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Organelles in focus

# Mitochondria in non-alcoholic fatty liver disease

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ABSTRACT

NAFLD is a common disease in Western society and ranges from steatosis to steatohepatitis and to end-stage liver disease. The molecular mechanisms that cause the progression of steatosis to severe liver damage are not fully understood. One suggested mechanism involves the oxidation of biomolecules by mitochondrial ROS which initiates a vicious cycle of exacerbated mitochondrial dysfunction and increased hepatocellular oxidative damage. This may ultimately pave the way for hepatic inflammation and liver failure. This review updates our current understanding of mitochondria-derived oxidative stress in the progression of NAFLD.

## 1. Introduction

Fat accumulation in the liver is pathognomonic for non-alcoholic fatty liver disease (NAFLD) (see Box 1). This steatosis can progress to inflammatory NASH, fibrosis, cirrhosis and hepatocellular carcinoma, ultimately culminating in liver failure. Non-alcoholic steatohepatitis (NASH) development may be negatively propagated by the predisposition of individuals to genetic factors. In fact, several different genetic loci, *PNPLA3*, *NCAN*, *GCKR* and *LYPLAL1*, have been identified as determinants of steatosis (Mehta et al., 2016). Sedentary lifestyles, dietary changes, epidemic obesity and type 2 diabetes further contribute to the worldwide increase in NAFLD, which currently affects 25% of the worldwide population.

Hepatic mitochondria are structurally and molecularly altered in NAFLD (Einer et al., 2017). As the cell powerhouse, a decline in mitochondrial function, concomitant with structural and molecular alterations, may provoke metabolic disturbances and may potentially contribute to NAFLD progression (Fig. 1A and B). However, the sequence of events and signaling pathways that link mitochondrial remodeling and dysfunction to stages of NAFLD progression remain unclear.

## 2. Physiology and pathology of mitochondria in NAFLD

### 2.1. Changes in mitochondrial metabolism in NAFLD (Fig. 2A and B)

#### 2.1.1. Steatosis

High-fat diets and the dysregulation of lipid metabolism cause the accumulation of hepatic free fatty acids (FFAs) and triglycerides (TGs) (Eccleston et al., 2011). Under these conditions, a metabolic shift is induced to overcome the hepatic FFA burden. This shift includes enhanced mitochondrial fatty acid oxidation (FAO), tricarboxylic acid (TCA) cycle induction and oxidative phosphorylation (OXPHOS) stimulation (Sunny et al., 2011). These pathways appear to be regulated by an increased expression of PPAR-α, which promotes FFA delivery to the mitochondria *via* CPT-1. Additionally, AMPK, which acts as the

*Abbreviations:* 8-OHdG, 8-hydroxy-2-deoxyguanosine; $\Delta y_m$, mitochondrial membrane potential; AMPK, AMP-activated protein kinase; apoB, apolipoprotein B; AST, aspartate transaminase; ALT, alanine transaminase; ATP, adenosine triphosphate; CPT-1, carnitine palmitoyl-transferase 1; DNA, deoxyribonucleic acid; ER, endoplasmic reticulum; ETC, electron transport chain; FAO, fatty acid oxidation; FFA, free fatty acids; Gpx, glutathione peroxidase; GSH, glutathione; HFD, high-fat diet; HNE, 4-hydroxy-2-nonenal; IL, interleukin; IR, insulin resistance; iNOS, inducible nitric oxide synthase; JNK, c-JunNH$_2$-terminal kinase; MDA, malondialdehyde; miR, microRNA; MPT, mitochondrial permeability transition; mtDNA, mitochondrial DNA; mtFAO, mitochondrial FAO; mtGSH, mitochondrial GSH; NADPH, nicotinamide adenine dinucleotide phosphate; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; NF-κB, nuclear factor kappa-B; NO, nitric oxide; NRF-2, nuclear respiratory factor 2; OXPHOS, oxidative phosphorylation; PGC-1α, peroxisome proliferative activated receptor-gamma coactivator-1α; PPAR-α, peroxisome proliferator activated receptor-α; RNS, reactive nitrogen species; ROS, Reactive oxygen species; SOD2, superoxide dismutase 2; TCA, tricarboxylic acid; TFAM, mitochondrial transcription factor A; TG, triglycerides; TLR, toll-like receptor; TNF-α, tumor necrosis factor- α; UCP2, uncoupling protein 2; UPR, unfolded protein response; VLDL, very low density lipoprotein

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**Box 1**
NAFLD and NASH facts.

