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Mitochondria, oxidative stress and nonalcoholic fatty liver disease: A complex relationship

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Abstract

According to the 'multiple-hit' hypothesis, several factors can act simultaneously in nonalcoholic fatty liver disease (NAFLD) progression. Increased nitro-oxidative (nitroso-oxidative) stress may be considered one of the main contributors involved in the development and risk of NAFLD progression to nonalcoholic steatohepatitis (NASH) characterized by inflammation and fibrosis. Moreover, it has been repeatedly postulated that mitochondrial abnormalities are closely related to the development and progression of liver steatosis and NAFLD pathogenesis. However, it is difficult to determine with certainty whether mitochondrial dysfunction or oxidative stress are primary events or a simple consequence of NAFLD development. On the one hand, increasing lipid accumulation in hepatocytes could cause a wide range of effects from mild to severe mitochondrial damage with a negative impact on cell fate. This can start the cascade of events, including an increase of cellular

reactive nitrogen species (RNS) and reactive oxygen species (ROS) production that promotes disease progression from simple steatosis to more severe NAFLD stages. On the other hand, progressing mitochondrial bioenergetic catastrophe and oxidative stress manifestation could be considered accompanying events in the vast spectrum of abnormalities observed during the transition from NAFL to NASH and cirrhosis. This review updates our current understanding of NAFLD pathogenesis and clarifies whether mitochondrial dysfunction and ROS/RNS are culprits or bystanders of NAFLD progression.

KEYWORDS

mitochondria, mitochondrial dysfunction, nonalcoholic fatty liver disease, nonalcoholic steatohepatitis, oxidative stress, ROS

1 | NAFLD DEFINITION, DESCRIPTION AND EPIDEMIOLOGY

Nonalcoholic fatty liver disease (NAFLD) has an increasing incidence, commonly attributed to unhealthy lifestyles. NAFLD diagnosis is based on the presence of hepatic steatosis documented by imaging or histology and exclusion of excessive alcohol consumption, chronic or acute liver diseases, and other secondary causes of steatosis. Moreover, NAFLD is frequently referred to as a hepatic manifestation of metabolic syndrome. The prevalence of NAFLD and nonalcoholic steatohepatitis (NASH) has increased over the past decades and exceeded 25% of the adult population worldwide. There are differences between regions but NAFLD cannot be considered a Western countries disease exclusively anymore. NAFLD prevalence ranges from 13% in Africa to 30% in Asia and 32% in the Middle East. Europe and North America have reported a prevalence of 24%. Regional leaders have even higher prevalence, such as 30% in China, 33% in India and 51% in Indonesia.[1,2] The incremental trends of the NAFLD epidemic in Western countries have slowed down compared to Asia but more significant numbers of patients with chronic disease account for a high incidence of progression into more advanced stage. It is reflected by an increase in disease burden measures related to cirrhosis and hepatocellular carcinoma (HCC) in patients with NAFLD or NASH compared to other chronic liver diseases. NASH has also gained importance as an underlying cause of end-stage liver disease and HCC among liver transplant candidates in Western countries.[3,4] Projections for the NAFLD epidemic up to 2030 have been reported recently. Estimates show that the total prevalence of NAFLD will increase by 13%-20% in Europe, 18% in the United States and 29% in China. The prevalence of NASH will increase by 43%-49% in Europe and China and 56% in the United States.

Genetic analyses of patients with fatty liver demonstrated that inherited predisposition also plays a vital role in the development and progression of hepatic steatosis.[5] Indeed, familial clustering and the result of twin studies showing greater concordance between monozygotic compared to dizygotic twins underscore the role of genetics in NAFLD.[6] In recent years, we have learned that carriers of the common adiponutrin a.k.a. patatin-like phospholipase domain-containing protein 3 (PNPLA3) variant p.I148M are at increased risk of developing NAFLD,[7] liver fibrosis and cirrhosis,[8] as well as hepatocellular carcinoma (HCC).[9] Other variants, for example, membrane-bound O-acyltransferase domain-containing 7 gene (MBOAT7) variant rs641738 C>T, transmembrane 6 superfamily member 2 gene (TM6SF2) variant p. E167K

or glucokinase regulatory protein gene (GCKR) variant p. P446L have also been shown to enhance the NAFLD risk.[10] PNPLA3, which is highly expressed on the intrahepatic lipid droplets, is involved in hydrolysis of triglycerides. This risk variant is most prevalent in Hispanics and is the least common in African Americans (17%). Previous studies indicate the PNPLA3 might have a lysophosphatidic acyltransferase activity[11] or function as a lipase.[12] It was also postulated that in hepatic stellate cells, PNPLA3 has retinyl-palmitate lipase activity.[13] TM6SF2 is, in turn, involved in the hepatic secretion of VLDL (very low-density lipoproteins). Carriers of the TM6SF2 p.E167K variant have lower circulating VLDL, resulting in diminished cardiovascular risk and increased lipid contents in the liver.[14] MBOAT7 possesses a lysophosphatidylinositol acyltransferase activity and is involved in anti-inflammatory processes by regulating arachidonic acid levels.[15] NAFLD-associated genetic variant of MBOAT7 was linked to lower expression of MBOAT7 and its decreased function.[16] This already somewhat complicated picture of NAFLD genetics gained recently another level of complexity: variants in two genes, namely mitochondrial amidoxime reducing component 1 (MARC1)[17] and hydroxysteroid 17-beta dehydrogenase 13 (HSD17B13),[18] were shown to have protective effects on liver status in the setting of NAFLD. Not surprisingly, polygenic risk scores, including the above-listed polymorphisms, have been developed (eg as shown lately by Bianco et al).[19] These polygenic scores facilitate stratification of patients with fatty liver according to their inherited predisposition. However, genetic analyses of patients with NAFLD have not gained much attention in the clinic.[5]

2 | DIAGNOSIS, CLASSIFICATION, STAGING AND NONINVASIVE BIOMARKERS OF NAFLD

2.1 | Diagnosis and classification

NAFLD diagnosis is based on the presence of hepatic steatosis documented by imaging or histology, and exclusion of excessive alcohol consumption or other secondary causes of steatosis. Clinical description broadens the perspective, and NAFLD is frequently referred to as a hepatic manifestation of metabolic syndrome. Liver biopsy is considered the gold standard in diagnosing NASH and differentiation between various stages of the disease characterized by steatosis, inflammation, steatohepatitis, fibrosis and cirrhosis. Noninvasive tests such as imaging studies and various biochemical indexes are less accurate and cannot replace biopsy. However, ultrasound examination is used to diagnose of hepatic steatosis in more than 90% of participants in extensive cohort studies.[20]

2.2 | Staging

Depending on the pathological stage of the disease, the risk of progression and complications of NAFLD increases. Simple steatosis without hepatocyte injury that is a predominant presentation of the disease is associated with a limited risk of progression into more advanced stages, including steatohepatitis, fibrosis and cirrhosis. By definition, at least 5% of hepatic steatosis is required to establish the diagnosis and less than 5% of fatty infiltration is not considered NAFLD. NASH is defined as fatty infiltration exceeding 5% with concomitant inflammation and hepatocyte injury. At the time of diagnosis, various fibrosis degrees are present, ranging from no fibrosis up to cirrhosis.[1] The staging of NASH is most commonly based on the pathological scores such as NAFLD Activity Score (NAS) or Steatosis Activity Fibrosis (SAF).[21,22] In NAS score, each feature adds points to 0-8 total including 0-3 points for steatosis (in the range of <5%, 5%-33%, 33%-66% and >66%), 0-3 for lobular inflammation and 0-2 for ballooning. The diagnosis is NASH ≥5 points and not-NASH ≤2 points and a borderline diagnosis of NASH for 3-4 points. Fibrosis is classified as stages 0-4. Disease activity variations assessed with NAS correlate with progression or regression of fibrosis.[23]

