- κ B, activator protein 1 (AP-1), and peroxisome proliferator-activated receptors (PPARs). This ultimate event leads to the production of inflammatory cytokines and chemokines, with all metabolic consequences observed in NAFLD (insulin resistance, steatohepatitis, fibrogenesis, etc.). Additional endogenous targets regulating the innate immune elements in NASH include CASP8 and FADD-like apoptosis regulator (CFLAR), tumor necrosis factor- α (TNF- α)-induced protein 3 (TNFAIP3), cylindromatosis (CYLD), transmembrane BAX inhibitor motif-containing 1 (TM6SF1), TNF receptor-associated factor 6 (TRAF6), TRAF1, TRAF3, dual-specificity phosphatase 14 (DUSP14), tripartite motif 8 (TRIM8), dickkopf-3 (DKK3), and TRAF5 (*see also* [22]).

Fig. 3. Chemical structure, name, formula, and molecular weight of fats accumulating in the steatotic liver: fatty acid (e.g., oleic acid), triglyceride, lecithin (lysophosphatidylcholine), ceramide, and cholesterol.

Fig. 4.

Key events in mitochondrial adaption and oxidative stress in NAFLD. The events contributing to mitochondrial oxidative metabolism and hepatocyte energy homeostasis include fatty acid β -oxidation, the tricarboxylic acid cycle (TCA), electron flow along the electron transport chain (ETC) electrochemical proton gradient generation, and ATP synthesis. In the absence of glucose, ketogenesis takes place. Dietary carbohydrates (Piero, NEFA is FFA) and dietary free fatty acids (FFA) provide two major sources which enrich the free fatty acid pool. Glucose, either from dietary sources or from hepatic glycogen, is converted to pyruvate during glycolysis. Pyruvate can either enter the mitochondrion or provide the substrate to the FFA pool via DNL. In the cytoplasm, FFA are first converted into fatty acyl-CoA, and carnitine-acyl-CoA is formed due to the mitochondrial carnitine palmitoyltransferase 1 (CPT-1); acyl carnitine is transported via the acylcarnitine/carnitine antiporter inside mitochondria where because of CPT-2, acyl-CoA is formed. In the mitochondrial matrix, acyl-CoA undergoes β -oxidation providing acetyl-CoA which is oxidized in the tricarboxylic acid (TCA) cycle to provide energy. During the TCA cycle, products are citrate, cis-aconitate, isocitrate, α -ketoglutarate, succinyl-CoA, succinate, fumarate, malate, and oxaloacetate. Ketone body synthesis is the consequence of prolonged starvation (or diabetes), when oxaloacetate is depleted due to gluconeogenesis. Then, acetyl-CoA does not enter the TCA cycle and, in the mitochondria, is converted to acetone, acetoacetate, and β -hydroxybutyrate, β -HB (ketone bodies). In case of excess FFA influx, hepatic fatty acid β -oxidation is insufficient, thus providing accumulation of lipotoxic intermediates. The process of mitochondrial β -oxidation generates NADH and FADH₂ with electron transport to the electron transport chain (ETC). Under normal conditions, the rate of electron flow through the ETC is limited by the rate of ATP turnover as well as by the rate of processes which make use of the electrochemical proton gradient. However, impairment of electron transfer across ETC results in the generation of reactive oxygen species (ROS).

Mitochondrial ROS also originate from reactions catalyzed by enzymes such as long acyl-CoA dehydrogenase (LCAD), very long-chain acyl-CoA dehydrogenase (VLCAD), glycerol 3-phosphate dehydrogenase (GPDH), α -ketoglutarate dehydrogenase (AKGDH), and pyruvate dehydrogenase (PDH). Abbreviations: ACC, acetyl-CoA carboxylase (ACC); ANT, adenine nucleotide translocator; CACT, carnitine-acylcarnitine transferase; CPT-1, carnitine palmitoyltransferase-1; CPT-2, carnitine palmitoyltransferase-2; DNL, de novo lipogenesis; ETF, electron transfer flavoprotein; ETFDH, ETF dehydrogenase; β -HB, β -hydroxybutyrate; PEP, phosphoenolpyruvic acid; TG, triglycerides; VLDL, very-low-density lipoprotein [48].

Fig. 7.