- In NAFLD 5% of the liver cells present micro- or macrovesicular steatosis.
- Obesity, diabetes, hyperlipidaemia and high blood pressure (features of metabolic syndrome) are NAFLD risk factors.
- 90% of NAFLD patients have at least one of the above mentioned features.
- There are no clinical symptoms associated to steatosis during the early development of NAFLD.
- 10-25% of NAFLD patients progress to inflammatory steatohepatitis (NASH).
- NASH is diagnosed by liver biopsy.
- NASH features include macrosteatosis, hepatocyte ballooning and lobular inflammation.
- These lesions define the NAFLD activity score (NAS) used to classify NAFLD grading.
- No drugs/therapies are approved for NAFLD treatment.
- Current treatment strategies for NAFLD patients aim at the amelioration of risk factors through lifestyle and dietary changes.

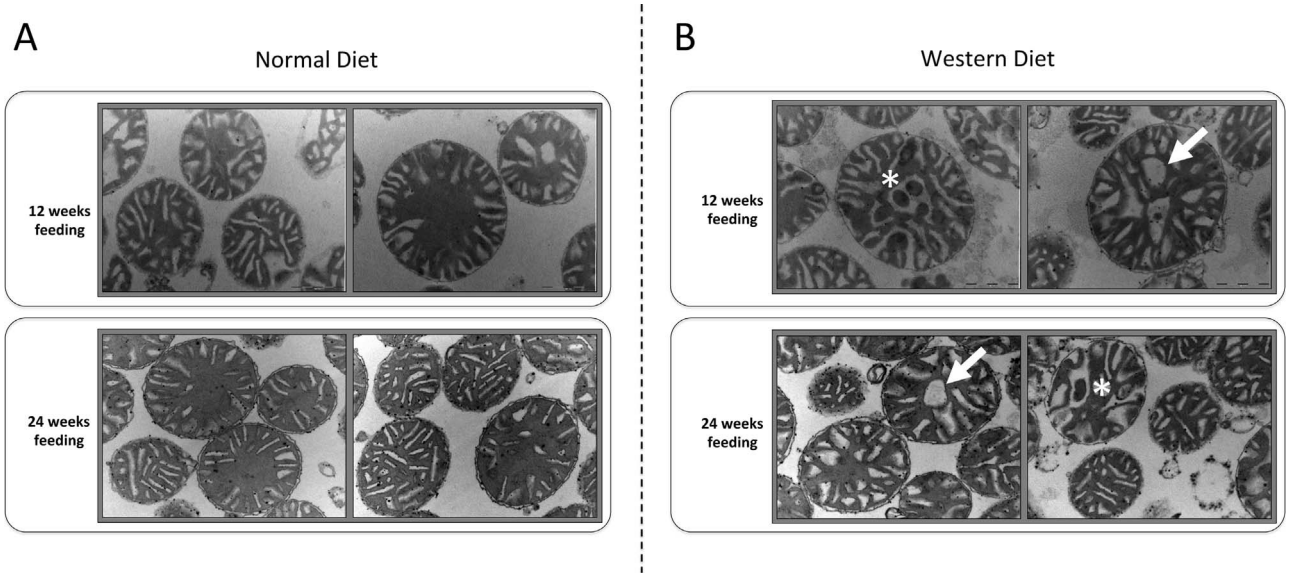


**Fig. 1.** Electron microscopy of mitochondria isolated from livers of C57BL/6NCrl mice fed either a normal (A) or high-fat (45% kcal from fat), high-fructose (23.1 g/l fructose, 18.9 g/l glucose) "Western diet" (Einer et al., 2017) (B) for 12 or 24 weeks, respectively. Such isolated mitochondria appeared intact, *i.e.*, without outer membrane disruptions. Mitochondria from normal diet fed mice (A) appeared with regular and elongated cristae structures. In contrast, many mitochondria from Western diet fed mice (B) had ballooned or rounded cristae (arrow) as well as condensed matrix structures (asterisk). These structural pecularities of the inner mitochondrial membrane may be accompanied by alterations in oxidative phosphorylation. Mouse liver mitochondria were isolated as recently reported by Schulz S. et al. PMID:25820715). Crude mitochondrial fractions were further purified by density gradient centrifugation at 9000 × *g* using an 18/30/60% PercollTM gradient system. The purified organelles were washed in isolation buffer without BSA and subsequently fixed with 2.5% glutaraldehyde (Science Services GmbH, Germany), postfixed with 1% osmium tetroxide, dehydrated with ethanol, and embedded in Epon. Ultrathin sections were negative stained with uranyl acetate and lead citrate and then analyzed by transmission electron microscopy.

cell's energy status sensor, inhibits *de novo* lipogenesis and increases FAO by decreasing malonyl-CoA levels and preventing CPT-1 inhibition (Rolo et al., 2012). Enhanced CPT-1 activity has been reported to protect NAFLD development. In fact, CPT-1 activation decreases serum markers of liver damage (AST, ALT, bilirubin, mtDNA) in treated NAFLD patients (Lim et al., 2010). Moreover, in early NAFLD, the up-regulation of UCP2 may protect cells from increased ROS levels (Serviddio et al., 2008). Therefore, increased mitochondrial activity appears to protect hepatocytes from the deleterious effects of FFAs deposition (Koliaki et al., 2015) .