2.3 | Noninvasive biomarkers of NAFLD

Considering the fact that the alteration of specific proteins or peptides in patients' serum may be related to a specific disease, several groups also try to identify novel diagnostic biomarkers characteristic for different stages of NAFLD. To avoid an invasive liver biopsy, new reliable, noninvasive biomarkers that identify the disease's progression are urgently needed. Unfortunately, liver enzymes per se are not reliable and accurate predictors of NAFLD. Circulating extracellular vesicles (EVs) and cell-derived small membrane-surrounded structures seem to be a promising NAFLD and NASH biomarker. Hepatocyte-derived EVs contain hepatocyte markers as asialoglycoprotein receptor 1 (ASGPR1) and bile acyl-CoA synthetase (SLC27A5). It has been demonstrated that the level of hepatocyte-derived EVs correlates with NASH severity.[24]

Many proteins[25] and metabolites[26] or lipidomic signatures[27,28] may act as NAFLD biomarkers at different stages of the disease. For more information about them, see the most recent reviews.[29,30] Several studies demonstrated that circulating molecules, such as microRNA (miRNA) and cell-free nuclear material DNA or RNA, can also be considered as potential promising biomarkers. Mitochondria contain their own extranuclear genome, mitochondrial DNA (mtDNA), a 16.5 kb circular DNA molecule present as multiple copies in cells.[31] MtDNA copies in single cells can range from hundreds

to thousands of copies depending on the cell's bioenergetic needs. Moreover, mtDNA can dynamically change in response to physiological stimuli and under disease conditions. As part of the mitochondrial cell cycle, cellular mtDNA is constantly replicated and replaced via degradation. However, if the mitochondrial life cycle is disrupted and degradation of damaged mtDNA is impaired, mtDNA can leak out of the mitochondria into the cytosol or the peripheral circulation. Due to its resemblance to bacterial genomes, this cell-free (cf) mtDNA can activate inflammation via the TLR-9 pathway, leading to the activation of TNF-alpha and a downstream inflammatory cytokine response.[32] Dysregulation of circulating mitochondrial DNA (mtDNA) has been widely reported in the literature, and circulating mtDNA has been proposed to be a minimally invasive biomarker of mitochondrial dysfunction.[32] Elevated levels of cell-free (cf)-mtDNA in diabetic patients and changes in both hepatic and circulating mtDNA have been reported in both animal models and human studies of NAFLD.[33,34] A link between oxidative stress and leukocyte mtDNA was suggested in a cross-sectional study of a Chinese cohort showing that elevated mtDNA copy numbers in NAFLD patients positively correlated with the oxidative stress marker 8-oxo-2'deoxyguanosine.[35] Leakage of damaged mtDNA into the periphery from both hepatic tissue and fat cells has been shown to cause enhanced inflammation.[34,36] Therefore, combining the impact of oxidative stress on inducing maladaptive mtDNA replication, together with the inflammatory properties of (cf)-mtDNA in circulation, suggest that circulating (cf)-mtDNA is a potential mediator of the chronic inflammation seen in NAFLD and could be a useful biomarker. Additionally, miRNAs, especially miR-122 and miR34a, are also considered a promising diagnostic biomarker for NAFLD.[29] However, robust cross-sectional and longitudinal human studies are needed to understand the time course of (cf)-mtDNA and miRNAs changes to evaluate their use as potential biomarkers of NAFLD.

2.4 | Dietary and animal models to study NAFLD development and progression

The complicated issue of NAFLD progression has been studied in in vitro model and in laboratory animals and humans. Studies performed in rodents or humans revealed the complexity of the factors involved in NAFLD development and progression. Several animal models and NAFLD-inducing diets are useful to study the pathogenesis of NAFLD progression to the more severe stages. Among the available diets, the most popular are choline-deficient (CD) diets, semisynthetic choline-deficient L-amino acid-defined (CDAA) diet, methionine- and choline-deficient (MCD) diet, high-fat (HF) diets, high-fat diets combined with choline deficiency, Western diets (WDs) and cholesterol-supplemented Western diet.

2.4.1 | Choline deficiency-based diets: Choline-Deficient (CD) Diets, Semisynthetic Choline-Deficient L-Amino Acid-Defined (CDAA) Diet, Methionine- and Choline-Deficient (MCD) Diets

Choline deficiency-based diets induce a significant increase in liver triglycerides in rodents after some weeks of feeding.[37,38] Moreover, in rats, moderate periportal micro- and macrovesicular liver steatosis can be visible already after 4 weeks and could be further worsened by a prolonged feeding up to 12 weeks[39] and slight signs of inflammation and fibrosis.[39] Animals feed with CD diet very often do not show significantly increased weight gain.[38,38] Moreover, an increased incidence (~15%) of hepatocarcinogenesis has been observed in rats fed with CD diets for 52 weeks.[38,40] Unfortunately, this model poorly reflects metabolic phenotype in patients. CDAA diet represents a variation of choline deficiency diet, which is also deprived of L-amino acids. Compared to the choline-deficient diet, this diet is more effective inducing steatotic phenotype and liver triglyceride content.[41] Longer feeding time with CDAA diet (up to 22 weeks) causes inflammation and pronounced fibrosis in mice[42] as well as increased body weight, plasma triglycerides and insulin resistance.[42,43] It has been observed that a combination of a CDAA diet with a fat-enriched diet (6-9 weeks of feeding) significantly worsens the fibrotic NASH phenotype in mice.[44] Combination of choline deficiency with methionine deficiency (MCD diet) is one of the most popular, rapid and reproducible nutritional rodent models of a NASH; however, as in the case of CD diet, the MCD model does not exhibit any of the metabolic features of human NAFLD. In mice, MCD diet causes weight loss (up to 40% in 10 weeks). The animals showed also low fasting blood sugar, peripheral insulin sensitivity, low serum insulin and decreased blood triglyceride and cholesterol levels.[45-47] In C57BL/6 mice, MCD diet induces rapid and severe lobular inflammation and hepatocyte ballooning (already after 2-8 weeks) and early-onset fibrosis at 8-10 week of feeding.[45] Macrovesicular steatosis, perisinusoidal fibrosis, hepatocyte ballooning, apoptosis and necroinflammation, as well as mitochondrial anomalies, are also characteristic for MCD-induced NASH phenotype.[48] Also as in the case of CD diet, its combination with HF diet shortens the time to 17 days to the appearance of extensive steatohepatitis with macro- and microvesicular steatosis and inflammatory foci.[49]