Potential targets at a mitochondria level in NAFLD. Several agents have been tested in cellular/animal models or at a very preliminary clinical stage, with few supportive evidence. Different levels of therapeutic targets include: (1) nuclear receptors and different signaling pathways; (2) enzymes acting in mitochondrial metabolism; and (3) pathways controlling reactive oxygen species (ROS) and oxidative stress. Abbreviations: AMPK, AMP-activated protein kinase; SIRT6, sirtuins; PGC-1α, peroxisome proliferator-activated receptor coactivator 1α; PPARs, peroxisome proliferator-activated receptors; ERRs, estrogen-related receptors; NRFs, nuclear respiratory factors. ANT, adenine nucleotide translocator; UCPs, uncoupling proteins; Cyt. C, cytochrome c; CPT-1, carnitine palmitoyltransferase 1; CPT-2, CPT-1, carnitine palmitoyltransferase 2; SOD2, superoxide dismutase 2; IDH2, isocitrate dehydrogenase 2 [179].

Fig. 8.

Main reports on liver tissue and mitochondrial adaptations by acute and chronic physical exercise, high aerobic capacity, and sedentary behaviors. Abbreviations: ECT, electron transport chain; ESP, heat shock proteins; MTP, mitochondrial permeability transition pore; SIRT, sirtuin. Adapted from [223]. Cartoons from <http://www.riskmanagement365.com/wp-content/uploads/2013/03/physical-exercise.jpg> and <http://johannesbrug.blogspot.com/2015/10/determinants-of-engaging-in-sedentary.html> [229].

Table 1

Most frequent causes of liver steatosis

• Nonalcoholic fatty liver (NAFLD)
• Alcoholic liver disease (ALD)
• Hepatitis C (in particular genotype 3)
• Lipodystrophy
• Wilson disease
• Starvation
• Parenteral nutrition
• Abetalipoproteinemia
• Drugs (e.g., methotrexate, tamoxifen, glucocorticoids, amiodarone, valproate, anti-retroviral agents for HIV)
• Acute fatty liver of pregnancy
• HELLP (hemolytic anemia, elevated liver enzymes, low platelet count) syndrome
• Reye syndrome
• Inborn errors of metabolism (LCAT deficiency, cholesterol ester storage disease, Wolman disease)
• Drug-induced liver disease

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Table 2

Species of toxic lipids accumulating in NAFLD

Lipid	Notes
Free fatty acids (FFAs)	<ul style="list-style-type: none"> • FFAs originate from adipose tissue during underlying insulin-resistant status, de novo lipogenesis (from carbohydrates), dietary sources, decreased export as TG in VLDL • FFAs are internalized by CD36, which increases during insulin-resistant status • Especially toxic: saturated FFA (e.g., palmitate, stearate) • Less toxic: monounsaturated FFA (e.g., oleate)
Triglycerides (TG)	<ul style="list-style-type: none"> • Accumulate as micro- and macro-droplets. • Originating from increased influx of FFA • Considered as a more inert form which can also have an initial protective effect against the ongoing lipotoxic injury [29, 34]
Lysophosphatidylcholine (LPC)	<ul style="list-style-type: none"> • Originating from phosphatidylcholine via the intracellular action of phospholipase A2 or from extracellular lecithin-cholesterol acyltransferase • Agent able to mediate intracellular damage (endoplasmic reticulum stress, activation of apoptotic pathways downstream of JNK), also interacting with palmitate [35, 36]
Ceramides	<ul style="list-style-type: none"> • Ceramides, belong to a family of lipid molecules, are involved in inflammation and cell toxicity via interaction with TNF-α. Inhibition of ceramide synthesis decreases steatosis, cell injury, and insulin sensitivity in animal models of NAFLD [37] • Ceramides generate from serine and palmitoyl-CoA (enzyme serine palmitoyltransferase) and also from sphingomyelin (enzyme neutral sphingomyelinase) • Mediate insulin resistance, cell toxicity, and pro-inflammatory effects (sequence IL-1 $\rightarrow$ ceramides $\rightarrow$ TNFα $\rightarrow$ inflammation) [37] • Can induce release of extracellular vesicles (EV) $\rightarrow$ cell-cell communication (also in NASH)
Free cholesterol	<ul style="list-style-type: none"> • Role in inflammation, fibrosis, and liver injury [38] • Target cells: hepatocytes, stellate cells, and Kupffer cells • One sequence: liver steatosis/NASH $\rightarrow$ increased expression sterol regulatory element-binding protein (SREBP)-2 $\rightarrow$ upregulation of HMG-CoA reductase $\rightarrow$ increased synthesis of free cholesterol (mitochondria) [39] $\rightarrow$ apoptosis $\rightarrow$ JNK-dependent pro-inflammatory pathways

Table 3

Hallmarks of cellular events involved in NAFLD/NASH progression. Adapted from [22, 40]

• Insulin resistance
• Steatosis
• Oxidant stress
• Mitochondrial dysfunction
• Endoplasmic reticulum stress
• Inflammation
• Apoptosis
• Fibrosis

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Table 4

General measures for all NAFLD patients.