#### 2.1.2. NASH

Despite the attempts of the liver to recover from fat accumulation, in the long run, mitochondrial adaptation is insufficient to prevent lipotoxicity due to continuous FFAs deposition. This was demonstrated in a choline-deficient NAFLD model, which exhibited an increase in OXPHOS efficiency at 12 weeks but had lost capacity at 16 weeks (Teodoro et al., 2008). At this later time point, the mitochondria presented with alterations in the ETC complexes and membrane potential ($\Delta\psi_m$), induced mitochondrial permeability transition (MPT) pore opening and reduced ATP synthesis (Teodoro et al., 2008). Accordingly, the capacity of the mitochondria to overcome the increased FFAs concentration was lost in more advanced stages of the disease. In these stages, disease progression was accelerated by CPT-1 downregulation, impaired mitochondrial FAO (mtFAO), and chronic ATP depletion caused by higher UCP2 expression in hepatocytes (Serviddio et al., 2008).

### 2.2. Mitochondrial participation in NAFLD progression to NASH

#### 2.2.1. Progression to NASH

NASH is characterized by an inflammatory state due to ROS and RNS overproduction, lipotoxicity and an increase in pro-inflammatory and profibrogenic cytokines. Oxidative stress and lipid peroxidation activate NF-κB to induce pro-inflammatory cytokines, including TNF-α, IL-1β, Il-6 and IL-8 (Carter-Kent et al., 2008; Rodrigues et al., 2017). Furthermore, circulating mitochondrial DNA (mtDNA) released from damaged hepatocytes of mice fed a HFD, caused TLR9 activation, triggering a pro-inflammatory cytokine response and ultimately liver inflammation (Garcia-Martinez et al., 2016). The transition to NASH can also be related to adiponectin levels. $Lepr^{db/db}$ mice fed a HFD develop NASH with concomitantly diminished hepatic adiponectin,