2.4.2 | High-fat and Western diets

A major advantage of these models is their high similarity to the metabolic profile observed in humans suffering from NAFLD. In contrast to the above-described diets, in the case of HF diets, the progression of NAFLD is visible only after extensive feeding time (>34 weeks) and is characterized by less pronounced signs of inflammation.[50] Only mice feed for a much longer time (approx. 50 weeks) developed increased inflammatory liver infiltration and minimal fibrosis. As in other diets, time of appearance NAFLD phenotype and degree of accompanying metabolic alterations depend on species, strain and sex of animals Moreover, high impact on observed NAFLD phenotype can have the FFA composition of HF diet (the content of saturated and unsaturated FFAs). For example, AKR/J mice fed with HF diet enriched in a trans fatty acid developed more pronounced steatosis and liver damage after 8-16 weeks compared to the mice feed with nonmodified HF.[51] Moreover, trans-fat-enriched HF diet significantly increased insulin resistance in mice.[51] In rats, trans-fat-enriched HF diet also develops a more pronounced NAFL profile in comparison to standard HF diet, but without differences in liver damage.[52] Western diets (WDs) mimic in rodents our Western dietary habits especially taking into account a high concentration of saturated fats and simple carbohydrates. It has been shown that a high intake of simple carbohydrates (fructose, sucrose or glucose) without combination with a high-fat diet is responsible for obesity and NAFL development in humans. Especially fructose, known for lipogenic properties, leads to visceral fat deposition, liver TG accumulation and insulin resistance.[53,54] The combination of fructose and a HF diet provided to mice for 8 weeks much faster induces steatosis than the HF diet alone. Such combined diet resulted in significant inflammation in the liver, however, without visible progression of liver damage.[55,56] Mice feed with sucrose-supplemented HF diet for 15 weeks exhibited similar NAFL pattern (as HF diet alone), but increased levels of AST and ALT suggested more serious liver damage.[57] A study by Bortolin et al[58] performed on rats showed that 16 weeks of feeding with HF diet combined with sucrose caused significantly more pronounced steatosis, increased liver triglycerides and obesity in comparison to the rats fed with the HF diet alone. In contrast to the study presented by Sampey et al,[59] Bortolin et al[58] observed that in their study, rats fed with HF diet alone had the same body weight or fat white deposits as a control chow-diet group. The authors explained the importance of choosing the right control diet for the comparative experiments.[59] In the literature, several other varieties of Western diets have been found in the context of NAFLD induction in rodents. For example, (a) the 'American Lifestyle-Induced Obesity Syndrome' (ALIOS) diet, being the combination of a HF diet (45 kcal% with 30% fat content from trans fatty acids) and fructose present in drinking water, induces significant steatosis, inflammation and liver damage in male C57BL/6 mice fed with this diet for 16 weeks. In mice, no fibrosis was observed in liver histology. However, the fibrogenic response in the liver could be detected at the molecular level[60]; (b) another example can be a combination of Western diet (HF diet—where 12% of FFA in which saturated combined with fructose in drinking water) supplemented with 2% cholesterol. The results observed for this diet were comparable to those for HF diet-fed only[61]; (c) HF diet supplemented with cholesterol and cholate. In rodents, this diet leads to the development of NASH-like liver phenotype, including MDBs and ballooned hepatocytes. However, observed weight loss, increased insulin sensitivity, and lower serum TG levels are opposite to what is observed NAFLD/NASH patients[48]; (d) Charlton's fast-food model. This diet is based on a high trans-fat-HF diet (40 kcal% of which 18% is trans-fat), 2% cholesterol and 20% sucrose present in the food.[62,63]

It is important to mention that the diet composition impacts the observed phenotype, NAFLD progression and its transition to NASH. Scientists should always consider an interplay between the genetic background, diet composition and health conditions, including gut microbiota.[64] Evidence shows that NAFLD is also associated with promoting abnormal gut microorganisms colonization, which may promote liver condition deterioration.[65] Mice fed with high-fat/high-cholesterol (HFHC) diet for 14 months suffered from gut dysbiosis, similar to the observed in hypercholesterolemic patients. Germ-free mice that have been gavage with stools from mice fed HFHC manifested hepatic lipid accumulation, inflammation and enhanced cell proliferation. This suggests that the microbiota condition may already affect liver response to the studied diet and may impact the disease progression.[66]

The rate of NAFLD manifestation and its progression in animal models also depends on their genetic background. Although most of the human NAFL predisposing genetic variants are not present and cannot mimic human disease in rodents, Newberry and colleagues recently created a mouse devoid of Tm6sf2 gene (human TM6SF2 variant rs58542926) to study its impact on the development and progression of NAFLD.[67] Tm6 LKO mice fed a high-fat diet for 3 weeks exhibited increased steatosis and fibrosis. This NAFLD phenotype was further exacerbated when mice were fed with high-fat/fructose diet for 20 weeks.[67] Among many different mice strains used in NAFLD studies, those naturally predisposed to diabetes type 2 (DM2) and NAFLD development, for example Lepob/Lepob (ob/ob) or Leprfa/Leprfa rat model (fa/fa, also known as Zucker rats) do not need any particular treatment to observe NAFLD-related changes in the liver.[68,69] Another genetic DM2 model is based on mutation in the Alms1 gene, which leads to the increased food intake, increased body weight and DM2. When these animals are fed

for 20-24 weeks with HFD, NASH phenotype with signs of fibrosis was observed.[70,71]

It is necessary to underline that the above-described dietary NAFLD models focus mostly on the metabolic situation observed in patients and may differ regarding clinical or morphologic aspects. Moreover, the accompanying diseases in humans can impact the rate of NAFLD progression, worsening liver function and prognosis. More information about the above-described diets, their impact on metabolism, mitochondrial function and their efficacy to induce a NASH phenotype, as well as features that are similar in rodents and humans, is summarized in other sources, for example in the review by Simoes et al (2019).[72]

3 | METABOLIC COMORBIDITIES OF NAFLD—MITOCHONDRIAL ABNORMALITIES

NAFLD is common among patients with metabolic syndrome. NAFLD prevalence rises with increasing body mass index (BMI) and the number of criteria defining metabolic syndrome.[20] Type 2 diabetes is an independent risk factor for severe steatosis and fibrosis. Contrarily, NAFLD has been shown to more than double the risk of type 2 diabetes.[73] The association between NAFLD and other conditions has been reported including obstructive sleep apnoea,[74] hyperuricaemia[75] and even neurodegenerative disease,[76] and the list is not exhaustive. Therefore, a new definition of metabolic dysfunction-associated liver disease (MAFLD) has been recently proposed and combines hepatic steatosis with overweight or obesity, type 2 diabetes or two or more metabolic abnormalities.[77]

3.1 | Mitochondrial abnormalities in early NAFLD

Steatosis per se represents a result of storing lipotoxic free fatty acids (FFAs) as stable intracellular triglyceride stores and seem to be an adaptive response of hepatocyte to excessive stress caloric supply. Hepatic lipid accumulation results from a balance between the mechanisms governing lipid intake and lipid clearance. The main pathways involved in these processes are the uptake of circulating lipids, de novo lipogenesis (DNL), fatty acid oxidation (FAO) and very low-density lipoprotein (VLDL) export.[78] In a NAFLD context, free fatty acids (FFAs) uptake is increased due to a higher amount of FFA influx from lipolysis in adipose tissue.[79,80] Accordingly, fatty acid transporter (FATP2 and FATP5) and translocase proteins (CD36) have been found increased in NAFLD and NASH patients.[81,82] Moreover, a higher FAs uptake and its intracellular transport inside hepatocytes is correlated with the up-regulation of fatty acid-binding proteins FABP1, FABP4 and FABP5,[83,84] which thereby promotes the storage of harmful FAs and subsequent steatosis. A study using stable isotope traces has showed that 60% of hepatic lipid accumulation is derived from adipose tissue lipolysis. However, other sources have to be taken into account as mentioned above, namely DNL (26%) and the diet (15%).[85] DNL is a condition associated with hyperglycaemia and hyperinsulinemia,[86,87] under the regulation of sterol regulatory element-binding protein-1c and carbohydrate responsive element-binding protein (ChREBP) in response to glucose and insulin.[88,89] Therefore, once active, these transcription factors induce de novo synthesis-associated FAs enzymes—acetyl-CoA carboxylase (ACC) and fatty acid synthase, as showed in NAFLD patients and in animal models.[90-92] Importantly, it was shown that knockout of both ACC-1 and ACC-2 isoforms caused a decrease in hepatic lipid accumulation, thereby protecting against the development of obesity, diabetes and NAFLD.[93,94] Interestingly, several works have associated ChREBP with higher mitochondrial oxidative phosphorylation efficiency and increased mitochondrial biogenesis.[95] Another lipogenic enzyme that was shown to have a critical role during hepatic lipid accumulation is stearoyl-CoA desaturase-1 (SCD-1).[96] By catalysing the biosynthesis of monounsaturated fatty acids, SDC-1 prevents the intracellular accumulation of saturated fatty acids, which are described to promote endoplasmic reticulum (ER) stress, cellular apoptosis and in later stages, fibrosis.[97-99]