Measure	Notes
Alcohol abstinence	<ul style="list-style-type: none"> • Alcohol consumption associated with progression of hepatic fibrosis in NAFLD [90] • Endogenous ethanol produced by intestinal bacteria induces mitochondrial dysfunction in NAFLD [91]
Vaccination for hepatitis A virus and hepatitis B virus	<ul style="list-style-type: none"> • In patients without serologic evidence of immunity • Other vaccination similar to the rest of the population
Reducing risk factors Lifestyles	<ul style="list-style-type: none"> • Reducing risk factors for cardiovascular disease [20] • Control of diabetes mellitus • Lipid lowering therapy • Diet (long-term caloric restriction rather than intermittent fasting) to improve insulin sensitivity [92] and to prevent oxidative damage [93, 94]. Avoid extra fructose in diet [95]. Mediterranean diet is beneficial [48, 96] • Physical exercise (also to achieve weight loss or to maintain ideal weight). In the rodent model, endurance training as therapeutic tool mitigated the clinical/anatomical-related features induced by Lieber-DeCarli diet, thus possibly contributing to control obesity and metabolic disorders [97] • Weight loss (5–7% in NAFLD; 7–10% in NASH) in overweight and obese patients [6]. Improvement in liver biochemical tests, liver histology, serum insulin levels, and quality of life [98–103]. Even liver fibrosis can improve in NASH with at least 10% weight reduction, but goal is difficult to achieve in the majority of patients or to maintain for a long time [101] • Bariatric surgery, if weight loss is insufficient and patients meet inclusion criteria. Decreased prevalence of NASH [104, 105]. Follow-up for potential worsening of fibrosis [106–112]

Table 5

Current and experimental therapies for NAFLD patients (Chemical formulas are depicted in Fig. 6a–d)