A

| | Model | Dose | Sample | Analysis | Mitochondrial response | PMID |
|---|---|---|---|---|---|---|
| Cell line | FL83B hepatocytes | mannitol-balanced glucose (33 mM) + leptin (25 ng/ml) | | Western blot, RT-PCR, immunohistochemistry, glucose uptake | increased mitochondrial fusion | 26119995 |
| | $HepG2^{HSS}$ | 300 μM oleic acid | | RT-PCR, Western blot, microscopy | HSS gene protected the cells from OA-induced lipotoxicity | 26108664 |
| | primary mice hepatocytes | 200 μM palmitate-BSA complex / Ad-DLP1-K38A | | Clark-oxygen electrode | mitochondrial fission plays a vital role in the progression of nonalcoholic fatty liver disease. | 25080922 |
| Rodent | $Tlr9^{KO}$ and Lysm-Cre $Tlr9^{fl/fl}$ | HFD | plasma, liver macrophages | FACS, mRNA quantification of inflammatory markers | increase in mtDNA. | 26808498 |
| | C57BL/6J | Leptin (25 ng/ ml), 30% FRD + leptin (1 mg/ kg .bw) | hepatocytes | Western blot, RT-PCR, immunohistochemistry, glucose uptake | increased mitochondrial fusion | 26119995 |
| | C57BL/6 | 40% high-fructose high *trans*-fat diet +BCAA | liver and plasma | GC-MS, Western blot, (NMR)-based metabolic flux analysis, metabolites and hormone measurements | BCAA (Branched-chain aminoacids) infusion resulted in elevated rates of gluconeogenesis, mitochondrial anaplerosis and pyruvate cycling | 26058864 |
| | $Wistar/C57BL/6J^{P}_{ck1^{lox+neo/lox+neo;}}$ $Wistar/C57BL/6J^{P}_{ck1^{lox/lox}}$ *Alb-Cre*$^{];}$ $Wistar/C57BL/6J^{P}_{ck1^{lox/lox}}$ | 60% HFD | liver | isotopomer analysis, LC-MS, GC-MS, HPLC, LC-MS/MS, Western blot, qPCR | hepatic anaplerotic/cataplerotic pathway induction in the liver might contribute to oxidative stress and inflammation | 26571396 |
| | $C57BL/6J^{HSS}$ | MCD/HFD + HSS gene | liver isolated mitochondria | Western blot, immunohistochemistry, spectrophotometry, ELISA | increased in CPT-1 activity | 26108664 |
| | B6SJL/129 | DLP1-K38A expression (induced by diet +DOX) + 60% HDF | liver | immunohistochemistry, electron microscopy, Western Blot | mitochondrial fission plays a vital role in the progression of nonalcoholic fatty liver disease. | 25080922 |
| | $B6.BKS(D)\text{-}Lepr^{db/J}$ | 71% Liquid HF | liver | Western blot, densitometric quantitation | adiponectin levels are related with the development of NASH through impaired in mitochondrial β-oxidation | 24464605 |
| | Leptin-deficient Ob/Ob | Standard diet | liver | histology, ELISA, Western blot, RT-PCR, isotopic labeling | mitochondrial dysfunction and upregulation in *the novo* lipogenesis | 23401753 |
| | 75% Balb/c and 25% B6D2F2 | SF+ (Acetyl-L-carnitine (ALC)+Lipoic acid (LA)) and HF + (ALC+LA) | liver | electron microscopy, ELISA, enzymatic assay, spectrophotometry, Western blot | enlarged mitochondrial was found in HF mice | 24176233 |
| | Leptin-deficient Ob/Ob mice | standard diet | adipocytes. mitochondria isolated from WAT, muscle and liver | FACS, Clark-oxygen electrode, electrophoresis, Western blot, Citrulline assay, isotopic labeling, confocal and immunoelectron microscopy | defective leptin–AMPK pathway is related with dysfunctional mitochondria | 21529143 |
| | Sprague-Dawley | 40% HFD | liver | ELISA electron microscopy, RT-PCR, Western blot | increased in liver mitochondrial biogenesis | 20629985 |
| | OLETF | standard diet | liver isolated mitochondria | isotopic labeling, histology, Western blot, fluorescence microscopy, enzyme activity assays,TEM | progressive mitochondrial dysfunction | 20347174 |
| | Wistar | HFD/methionine and choline deficient diet (MCD) | liver isolated mitochondria | histology, Clark-oxygen electrode, TPP+ electrode, HPLC, Western blot, RT-PCR, spectrofluorometry | upregulation of UCP-2 | 18308829 |
| | Sprague-Dawley | 71% HFD + endurance training (ET) | liver isolated mitochondria | MS, EM, TLC, Clark electrode, $TPP^{+}$ electrode | Loss of cristae, intra-mitochondrial granules, swelling, increased mitochondrial membrane composition of PE and PA and decreased PIES, CL and PC/PE, decreased RCR, $\Delta\Psi_m$ and uncoupling respiration. | 25063232 |
| | C57BL/6J | HFHF (40%/22%) +2% chol | liver | NMR, MS, RT-PCR | increased mitochondrial TCA cycle activity, inefficient FAO and accumulation of toxic lipid intermediates | 26814015 |

**Fig. 2.** Mitochondrial metabolism and related mechanisms studied in the context of NAFLD. (A) – Studies using animals and *in vitro* models; (B) – Studies involving human subjects.

B

| Study's PMID | Year | No. of patients | Sample, Analysis | Mitochondrial response |
|---|---|---|---|---|
| 26808498 | 2016 | 3 groups of subjects: lean, 8 obese but normal ALT, 8 obese and high ALT | plasma; FACS, mRNA quantification of inflammatory markers | increase in total DNA and mtDNA, but not nuclear DNA |
| 26058864 | 2015 | 94 with insulin sensitivity | plasma; nuclear magnetic resonance (NMR)-based metabolic flux analysis, GC- and LC-based mass spectrometry | BCAA infusion resulted in elevated rates of gluconeogenesis, mitochondrial anaplerosis and pyruvate cycling |
| 25955209 | 2015 | 16 OBE NAFL+, 18 OBE NAFL -, 7 OBE NASH | liver isolated mitochondria; mitochondrial respiration, immunoblotting, oxidative stress (CAT, 8-OH-dG), RT-PCR | early stages of NAFLD show hepatic mitochondrial flexibility that is lost in NASH |
| 26140000 | 2015 | 19 undergoing bariatric surgery. | liver; MRI and MRS analysis, NMR, cholesterol and triglyceride determination by isopropyl alcohol-hexane method, enzyme-linked immunosorbent assays | improvements in glucose and lipid metabolism |
| 20571306 | 2010 | 45 NAFLD | blood; RT-PCR, ELISA | increased peripheral mitochondrial DNA copy number and reducing tendency of internal oxidative stress |
| 18308829 | 2008 | 10 NASH | liver isolated mitochondria; histology, Clark-oxygen electrode, TPP+ electrode, HPLC, Western blot, RT-PCR, spectrofluorimetry | upregulation of UCP-2 |