A major transcriptional factor involved in regulating hepatic lipid metabolism is peroxisome proliferator-activated receptor-α (PPAR-α). Upon binding to FAs, activated PPAR-α promotes FAs consumption through FAO and ketogenesis.[100] Although different NAFLD studies have reported FAO either increased, unchanged or decreased,[101-105] there is evidence that liver mitochondria are able to boost FAO in order to compensate for hepatic fat accumulation (https://doi.org/10.1016/j.livres.2019.06.001). Additionally, the export of triglycerides in the form of VLDL particles also contributes to decreasing fat content. However, this process tends to stabilize and fail to prevent steatosis when hepatic total fat content reaches 10%.[106]

Of note, mitochondria are not passive players in these scenarios, and they actively respond with several alterations to an increased lipid accumulation. During the early phase of adaptive responses to excessive lipids accumulation in hepatocyte cytosol, increased mitochondrial fatty acid oxidation (mFAO),[107] induction of tricarboxylic acid (TCA) cycle and stimulation of oxidative phosphorylation (OXPHOS)[108] could serve as a protective strategy to keep/control low-level FFA in the cytosol. Moreover, 5′ AMP-activated protein kinase (AMPK), energy status sensor, inhibits de novo lipogenesis and increases fatty acid oxidation by decreasing malonyl-CoA levels and preventing carnitine palmitoyltransferase 1

(CPT-1) inhibition.[109] Mitochondrial adaptation in NAFL can also be manifested as an increased mitochondrial mass in the liver.[110] At the mitochondrial level, increased FAO is correlated with higher reactive oxygen species production, which may contribute to mitochondrial oxidative damage and subsequent mitochondrial impairment.[111,112] In steatotic livers, the activity of complex I is reduced approximately by 35% and is accompanied with increased H_2O_2 generation. The inhibition of complex I can be explained by oxidization of cardiolipin, which is required for the proper function of complex I.[113,114]

Mitochondrial dysfunction can be also related to de-regulation of lipid homeostasis, for example caused by carnitine palmitoyltransferase 1 (CPT-1) inhibition in the presence of higher malonyl-CoA levels generated at the DNL pathway.[115] Such de-regulation is associated with the accumulation of lipid-derived toxic metabolites such as ceramides, diacylglycerols and dicarboxylic acids. These molecules are known to interfere with the mitochondrial function, insulin signalling pathway and the induction of pro-inflammatory cytokines and ER stress. In particular, ceramides and dicarboxylic acids can inhibit electron transport chain and deplete cellular ATP levels, being ceramides depletion associated with higher OXPHOS complexes activities.[116] Along NAFLD development, there is a direct correlation between mitochondrial FAO dysfunction and the development of hepatic steatosis,[117,118] being PPAR-α found downregulated with disease progression and its severity.[119,120]

3.2 | Mitochondrial involvement in progression to NASH

Several factors have been proposed to participate in the pathogenesis of NAFLD. The most important among them seem to be genetic factors, nutrition habits, lipogenesis, insulin resistance, gut microbiota, inflammation, oxidative stress and mitochondrial/metabolic remodelling. However, our knowledge about the factors responsible for the transition mode in each stage of NAFLD is still incomplete. Garcia-Martinez et al (2016) highlighted the potential involvement of mitochondria in the disease progression to NASH. The authors suggested mtDNA, considered a pro-inflammatory molecule[121,122] when released from fatty liver-damaged hepatocytes, causes liver inflammation by TRL-9 activation. In this scenario, mtDNA-induced liver inflammation could be an important factor responsible for the transition to NASH.[34] Interestingly, Fu et al[123] proposed that replacement of dysfunctional mitochondria by exogenous HepG2-derived mitochondria may recover hepatocyte function in high-lard-fat- and high-cholesterol feed mice. The experimental approach used by authors comprised intravenous injection of mitochondria isolated from HepG2 cells; however, how

mitochondria entered the cells in different tissues and were able to maintain the integrity and restore metabolic activity was not explained.[123]

Metabolic changes resulting from increasing hepatocyte FFA influx can be harmful and damage mitochondria through several mechanisms, including mitochondrial uncoupling and the induction of the mitochondrial permeability transition pore (mPTP) opening and oxidative stress. Chronic FFAs overload and disease progression are responsible for decreased CPT-1-mediated FFA transport into mitochondria and defective mFAO. Alterations in mitochondrial respiratory chain complexes' level and activity observed in different NAFLD models[112,124,125] translate into decreased ATP level. Decreased ATP level could be responsible for the induction of endoplasmic reticulum (ER) stress and unfolded protein response (UPR) activation, which stimulates de novo lipogenesis pathways and further aggravates liver steatosis.[126] Progression of NAFL to NASH is also accompanied by increased mitochondrial cholesterol accumulation,[127] leading to the cholesterol-induced alterations in the inner mitochondrial membrane's permeability. Such alterations in the properties of mitochondrial membranes can be a cause of mitochondrial glutathione (GSH) depletion described in NASH patients.[128] In patients with more advanced forms of NAFLD decreased of mtDNA levels have been observed.[34,129]