Antioxidants	Notes
Vitamin E	<ul style="list-style-type: none"> • Potent antioxidant agent [116] • Used in patients with biopsy-proven NASH and fibrosis stage 2 without diabetes mellitus. High doses (800 IU/day, improved steatosis, fibrosis) [20]
Antiapoptotic agents	Notes
Emricasan	<ul style="list-style-type: none"> • Pan caspase inhibitor might inhibit liver injury, inflammation, and fibrosis [117, 118]. More studies expected
Metabolic	Notes
Metformin	<ul style="list-style-type: none"> • Insulin sensitizer • Initial treatment in patients with NAFLD and diabetes mellitus. No improvement of liver histology [20, 119, 120]
Pioglitazone, Rosiglitazone (Peroxisome proliferator-activated receptors, PPARs)	<ul style="list-style-type: none"> • Thiazolidinediones (PPARγ) – Pioglitazone improves liver histology (fibrosis as well as inflammation and steatosis) [116, 121–125]. Increased risk of weight gain, heart failure, and osteopenia, risk of fractures [126]. Less potent PPARγ-agonism than rosiglitazone, but potential effects on mitochondria (see text). – Rosiglitazone has stronger PPARγ agonism with effects in NASH [127–129] – The European Association for the Study of the Liver (EASL) and the American Association for the Study of Liver Diseases (AASLD) [20, 130] 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 [123]. However, it has no indication to treat NAFLD without biopsy-based proof of NASH [20, 130]. Both pioglitazone and rosiglitazone share similar concerns for treating patients with NASH in clinical practice [131]
Liraglutide (GLP-1 receptor agonists)	<ul style="list-style-type: none"> • Glucagon-like peptide (GLP-1) is an intestinal hormone originating through the proteolytic processing of proglucagon. GLP-1 stimulates insulin secretion and inhibits secretion of glucagon. Upon arrival in the terminal ileum, BA activate the G-protein-coupled receptor (GPBAR-1) and stimulate the secretion of peptide YY (PYY), glucagon-like peptide 1 (GLP-1), and glucagon-like peptide 2 (GLP-2) by the intestinal L cells [132]. GLP-1 has important metabolic effects on glucose metabolism [133]. In particular, GLP-1 decreases postprandial glucagon production while enhancing insulin secretion. This step is associated with improved insulin resistance (including the decrease of excess TG lipolysis and FFA influx in the liver). Additional GLP-1 central actions are involved in decreasing appetite and delaying gastric emptying. GLP-1, acting as insulin sensitizer, displays an anti-NASH activity [134]. GLP-1 receptor agonists reduce liver steatosis and inflammation and improve the phenotype of hepatic stellate cells with antifibrotic effects • Liraglutide (GLP-1 receptor agonist) in the LEAN Phase II trial is effective in patients with NASH in terms of weight loss, resolution of steatohepatitis, and less progression of fibrosis, compared with placebo. Gastrointestinal adverse effects are diarrhea, constipation, and appetite loss [135] • Semaglutide is another GLP-1 receptor agonist that is currently tested in phase II clinical trial.
Acetyl-CoA carboxylase (ACC) inhibitor (Regulation of DNL)	<ul style="list-style-type: none"> • During <i>de novo</i> lipogenesis, ACC is the enzyme governing the transformation of acetyl-CoA to malonyl-CoA. • There are two isoforms of ACC: cytoplasmic ACC1 and mitochondrial ACC2. Malonyl-CoA produced by ACC2 suppresses the mitochondrial FFA transfer protein CPT-1. This step leads to the inhibition of β-oxidation of fatty acyl-CoA. By contrast, if ACC is inhibited, mitochondrial FFA β-oxidation is increased. Animal studies are controversial [136–138]. GS-0976 (a potent ACC inhibitor) used in clinical trial for 12 weeks significantly reduced hepatic steatosis and fibrosis marker TIM1 in patients with biopsy-proven NASH and F1–F3 fibrosis [139]. Serum TG levels increased, however. Likely, there was a compensatory increase in sterol regulatory element-binding protein 1 activity, with TG accumulation from peripheral FFAs [140]
Targeting ATP-citrate lyase (ACLY)	<ul style="list-style-type: none"> • ATP-citrate lyase (ACLY) catalyzes the cleavage of citrate to generate oxaloacetate and acetyl-CoA, in response to excess nutrients. It might become a therapeutic target for treatment of NASH [141]
Obeticholic acid (OCA) (Activation of farnesoid X receptor, FXR)	<ul style="list-style-type: none"> • Liver farnesoid X receptor (FXR) is a BA nuclear receptor highly expressed in the liver and small intestinal mucosa with key role in glucose and lipid metabolism [27]. In particular, FXR activation activates the inhibition of lipogenesis and gluconeogenesis, restitution of insulin sensitivity, and suppression of BA synthesis [142] • OCA (6-ethylchenodeoxycholic acid) is the lipophilic synthetic variant of CDCA. OCA is FXR semisynthetic agonist with 100-fold higher potency than that of chenodeoxycholic acid and promotes FFA oxidation and hepatic glycogen synthesis [28, 143, 144]. FXR is downregulated in NAFLD and OCA is a potent FXR activator [145]. OCA was tested in a randomized, multicenter double-blind placebo-controlled, parallel group clinical trial at 25 mg/day orally for 72 weeks. OCA improved liver histology of NASH without worsening of fibrosis in 45% of the treated patients vs. 21% of the placebo group. The liver enzymes serum alanine aminotransferase and aspartate aminotransferase concentrations decreased during OCA treatment [28]. In the FLINT trial, pruritus appeared in 23% of OCA-treated patients and its long-term safety and tolerability is still unclear. In addition, OCA at 25 mg/daily increased low-density lipoprotein (LDL) cholesterol in some patients [28]. Other trial, REGENERATE and REVERSE are in progress. • Mitochondrial effects of OCA are anticipated (see below) • Other non-bile acid FXR agonists (LJN452 and GS9674) will be investigated in clinical trials.
Aramchol (Inhibition of stearyl coenzyme	<ul style="list-style-type: none"> • Arachidyl-amido cholanoic acid (Aramchol) is the fatty acid–bile acid conjugate and inhibits stearyl coenzyme A desaturase 1 (SCD1), an endoplasmic reticulum enzyme that catalyzes the rate-limiting step in the formation of monounsaturated fatty acids (MUFAs) from saturated FA, specifically oleate and palmitoleate from stearyl-CoA and