**Fig. 2.** (*continued*)

which is associated with adipose tissue inflammation and hepatic mitochondrial dysfunction (Handa et al., 2014). The increased levels of cytokines activate Kupffer and stellate cells, which induce collagen deposition and liver fibrosis (Yin et al., 2015). The subsequent activation of the caspase cascade helps establish a chronic injury that ultimately results in end-stage liver disease and cell death (Handa et al., 2014).

#### 2.2.2. Mitochondrial involvement in NASH progression

Increased levels of the microRNA miR-21 have been reported in the liver of NASH patients and in animal models of NASH, with a concomitant increase in caspase-2 levels (Rodrigues et al., 2017). Activation of miR-21 through the mTOR/NF-κB pathway inhibits PPAR-α and exacerbates mitochondrial dysfunction and hepatocyte injury. In this state, the cell death causing opening of the MPT pore seems to play a critical role in hepatocyte cell death, as demonstrated using MPT inhibitors (Yin et al., 2015). Mitochondrial dysfunction in NASH decreases cellular ATP level, which may cause ER stress with the unfolded protein response (UPR) activation. The UPR is linked to the activation of *de novo* lipogenesis pathways and further aggravates steatosis (Lee et al., 2017). Recent studies have shown that prolonged endoplasmic reticulum (ER) stress or chronic activation of the UPR also induces hepatocyte death and inflammation by the CHOP-dependent signaling pathway (Willy et al., 2015). Alterations in the abundance and activity of OXPHOS proteins (*e.g.*, complex I, III and V) and antioxidant enzymes have been described during mitochondrial dysfunction in animal models of NAFLD (Eccleston et al., 2011; Rector et al., 2010). In fact, increased protein carbonylation has been observed in HFD-treated animals and in NAFLD patients. At the cellular level, these modifications may instigate the accumulation of misfolded proteins, thereby triggering ER stress and the UPR response (Willy et al., 2015). Moreover, incorrect protein folding, *e.g.*, in apoB, an essential protein for very-low-density lipoprotein (VLDL), may impair lipid export from the liver and exacerbate steatosis in mice (Uchiyama et al., 2006).

Increased mitochondrial cholesterol accumulation is also related with the progression of steatosis to steatohepatitis. In NASH patients, the depletion of mitochondrial GSH (mtGSH) has been linked to the higher accumulation of cholesterol (Gan et al., 2014). This may be caused by the impaired transport of mtGSH from the cytosol to the mitochondria due to cholesterol-induced alterations in membrane permeability. High cholesterol has also been shown to sensitize *ob/ob* mice hepatocytes to TNF- and Fas-induced apoptosis and to cause mitochondrial GSH depletion (Mari et al., 2006).

### 2.3. Is mitochondria-related oxidative stress a key player in NAFLD pathology?

#### 2.3.1. Mitochondria and ROS in NAFLD (Fig. 3A and B)

In NAFLD, increased mitochondrial FAO and TCA cycle stimulation results in the enhanced supply of reducing equivalents to the electron transport chain (ETC). This over-reduction of the respiratory complexes promotes superoxide production (Aharoni-Simon et al., 2011). While complex I and III are considered major sites of superoxide, recent studies have suggested that other mitochondrial enzymes are also involved in this potentially detrimental process. Both 2-oxoglutarate dehydrogenase and glycerol 3-phosphate dehydrogenase may be necessary to maintain mitochondrial redox potential (Quinlan et al., 2013). Superoxide is enzymatically converted to hydrogen peroxide, which may cause mitochondrial damage and/or initiate signaling responses. To a lesser extent, extra-mitochondrial reactions may contribute to the elevated ROS/RNS production in NAFLD. The enzymes mediating these reactions include NADPH oxidase, xanthine oxidase and inducible nitric oxide synthase (iNOS) (Mantena et al., 2009). Collectively, these mechanisms may provoke a surplus of ROS (*i.e.*, oxidative stress) in NAFLD. Under normal conditions cells efficiently counteract physiological ROS formation through their antioxidant defense system and by triggering metabolic adaptations that reduce substrate delivery to the TCA cycle. In NAFLD, however, parallel to the increased mitochondrial ROS production, the diminished expression and activity of ROS detoxification mechanisms (*e.g.*, SOD2, catalase or GSH) have also been reported from *in vitro* and *in vivo* experiments (Besse-Patin et al., 2017) .