It is not surprising that alterations in mitochondrial morphology and function can impact liver physiology. The direct link between remodelling of mitochondrial structure, metabolic dysfunction and clinical phenotype development has been repeatedly demonstrated in several pathologies. In the range of adaptive response to the excessive FFA accumulation in the liver, in the initial NAFLD stages, observed metabolic changes are associated with an increase in mitochondrial mass, with or without increased mitochondrial fatty acid oxidation. Koliaki et al[130] described that maximal respiration rates measured in isolated mitochondria from obese patients with or without NAFL were 4.3- to 5.0-fold higher than lean individuals. This was also confirmed by Sunny et al,[108] who showed that mitochondrial oxidative metabolism was increased in the livers of subjects with elevated intrahepatic triglycerides. The increase in mitochondrial oxidative metabolism involved a 2~fold induction of oxidative fluxes through the TCA cycle. This finding also demonstrated that even with a large accumulation of triglycerides in the liver, the TCA cycle is functional. Interestingly, increase TCA fluxes were associated with gluconeogenesis, which, according to the authors, could account for the increased energy demand observed in individuals with NAFLD. The results from this study also dismiss the notion that mitochondrial dysfunction is a primary event in the progression from steatosis to more severe forms. Still, mitochondrial substrate overload could contribute to ROS generation or possibly to a cellular metabolic unbalance, which can prime hepatocytes
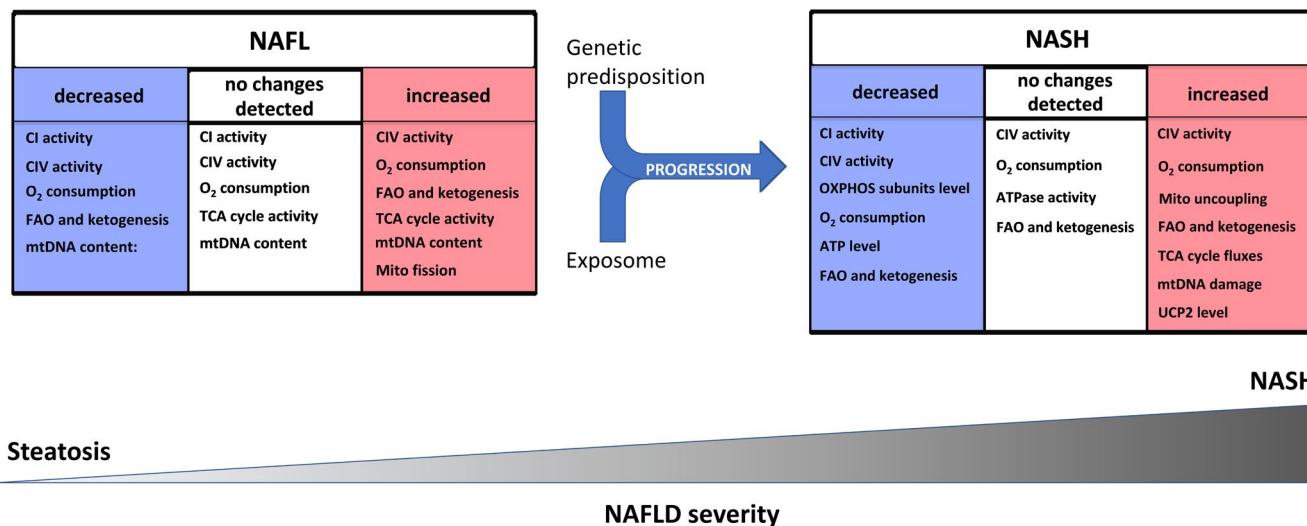
for a pro-inflammatory state. Interestingly, even with increased mitochondrial substrate oxidation via the TCA cycle, lipotoxicity and incomplete fat oxidation typical of NAFLD progression to NASH were not avoided,[131] contributing to inflammation and fibrosis. Agreeing with a progressive failure to maintain an effective lipid oxidation profile with increase intrahepatic steatosis is the fact that mitochondrial biogenesis is inhibited and decreases with progression towards NASH. Interestingly, simultaneously with altered mitochondrial biogenesis, mitochondrial mass increases,[110] although such mitochondria were swollen and showed a loss of cristae structure and were characterized by 31%-40% lower maximal respiration and mitochondrial uncoupling.[130] This suggests that higher mitochondrial mass detected in NASH patients could result from defective removal of damaged mitochondria.[130]

It has been demonstrated that incubation of HepG2 cells with saturated fatty acids (a model resembling NASH) causes mitochondrial abnormalities accompanied with inhibition of mtDNA gene expression and accelerated degradation of respiratory chain subunits.[132] Sequential exposure of hepatocytes to high concentrations of fatty acids and TNF-α mimic in vitro the progression of NAFLD from simple steatosis to steatohepatitis. In such a condition, the damage could be observed not only at a mitochondrial level but also elsewhere in the hepatocyte. Among them are increased apoptosis, reduced hepatocyte viability, increased oxidative stress, reduction in lipid droplet size and up-regulation of IkappaB kinase beta-interacting protein and adipose triglyceride lipase expressions.[133]

More details about the interplay between mitochondrial dynamics and NAFLD can be found in a recent review by Longo et al.[134] In more advanced disease stages, the presence of megamitochondria (giant mitochondria) was also described.[135] Their presence has been also reported in other tissues with a high degree of metabolic activity.[136] However, it is still unknown whether the presence of megamitochondria in the liver is an adaptation or a consequence of NAFLD development.[135] Up to now, there is no clear explanation for the progressive decline of OXPHOS during NASH. Begriche et al proposed possible mechanisms explaining OXPHOS dysfunction: lipotoxicity, oxidative stress and effect of interferons, adiponectin and forkhead box protein O1 (FoxO1). For a detailed description of relations between abovementioned factors and mitochondrial dysfunction, see their comprehensive review.[137] A summary of the described alterations in the mitochondrial function has been presented in Figure 1.

4 | ROS AND RNS CULPRITS OR BYSTANDERS OF NAFLD PROGRESSION

Under physiological conditions, ROS are continuously produced in the liver due to intracellular metabolism, although kept under a certain threshold for redox signalling pathways, for example cell proliferation and differentiation.[138] Moreover, ROS like H_2O_2 regulates the expression of many genes, including AP-1, CREB, HSF1, NRF2, HIF-1, TP53,

NAFL		
decreased	**no changes detected**	**increased**
CI activity	CI activity	CIV activity
CIV activity	CIV activity	O₂ consumption
O₂ consumption	O₂ consumption	FAO and ketogenesis
FAO and ketogenesis	TCA cycle activity	TCA cycle activity
mtDNA content:	mtDNA content	mtDNA content
		Mito fission

Genetic predisposition → PROGRESSION → Exposome

NASH		
decreased	**no changes detected**	**increased**
CI activity	CIV activity	CIV activity
CIV activity	O₂ consumption	O₂ consumption
OXPHOS subunits level	ATPase activity	Mito uncoupling
O₂ consumption	FAO and ketogenesis	FAO and ketogenesis
ATP level		TCA cycle fluxes
FAO and ketogenesis		mtDNA damage
		UCP2 level

Steatosis ——————————→ NASH

NAFLD severity

FIGURE 1 Outcomes regarding mitochondrial parameters and related metabolic processes in NAFLD. The figure was prepared initially based on Begriche et al[58] and Simoes et al[66] and updated accounting for subsequent studies. Disease stage is according to Begriche et al[58] and Koliaki et al.[54] ACC, acetyl-CoA carboxylase; FAO, fatty acid oxidation; FAS, fatty acid synthase; OXPHOS, oxidative phosphorylation; PPARγ, peroxisome proliferator-activated receptor γ; SCD-1, stearoyl-CoA desaturase-1; SREBP-1c, sterol regulatory element-binding protein-1c; TCA, tricarboxylic acid cycle; UCP-2, mitochondrial uncoupling protein 2; β-HAD, β-hydroxyacyl-CoA dehydrogenase. Published reports used for Figure 1 preparation are available and listed in Supplementary Material

NF-κB, NOTCH, SP1 or SCREB-1.[139,140] Due to their chemical structure, ROS are divided into two main categories: free radicals (superoxide radical ($O_2^{.-}$), hydroxyl radical ($HO^.$), nitric oxide ($NO^.$, that we will describe in more detail below), nitrogen dioxide ($NO_2^.$), carbonate radical anion ($CO_3^{.-}$), and alkoxyl/alkyl peroxyl ($RO^./ROO^.$)) and nonradicals (hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl), and peroxynitrite ($ONOO-$)/peroxynitrous acid (ONOOH)). For a detailed description of chemical ROS features, we refer to a comprehensive review.[141] Within the different species, $O_2^.$, $NO^.$ and H_2O_2 are the primary molecules produced within the cell. These low reactivity molecules can readily generate other ROS species (HOCl, $ONOO-$ and ONOOH), which ultimately can form $NO_2^.$, $CO_3^{.-}$ and $RO^./ROO^.$, known as powerful inducers of cellular oxidative damage.