Antioxidants	Notes
A desaturase 1, SCD1)	<p>palmitoyl-CoA (the rate-limiting step in hepatocyte lipogenesis) [146]. SCD1 modulates liver fatty acid metabolism, and SCD1 inhibition decreases the synthesis and increases β-oxidation of fatty acids. This enzyme plays a role as the rate-limiting step in the synthesis of monounsaturated fatty acids (MUFAs), which are the major fatty acids of TG, cholesteryl esters, and membrane phospholipids.</p> <ul style="list-style-type: none"> In vitro models show that aramchol provides 70% to 83% inhibition of SCD1. This step decreases hepatic storage of TG and fatty acid esters. Aramchol was used as the in a phase IIa trial in 60 patients with biopsy-proven NAFLD for three months. This drug was safe, well tolerated, and significantly reduced liver fat content (magnetic resonance spectroscopy) in patients with NAFLD. The anti-steatotic effect occurred in a dose-dependent manner with a trend of metabolic improvements. Effects on inflammation and fibrosis need further investigation [147, 148] Effects might also be evident at the level of mitochondria in the liver (see below)
Fibroblast growth factor-19 (FGF-19)	<ul style="list-style-type: none"> The terminal small intestine upon effect with bile acids releases FGF-19 [27, 149]. FGF-19 is a regulator of energy expenditure [150], and acts on liver FXR via the FGFR4β-Klotho receptor [27]. Human studies show that FGF-19 decreases hepatic fat and liver enzymes in patients with biopsy-confirmed NASH [151]
Fibroblast growth factor-21 (FGF-21)	<ul style="list-style-type: none"> FGF-21 is expressed mainly in the liver and is a potent activator of glucose uptake on adipocytes [152]. FGF-21 displays insulin-sensitizing and antifibrotic effects in the liver. Tested in animal models and in a short-term trial in humans [153]
Elafibranor	<ul style="list-style-type: none"> Elafibranor (GFT505) is dual peroxisome proliferator-activated receptor (PPAR) α/δ agonist. In the animal model, elafibranor exerts antidiabetic effects in db/db mice without PPARγ-associated adverse cardiac effects [154, 155]. Elafibranor in a short-term trial (4–12 weeks) decreased ALT [154, 156]; at a dose of 80 mg ($N=93$) and 120 mg ($N=89$) daily for 12 months the drug was used in a randomized controlled trial in patients with biopsy-proven NASH, leading to histologic improvement, and without worsening of fibrosis. Liver enzymes, glucose and lipid profiles, and systemic inflammatory markers were also improved [157]. Elafibranor has potential effects on mitochondria (see below)
Resmetirom (Thyroid Hormone Receptor (THR) β)	<ul style="list-style-type: none"> Resmetirom is hepatic thyroid hormone receptor (THR)β-selective agonist [89] with anti-steatogenic effects [158] MGL-3196 treatment for 12 weeks decreased the amount of liver fat by approximately 40%, with few adverse reactions [159] The effect of VK2809 in patients with NAFLD and hyperlipidemia is being investigated
Immune response	Notes
Selonsertib (GS-4997)	<ul style="list-style-type: none"> The innate immune response plays a key role in the pathogenesis of NASH and involves the pathways such as (ASC-1)-JNK, MAP kinase, ERK, and NFκB. Activated ASK1 induces downstream signaling transduction, leading to inflammation, apoptosis, and fibrosis [23, 160]. ASK1 becomes a therapeutic target in NASH Selonsertib is a selective inhibitor of ASK1 and short-term clinical trial exist in NASH patients [161] Experimental agents acting as ASK1 inhibitors are CASP8 and FADD-like apoptosis regulator (CFLAR) and TNF-α-induced protein (TNFAIP)3 [162, 163]. Studies are in progress
Cenicriviroc	<ul style="list-style-type: none"> Cenicriviroc is a chemokine receptor antagonist targeting C-C motif chemokine receptor 2 and 5 with effect on innate immunity, activation of stellate cells and fibrosis. Potential beneficial effects in NASH and inflammation are under investigation [164]
Cylindromatosis (CYLD)	<ul style="list-style-type: none"> TAK1 belongs to the MAP3K family within the innate immunity signaling transduction (JNK–p38, NF-κB signaling pathways [165]) CYLD inhibits TAK1 activation through its deubiquitinase function (i.e., inhibits TAK1 overactivation without deletion) and might be effective in suppressing NASH progression [166]
Antifibrotic agents	Notes
ND-L02-s0201 anti-heat shock protein 47 (HSP47)	<ul style="list-style-type: none"> With ongoing liver injury, both fibrogenesis and fibrolysis play a key role in the progression of NASH. A rational approach includes the inhibition of fibrogenesis, as well as the acceleration of fibrinolysis. Hepatic stellate cells play a role in this respect, and an ongoing trial includes a phase 1b/2, open label, randomized, repeat dose, dose escalation study to evaluate the safety, tolerability, biological activity, and pharmacokinetics of ND-L02-s0201 injection, a vitamin A-coupled lipid nanoparticle containing siRNA against HSP47, in subjects with moderate to extensive hepatic fibrosis (METAVIR F3–4) (ClinicalTrials.gov number: NCT02227459). The mechanism includes apoptosis of hepatic stellate cells
Belapectin (Inhibitor of galectin)	<ul style="list-style-type: none"> Galectin acts as a fibrogenic factor. In a subgroup analysis of patients without esophageal varices, 2 mg/kg belapectin did reduce hepatic vein portal gradient (HVPG) and development of varices. ClinicalTrials.gov number: NCT02462967. [167]
Agents acting at extrahepatic levels	Notes
BAR502	<ul style="list-style-type: none"> Animal studies show that BAR502 is a molecule with dual activity as a ligand for FXR and TGR5 (a G protein-coupled receptor specific for BA). BAR502 leads to browning of white adipose in NASH [168]
Probiotics	<ul style="list-style-type: none"> The intestinal microbiota and intestinal permeability appear to play an important role in NAFLD. Future therapies might therefore include the diagnosis of intestinal dysbiosis, as well the use of single or combined probiotics [169]
Uncertain therapies	Notes
Atorvastatin	<ul style="list-style-type: none"> Improvement of liver enzyme aminotransferase levels [170–172]