Thus, a surplus of ROS/RNS and a reduced antioxidant defense capacity may develop in NAFLD. Table 2 lists the most recent works in cell culture, animal models or human patients that report on mitochondrial ROS production and its causal role in the oxidative damage of NAFLD. Notably, a pro-oxidative state appears to precede extensive

A

| | Model | Treatment | Sample | Analysis | Mitochondrial response | PMID |
|---|---|---|---|---|---|---|
| Cell line | H4IIEC3 | 2% palmitate or oleate | | fluorimeter, spectrophotometer | increased ROS (no contribution of NADPH oxidase or xanthine oxidase); increased protein carbonyl levels | 19332540 |
| | *Jnk1*$^{-/-}$ primary hepatocytes | 20-40μM LDL | | fluorimeter, spectrophotometer, | increased ROS, depletion of GSH | 25064435 |
| | C3A | oleate, octanoate, lactate, pyruvate, ammonia treated | | enzyme activity assay, FACS, fluorescence microscopy, fluorimeter | increased ROS | 22429485 |
| | HepG2 SIRT3$^{KO}$ | 25mM glucose | | Seahorse analyser, fluorimeter | increased ROS | 20647045 |
| | HepG2 SIRT3$^{+/+}$ | 0.5mM palmitate | | confocal microscope, RT-PCR | increased MnSOD activation and decreased superoxide levels | 28437863 |
| | FaO HEVC | 0.75mM oleate/ palmitate + phenolic compounds | | fluorimetric analysis, spectrophotometer, Western Blot | decreased oxidative stress | 28526925 |
| | HepG2$^{ALCAT1+/+}$ | | | TBARS kit, fluorimeter, RT-PCR | increased oxidative stress; increased lipid peroxidation | 25203315 |
| | H4IIEC3 | 400 μM palmitate | | fluorimeter, Oroboros Oxygraph-2K, $^{13}$C-MFA | palmitate induce oxidative stress | 25061559 |
| Rodent | OLETF | standard diet | liver isolated mitochondria | enzyme activity assay, fluorimeter, Western blot | decreased antioxidant capacity (decreased SOD activity and increased GSSH levels); increased ROS | 20347174 |
| | Wistar | choline-deficient diet | liver isolated mitochondria | spectrophotometer, Clark-oxygen electrode, enzymatic activity assay, Western blot | increased protein oxidative damage | 18765303 |
| | C57BL/6J | 60% HFD + apigenin (flavonoid) | liver | RT-PCR, enzymatic activity assay, spectrophotomoter | decreased expression of genes involved in oxidative stress | 28414138 |
| | C57BL/6J catalase$^{KO}$ | 60% HFD | liver | lipid peroxidation assay, RT-PCR, Western blot | catalase deficiency accelerates oxidative stress; increased lipid hydroperoxides; increased 8-oxo-dG; decreased MnSOD expression; | 28461774 |
| | Sprague-Dawley | 60% HFD +STZ | liver isolated mitochondria | fluorescence microscopy, enzyme activity assay, RT-PCR | increased ROS | 25877002 |
| | C57BL/6J | 40% HFD | liver | spectrophotometer, RT-PCR | upregulation of oxidative stress (FAO and CYP2E1 contribution with no alterations in NADPH oxidase); increased protein carbonyl levels; decreased levels of anti-oxidant genes | 18640384 |
| | C57BL/6J | 60% HFD +rutin (flavonoid) | liver | fluorimeter, enzyme activity assay, RT-PCR, ELISA | rutin restored SOD activity and decreased oxidative damage | 28577437 |
| | 129/Svj CYP2E1$^{KO}$ | 60% HFD | liver | spectrophotometer, ELISA, Oxy-blot assay kit | increased mRNA and protein CYP2E1 levels; increased lipid peroxidation, protein carbonylation, nitration and glycation | 22668639 |
| | Sprague Dawley ALCAT1$^{KO}$ | HFD | liver | TBARS assay, fluorimeter | increased oxidative stress and lipid peroxidation | 25203315 |
| | C57BL/6J | 45% HFD +0.2%cholesterol | liver | fluorimeter, Western blot, enzyme activity assay, RT-PCR | no alterations in oxidative stress markers | 26391864 |
| | Wistar | 60%HFD + 10%HSD + green tea | liver | RT-PCR, fluorimeter, enzyme activity assay | increased oxidative stress, increased lipid peroxidation, decreased antioxidant capacity; tea treatment reduced oxidative stress and increased total antioxidant capacity, reduction in lipid peroxidation | 27866076 |
| | C57BL/6J PGC1-α$^{KO}$ | 45% HFD +30% d-fructose | liver | RT-PCR, fluorimeter | increased lipid peroxidation, increased oxidative stress, reduced mitochondrial enzymes (SOD2 and Prdx) involved in ROS detoxification | 27658772 |
| | Wistar | methionine-choline deficient diet | liver | TBARS, Western blot, immunohistochemistry, TBARS, enzyme activity assay | increased ROS levels (contribution of NADPH oxidase); increased peroxidated proteins around lipid droplets; decreased GSH content; moderated reduction of SOD2 after 3 weeks of treatment | 26881047 |
| | C57BL/6 | 71% HFD | liver isolated mitochondria | fluorimeter, 2D IEF/SDS-PAGE, immunoblotting | increased ROS followed by a reduction associated with UCP-2 and increased state 4 respiration, impaired NO metabolism | 20919931 |
| | C57BL/6J | 48% HFD | liver isolated mitochondria | Seahorse analyzer, enzyme activity assay, fluorimeter, LC-MS/MS | increased ROS production, decreased antioxidant enzymes levels | 27694529 |
| | Sprague-Dawley | HFHS (24%/32%) | liver | enzyme activity assay, gel electrophoresis, TBARS, fluorimeter | increased lipid peroxidation, increased protein oxidation, no differences in the activity of antioxidant enzymes | 25282656 |
| | C57BL/6J | 45% HFD + 3g/kg glucose | liver | Immunohistochemistry, Western blot, RT-PCR, fluorimeter | increased lipid peroxidation | 26464382 |
| | C57BL/6J | 35% and 71% HFD | liver isolated mitochondria | immunoblotting, immunofluorescence, spectrophotometer, immunohistochemistry | increased iNOS and CYP2E1 protein levels; increased mitochondrial protein modifications | 18752470 |
| | C57BL/6J | 60% HFD | liver isolated mitochondria | TG and MDA assay, RT-PCR, Western blot | reduced MDA levels, upregulation of catalase and SOD2, mitochondria oxidative stress reduction | 26666995 |
| | Sprague–Dawley | 60% HFD +STZ | liver isolated mitochondria | fluorescence microscopy, enzyme activity assay, RT-PCR, Western blot | hepatic ROS overproduction associated with T2DM in NAFLD | 25877002 |
| | Wistar | 60% HFD + lipoic acid (antioxidant) | liver isolated mitochondria | RT-PCR, Western blot, fluorimeter, enzyme activity assay, TBARS | reduced oxidative damage in mtDNA | 22327056 |
| | C57BL/6J $^{Lep(-/-)}$ | I) ob/ob, II) Mn[III] tetrakis, III) IgG1; IV) anti-TNF; V) uric acid | liver | spectrophotometer, immunoprecipitation | iNOS expression might enhance peroxynitrite formation | 16941682 |
| | *fa/fa* Zucker rats | 60% HFD | liver isolated mitochondria | enzyme activity assays, spectrophotometer, Western blot, ELISA | increased MDA and protein carbonyl levels; decreased GSH, Gpx, SOD and catalase activities; increased NADPH oxidase activity; decreased CYP2E1 expression | 15522905 |