To overcome nitroso-oxidative stress, cells possess a vast panel of enzymatic and nonenzymatic antioxidant defence systems. Enzymatic mechanisms include superoxide dismutase (SOD1-3), which converts $O_2^{.-}$ into H_2O_2, which can be further converted into H_2O and O_2 by catalase. Moreover, H_2O_2 is also converted into H_2O by the action of glutathione peroxidase (GPX) or peroxiredoxin (PRDX), with oxidized glutathione (GSSG) converted back to its reduced form (GSH) by the action of glutathione reductase (GR). The cell's redox status regulation can be restrained due to GSH depletion and GSSG accumulation, as shown in NAFLD/NASH primary hepatocytes and NAFLD rodent models.[125,127] H_2O_2 can also be reduced by the action of PRDX, using reduced thioredoxin as the electron donor. Lastly, glutathione-S-transferase can detoxify xenobiotic compounds through its conjugation with GSH. RNS/ROS can freely diffuse within various cell organelles; their signalling or damage effects within specific cell compartments constraints are limited to their half-life.[142]

Taking into account several controversies, mitochondria are considered one of the important sources of ROS during pathological conditions.[143,144] So far, several distinct sites of ROS production in mammalian mitochondria have been identified. NADH:ubiquinone oxidoreductase (complex I) is considered a major source of reactive oxygen species in mitochondria. Several sites of $O_2^{.-}$ production in mitochondrial complex I have been proposed including flavin,[145-147] bound reduced nucleotide,[148] FeS clusters N2[149] and N1a,[150] and a semiquinone radical.[151,152] Complex II is another source of mitochondrial ROS, involving the release of electrons from the flavin site or in a RET mode from a reduced ubiquinone pool.[153] Interestingly, a mutation in complex II might also result in $O_2^{.-}$ overproduction.[154] At Complex III, $O_2^{.-}$ can be generated from the ubisemiquinone site and released to the intermembrane space, which can permeate into the matrix in the form of H_2O_2.[155] Other mitochondrial enzymes that have been associated with ROS production are glycerol 3-phosphate dehydrogenase considered as donor of electrons

to ETC; α-ketoglutarate dehydrogenase and pyruvate dehydrogenase involved in TCA cycle.[156] In addition, the flavoprotein acyl-CoA dehydrogenase can produce ROS during oxidation of lipid-derived substrates.[157,158] Other documented sources of ROS in mitochondria include two other enzymes: monoamine oxidase and dihydroorotate dehydrogenase.[159,160] It is important to underline that also microsomes (cytochrome P450, diamine oxidase), peroxisomes (enzymes involved in fatty acid oxidation) and enzymes in the plasma membrane (like eg NADPH oxidase and lipoxygenase) have been identified as ROS generators.

Increased mitochondrial activity in the early phase of NAFL protects hepatocytes from lipotoxicity, while under excessive lipid influx increased activity can extensively generate ROS.[137] Increased ROS generation is caused by the fact that continuous supply of reduced substrates to the electron transport chain (ETC) promotes the leak of electrons from reduced flavin mononucleotide (FMN) or by reverse electron transfer (RET) at Complex I, resulting in $O_2^{.-}$ release into the mitochondrial matrix.[161] Several reports have highlighted that accelerated β-oxidation of short-, medium- and long-chain saturated FAs with augmented CPT-1α gene expression causes excessive mitochondrial electron flux, resulting in increased mitochondrial $O_2^{.-}$ production in in vitro and in vivo models of steatosis.[112,162] Moreover, UCP-2 up-regulation can protect hepatocytes from deleterious ROS effects.[163] Additionally, the nuclear factor erythroid-derived 2 like 2 (NRF2) in response to the elevated level of $O_2^{.-}$, activates expression of a compensatory antioxidant defence response.[164] A mitochondrial pro-oxidant state can overwhelm the mitochondrial antioxidant system (decreased SOD, GPX1 activity, and GSH/GSSG ratio levels) and result in oxidative injury in different mitochondrial structures containing proteins and lipids,[165] depletion of mtDNA copy number and higher mtDNA damage during the progression of NAFLD towards NASH and beyond.[166,167] Damaged mitochondria with loss of FAO and respiratory complex activities were described in later stages of the disease.[163] At high concentrations, some ROS, such as the hydroxyl radical, a highly reactive molecule, can cause oxidative modification of lipids (lipid peroxidation—malondialdehyde (MDA) and 4-hydroxy-2-nonenal (HNE)),[168] proteins (carbonylation and nitration)[169] and nucleic acids (mtDNA depletion and DNA damage—8-hydroxy-2'-deoxyguanosine (8-OHdG)).[130] The notion of nitroso-oxidative stress involves different markers ranging from alterations in ROS/RNS production to oxidative damage and altered antioxidant enzymes' activity. Several pieces of evidence suggest that the accumulation of hepatocyte damage is in the origin of the maladaptive response of hepatocytes to fat accumulation, thereby leading to hepatic metabolic impairment and NASH.[34,170]

In the context of hepatic steatosis, there is a pro-oxidant state with activation of ROS-generating mechanisms while

the levels/ activities of antioxidants are impaired, as identified in NAFLD animal models and NAFLD/ NASH clinical cohorts.[125,171,172] Some data suggest that, NAD^+-dependent deacetylase, sirtuin 3 (SIRT3) is implicated in the modulation of mitochondrial ROS response and in the modulation of hepatocyte susceptibility to cell death/ autophagy in the presence of a high-fat (HF) diet.[173,174] One pathway by which oxidative stress might cause the disruption of the mitochondrial network and function is through cardiolipin (CL) oxidation.[114] CL, an exclusive mitochondrial phospholipid, is highly sensitive to unsaturated bond-ROS attack, and its oxidation may lead to cytochrome c release from mitochondria, thereby triggering apoptotic cell death.[166]

It has been proposed that the accumulation of ROS-related damages is in the origin of the maladaptive response of hepatocytes to fat accumulation, thereby leading to hepatic metabolic impairment and NASH.[34,170] Nitric oxide (NO·) is a RNS/ROS whose contribution for NAFLD progression is still obscure, ranging from the possible culprit, or at least contributor, to liver/vascular protectant in NAFLD. The role of (NO·) in NAFLD-related inflammation has been previously described.[175] Moreover, an increase in NO· production in NASH patients compared to more benign NAFLD phenotypes was observed suggesting that it can be considered as an inflammation marker in patients progressing from liver steatosis to steatohepatitis.[175] In contrast, Gu et al described NO· as a protective agent for NAFLD progression towards more severe disease forms.[176] However, this observation is quite controversial because an increase, rather than a decrease of NO· production was shown to accompany NAFLD progression.[177] Indeed, NO·-mediated nitration of hepatocellular proteins, visualized by 3-nitrotyrosine level, is markedly induced in the inflamed liver tissue from patients with chronic liver disease.[178] Interestingly, NAFLD has been linked with altered blood pressure, suggesting endothelial NO· synthase (eNOS) dysfunction in the disease's pathogenesis. Chronic inhibition of eNOS via N(ω)-nitro-L-arginine methyl ester (L-NAME) increased liver injury in a rat model of obesity, insulin resistance and NAFLD.[179] Indeed, NAFLD patients show a marked eNOS dysfunction in platelets and liver tissue,[180] associated with S1177 phosphorylation.[181]