Antioxidants	Notes
Omega-3 fatty acids	• Some beneficial effects in the animal model of NAFLD induced by a high-fat diet [173]. Improvement in hepatic steatosis and aspartate aminotransferase levels [174, 175]
Aspirin	• Studies have documented less fibrosis and evolution to NASH [176]

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Table 6

Therapeutic strategies proposed to ameliorate mitochondrial function in NAFLD (Chemical formulas are depicted in Fig. 6a–d)

General measures	Notes
Lifestyles	<ul style="list-style-type: none"> • Moderately hypocaloric diet with physical exercise contributes to improve mitochondrial function and alleviate inflammation [57, 178, 179]
Antidiabetic drugs	<ul style="list-style-type: none"> • Thiazolidinediones (pioglitazone) [180]. • Metformin [181] • Liraglutide [182] • Elafibranor [154, 157]
Bile acids	<ul style="list-style-type: none"> • Obeticholic acid [28, 143, 144] • Ursodeoxycholic acid [183]
Agents acting as antioxidants, on nuclear receptors or mitochondrial metabolism	<ul style="list-style-type: none"> • Vitamin E (α-Tocopherol) [20] • Tempol [184]^a • Resveratrol [185–188]^a • Mitoquinone (Mito-Q) and Mitovitamin E (MitoVit-E) [189–191]^a • Silymarin (major component is Silybin) [70, 192, 193] • Corilagin [194]^a • Anthocyanins (i.e., Cyanidin) [195, 196]^a • Dihydromyricetin [197]^a • Berberine [198]^a • Hydroxytyrosol [173]^a • Cysteamine [199, 200]^a • Pentoxifylline [201–203]^a • Avocado oil [204–206]^a
Mitotherapy	<ul style="list-style-type: none"> • Mitotherapy implies exogenous mitochondria tagged with green-fluorescence protein (GFP) and retrieved in mouse liver, lungs, brain, muscle, and kidneys [207, 208]. Improved energy production may restore hepatocyte function. The approach is highly experimental [56]^a
Miscellanea	<ul style="list-style-type: none"> • Aramchol [147, 148]^a • Baicalin [209]^a • Nitro-oleic acid [210]^a • Carboxyatractyloside [211]^a • Genistein [212]^a • GS-0976 (Acetyl-CoA carboxylase (ACC) inhibitor)^a

^aHighly experimental. Further evidence is required