**Fig. 3.** Mitochondrial ROS production and related mechanisms studied in the context of NAFLD. (A) – Studies using animals and *in vitro* models; (B) – Studies involving human subjects.

B

| Study's PMID | Year | No. of patients | Sample, Analysis | Mitochondrial response |
|---|---|---|---|---|
| 27596100 | 2016 | 143 with NAFLD<br>102 with NASH | liver biopsy; sequencing, SNP profiling | mitochondrial haplogroup L modulates oxidative stress and the efficiency of OXPHOS, being less prevalent in NASH patients |
| 14556645 | 2004 | 31 (with NAFLD or NASH) | liver; enzyme activity assays, FRAP assay, Western blot | increased protein carbonyl levels; decreased GSH, SOD and catalase activities; increased CYP2E1 activity (in NASH patients) |
| 25955209 | 2015 | Obese insulin-resistant:<br>18 without NAFLD or NASH<br>16 with NAFLD<br>7 with NASH | liver biopsy; TBARS assy, enzyme activity assay, immunoblotting, RT-PCR | increased lipid peroxidation in all groups; increased ROS and 8-OH-deoxyguanosine levels in NASH group; decreased activity of catalase in NASH group |

**Fig. 3.** (*continued*)

mitochondrial damage and the subsequent mitochondrial impairment in NAFLD pathology (Koliaki et al., 2015).