Although excessive cellular ROS and /-or oxidative damage levels in NAFLD are associated with a compromised mitochondrial structure and function, most NAFLD studies lack experimental evidence to support the primary role of mitochondria as an essential source of ROS.[125,166,182] In fact, a comparative study investigating the contribution of different cellular organelles to ROS production demonstrated that endoplasmic reticulum (ER) and peroxisomes produce more H_2O_2 than mitochondria in rodents liver.[183] Furthermore, recent studies by Einer et al[55] described a mitochondrial adaptation linked to fat accumulation without evidence of mitochondrial oxidative stress markers during

steatosis. This work was supported by another study in which it was shown that oxidative stress in steatotic mice was caused by peroxisomes and not by mitochondria, in which no or very reduced ROS production and oxidative damage were observed.[184] These observations are consistent with the up-regulation of peroxisome proliferator-activated receptor alpha (PPAR-α) and ACOX genes, as well as with higher levels of peroxisomal-related proteins in livers of HF fed-mice[184,185] and in NAFLD patients.[186,187] Furthermore, a higher peroxisomal β-oxidation rate is responsible for A/J mice resistance to diet-induced steatosis and obesity.[188] This included the up-regulation of genes involved in peroxisomal structure (Pex11a), VLCFAs uptake (Abcd3), FAO (Slc27a2, Acsl4, Ehhadh, Ech1, Crat) and detoxification (Cat, Alas1). Accordingly, an increased peroxisomal oxidative activity seems to represent an alternative pathway to support mitochondrial function, especially when its oxidative capacity is overloaded, as described in high-fat diet-induced steatosis.[189] This role might explain the proximity between peroxisomes and lipid droplets in the cytosol.[190] However, peroxisomal FAO is associated with an exacerbation of oxidative stress in microvesicular steatosis.[191] Thus, cellular organelles that can counteract lipid accumulation, such as peroxisomes and microsomes or even cytosolic ROS-generating enzymes as NADPH oxidase (NOX) xanthine oxidase, have emerged as central ROS producers during the onset of NAFLD.

Another example can be the 66 kDa isoform of Shc (p66shc), an adaptor and redox protein that has been already linked to the lipid metabolism regulation by numerous reports.[192] It has been demonstrated that p66Shc participates in various mechanisms of liver injury.[193,194] In livers of NAFLD patients, expression levels of apoptosis regulating proteins p53, p21 and p66Shc were significantly increased.[195] p53/p66Shc-associated pathway was shown to play a significant regulatory role in NASH progression.[195] Similar observation comes from rodent NAFLD model.[196] Zhang et al[196] described a correlation between increased hepatic p66shc expression and upregulated expression of senescence markers: heterochromatin protein-1-beta (HP1b), p16, p21 and p53. Slowed down steatosis development has been observed for p66Shc knockdown condition, and on the other hand, overexpression of p66Shc promoted senescence and steatosis in L02 cells.[196] Moreover, targeting the p66Shc—cytochrome c cascade by catalpol can prevent the development of NAFLD complications, which could be mediated by a specific microRNA, miR-96-5p effect.[197] Interestingly, it has also been demonstrated that the sirtuin 1 (SIRT1)/p66Shc anti-apoptosis pathway is a good target to prevent NAFLD-related injury.[198] A negative effect of p66Shc activation on liver mitochondria function can be additionally prevented by exercise which promotes liver mitochondria adaptive remodelling and hepatocyte renewal.[199] Recently, it was also shown that the p66Shc oxidative and ER stress pathway can be a

potential therapeutic target in NAFLD as Isosteviol (ISV) prevents FFA-/HFD-induced hepatic injury. In rat model, ISV specifically inhibits expression, activation and translocation of p66Shc to mitochondria protecting against FFA-/HFD-induced hepatic injury.[198]

Peroxisomes are responsible for α-oxidation of branched-chain FAs and β-oxidation of very-long-chain FAs (VLCFA) and other processes as amino acid metabolism and biosynthesis of glycerophospholipids and bile acids. These organelles act as a chain shortening system, producing the short and medium FAs further diverted to mitochondria to complete their oxidation. Acyl-CoA oxidases (ACOX), D-amino acid oxidase, D-aspartate oxidase, L-α-hydroxy acid oxidase, polyamine oxidase and xanthine oxidase can generate ROS in peroxisomes.[200] Peroxisomes can further detoxify ROS by the most proeminent enzyme—catalase or by other peroxisomal resident antioxidant enzymes. Importantly, catalase silencing caused significantly more lipid accumulation, oxidative stress and inflammation in high-fat fed mice when compared to matched controls.[201]

A defective peroxisomal β-oxidation and a sustained peroxisome proliferator-activated receptor alpha (PPAR-α) activation seem to contribute to cytochrome P450 (CYP4A) induction and hepatic oxidative injury.[202] These observations provide evidence that if a VLCFAs oxidative pathway as peroxisomal FAO is defective or insufficient, the microsomal pathway may act as another alternative oxidative pathway. In ω-oxidation of VLCFA in microsomes, the first step of the reaction is catalysed by oxidoreductase CYP2E1/CYP4A enzymes in the presence of NADPH and O_2 with the generation of O_2^{-} and H_2O_2 as by-products.[203] Even though ω-oxidation is considered a minor FAO pathway under basal conditions,[204] up-regulation of this pathway may occur in the context of hepatic fat accumulation in rodents and humans.[205,206] Moreover, CYP2E1-mediated oxidative stress could induce insulin resistance development and inflammation, thereby aggravating NAFLD severity.[207] Although there are some discrepancies regarding the participation of CYP2E1 and NOX in cellular ROS production,[208,209] a few other studies have also confirmed the role of NOX in oxidative stress of fatty liver rats and NASH patients.[208,210] It was shown that hepatocyte NOX4 deletion reduced oxidative stress, improved insulin sensitivity and decreased liver inflammation and fibrosis in a NASH-induced mouse model.[210]
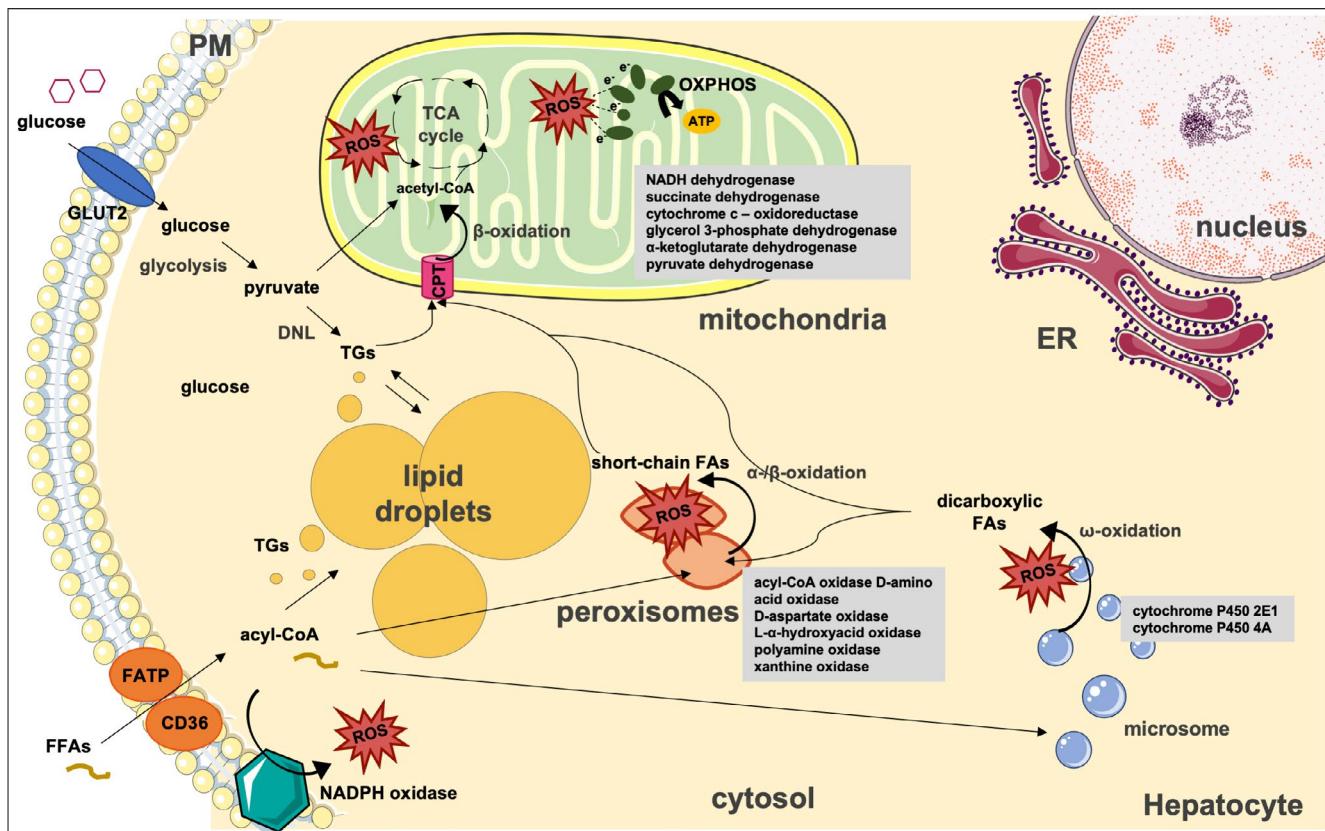
Mitochondria, peroxisomes, microsomes and RNS/ROS-generating enzymes such as NOX seem to play a role in ROS production during an early stage of NAFLD. Control of RNS/ROS for improving NAFLD/NASH phenotype may require a multi-organelle targeting approach. A potential clue for the role of ROS in NAFLD progression arises from the therapeutic use of antioxidants. Antioxidants such as sterol ester of alpha-linolenic acid,[211] physalin B,[212] N-acetyl cysteine,[213] or vitamin E and vitamin C[214] showed beneficial effects against liver injury, including fibrosis, in the context of NAFLD. Still, a meta-analysis of randomized trials for treating NAFLD found that those including antioxidants gave mixed results.[215] Interestingly, not only coenzyme Q10, a component of the mitochondrial respiratory chain, prevented some of the hallmarks of the NAFLD phenotype,[216] but its mitochondrial-targeted form, MitoQ (or mitoquinone), also showed some benefits in multiple models of fatty liver.[217,218] This demonstrates that ROS (and probably RNS) do have an essential role in NAFLD progression and that mitochondrial-derived ROS can play, at least in part, a role in key time-points of the disease progression. A schematic representation of hepatocyte cellular pathways involved in ROS generation in a NAFLD context is presented in Figure 2.

For example, vitamin E—a fat-soluble antioxidant, was analysed in the PIVENS trial published in 2010. Histological assessment of patients taking part in PIVENS study showed that vitamin E at the dose of 800 IU/day led to reduced hepatocyte ballooning ($P = .005$) and lobular inflammation ($P = .02$) but had no significant effects on liver fibrosis.[219] The subsequent TONIC trial involving 173 children and adolescents in NAFLD disappointingly showed that vitamin E (400 IU/twice daily for 96 weeks) did not reduce liver steatosis, fibrosis or lobular inflammation.[220] According to current recommendations, vitamin E can be considered as a short-term therapy in patients with biopsy-proven NASH. However, safety concerns (eg increased all-cause mortality[221] as well as enhances risk for prostate cancer[222]) need to be discussed with the patient before starting the therapy. The trials investigating resveratrol—a polyphenol with antioxidant properties found in fruits and vegetables, did not show major effects on fatty liver.[223] To date, obeticholic acid (OCA) is one of the few drugs that showed beneficial effects on NASH. Interim analysis of a phase 3 trial demonstrated that OCA at a 25 mg dose can significantly improve fibrosis and components of NASH.[224] However, these results have not led to the approval of OCA as therapy for patients with fatty liver. Given the paucity of approved and effective drugs for NASH, including antioxidants, current recommendations for patients with hepatic steatosis include lifestyle changes, weight loss and correction of risk factors for progressive NAFLD.

5 | CONCLUSION

Several contradictory observations describing mitochondrial response at different NAFLD stages reported in the literature make it very difficult to define with certainty the time course of changes in mitochondrial parameters during NAFLD progression. Fromenty's group in their elegant review proposed several factors that could be responsible for the observed discrepancies between the studies investigating mitochondrial function along with the NAFLD progression.[137] Among them

FIGURE 2 Schematic representation of hepatocyte-related organelles involved in ROS generation in a NAFLD context. Circulating levels of glucose and FFAs are taken by hepatocytes. In an early NAFLD stage, hepatic adaptation includes a series of molecular pathways in order to cope with the excess of available nutrients. One main pathway involved is the activation of FAO in mitochondria but also in peroxisomes, microsomes and NADPH oxidase. Despite the protective role of FAO by decreasing fat accumulation, the up-regulation of this pathway induce the production of ROS. When present in excess, ROS play a role as culprits in the induction of hepatic oxidative damage of molecules and organelles, thereby contributing to organelles malfunction (eg mitochondrial dysfunction) and further disease progression. ATP, adenosine triphosphate; CD36, cluster of differentiation; CPT, carnitine palmitoyltransferase; DNL, de novo lipogenesis; ER, endoplasmic reticulum; FAO, fatty acid oxidation; FAs, fatty acids; FATP, fatty acid transporter protein; FFAs, free fatty acids; GLUT2, glucose transporter 2; NADH, nicotinamide adenine dinucleotide; NADPH, nicotinamide adenine dinucleotide phosphate; OXPHOS, oxidative phosphorylation; PM, plasma membrane; ROS, reactive oxygen species; TCA, tricarboxylic acid cycle; TGs, triglycerides

are imperfect histological classification, nutritional and genetic factors and methodology used.[137] Moreover, ambiguous results from antioxidant administration may result from different treatment protocols, the specific disease stages at which the treatment is initiated, and the antioxidant molecule's potential to have a multi-targeting activity, for example by reducing inflammation in the liver.

Hence, based on our recent knowledge, it is difficult to answer whether mitochondrial abnormalities and or mitochondrial/peroxisomal-related oxidative stress are culprits or bystanders of NAFLD development and progression. Further carefully designed mitochondrial studies involving patients at different NAFLD stages and animal models of NAFLD are necessary to demonstrate the relationship between mitochondrial bioenergetics, oxidative metabolism, metabolic syndrome, oxidative stress and progression of NAFL to more severe NAFLD stages.

CONFLICT OF INTEREST
The authors declare no conflict of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.