### 2.3.2. Oxidative damage in mitochondria in NAFLD

Aside from enzymatic inactivation, oxidative stress is also linked to mtDNA alterations. MtDNA is sensitive to oxidative damage due to its proximity to the sites of ROS production and lack of histones or DNA repair systems. NAFLD is characterized by mtDNA depletion and increased hepatic levels of 8-hydroxy-2′-deoxyguanosine (8-OHdG), a marker of oxidized DNA (Koliaki et al., 2015). Moreover, oxidative damage to nuclear DNA may also amplify mitochondrial impairment by compromising the transcription of critical mitochondrial proteins. As a result, the expression levels of key regulatory factors involved in mitochondrial metabolism and organelle biogenesis, namely, PGC-1α, TFAM and NRF-2, have been reported to be reduced in NAFLD (Aharoni-Simon et al., 2011; Koliaki et al., 2015).

ROS can "attack" polyunsaturated fatty acids, leading to the production of aldehyde by-products, namely, MDA and HNE (Yin et al., 2015), that can diffuse from their site of origin, amplifying the effects of oxidative stress. Importantly, cardiolipin, a specific inner mitochondrial membrane phospholipid, is very susceptible to oxidative damage. In the presence of oxidized cardiolipin, altered membrane fluidity is associated with the destabilization and loss of ETC complex activity and the induction of MPT pore opening (Li et al., 2010). Moreover, the release of cytochrome c from cardiolipin into the cytosol can induce the caspase-mediated apoptotic pathway and trigger cell death (Kagan et al., 2005).

Finally, in NAFLD, ROS may be associated with ETC disruption, outer mitochondrial membrane permeabilization, altered $\Delta\psi_m$ and changes in mitochondrial structural integrity (Rector et al., 2010). Oxidative stress increases protein oxidation and lipid peroxidation and induces mitochondrial genome alterations. These mechanisms may thereby cause vicious cycle of mitochondrial oxidative damage and mitochondria-originating oxidative stress (Mantena et al., 2009).

### 2.3.3. Antioxidative treatment in NAFLD

Since the above studies have repeatedly reported oxidative mitochondrial damage, it is of interest to determine whether antioxidative treatments have a beneficial effect in NAFLD. In NAFLD animal models, the administration of lipoic acid resulted in preventive, therapeutic effects on hepatic steatosis by inhibiting *de novo* lipogenesis and by promoting a reduction in oxidative stress. Increased antioxidant enzyme (SOD2, GPx, GSH) abundance, reduced ROS production and increased mtDNA copy numbers have been reported (Geng et al., 2017; Valdecantos et al., 2012). Antioxidant ginkgolide A (GA) treatment in HFD mice increased the levels of anti-apoptotic Bcl-2, while a decrease in Bax, phosphorylated JNK, and cleaved caspase-3 and −9 levels were observed in the animal livers. Moreover, GA treatment also protected hepatocytes from inflammation (Jeong et al., 2017). Oxidative stress and lipid peroxidation are known factors that activate NF-κB to induce the increased production of pro-inflammatory cytokines. These factors contribute to the leukocyte recruitment, necro-inflammation, insulin resistance (IR) and fibrogenic factor release that ultimately cause end-stage liver disease (Rodrigues et al., 2017). Studies in various cell lines have shown that phenolic compounds reduce ROS and, therefore, may slow the progression of steatosis to fibrosis by reducing inflammation (decreased NF-κB phosphorylation) and endothelial cell migration (decreased NO release) (Jeong et al., 2017; Vergani et al., 2017).

## 3. Future outlook

NAFLD prevalence has doubled over the last 20 years and now affects approximately one-quarter of the worldwide population. Unfortunately, the sequence of events observed in NAFLD progression is still not clearly understood, which limits the development of efficient therapies to counteract the spectrum of progressive liver disorders. Since oxidative stress is considered a key pathological feature of NAFLD progression, therapeutic approaches have focused on antioxidative compounds to counteract ROS. Studies with NAFLD mice have shown that HFD-induced effects, such as steatosis, early mitochondrial dysfunction and dysregulated oxidative balance, can be prevented in the presence of phenolic compounds (Geng et al., 2017; Valdecantos et al., 2012). Moreover, these types of compounds also limit pathological features such as apoptosis, inflammation and cell migration, which are typical for more advanced stages of NAFLD (Jeong et al., 2017; Vergani et al., 2017). However, despite these promising results, there are currently no effective treatments for the pathological alterations in NAFLD patients. Future studies are required to determine the efficacy of pharmaceuticals that target mitochondrial dysfunction in NAFLD.

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