Synergistic interaction of fatty acids and oxysterols impairs mitochondrial function and limits liver adaptation during nafld progression

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\textbf{A B S T R A C T}

The complete mechanism accounting for the progression from simple steatosis to steatohepatitis in nonalcoholic fatty liver disease (NAFLD) has not been elucidated. Lipotoxicity refers to cellular injury caused by hepatic free fatty acids (FFAs) and cholesterol accumulation. Excess cholesterol autoxidizes to oxysterols during oxidative stress conditions. We hypothesize that interaction of FFAs and cholesterol derivatives may primarily impair mitochondrial function and affect biogenesis adaptation during NAFLD progression. We demonstrated that the accumulation of specific non-enzymatic oxysterols in the liver of animals fed high-fat+high-cholesterol diet induces mitochondrial damage and depletion of proteins of the respiratory chain complexes. When tested in vitro, 5α-cholestan-3β,5,6-epoxy-triol (triol) combined to FFAs was able to reduce respiration in isolated liver mitochondria, induced apoptosis in primary hepatocytes, and down-regulated transcription factors involved in mitochondrial biogenesis. Finally, a lower protein content in the mitochondrial respiratory chain complexes was observed in human non-alcoholic steatohepatitis. In conclusion, hepatic accumulation of FFAs and non-enzymatic oxysterols synergistically facilitates development and progression of NAFLD by impairing mitochondrial function, energy balance and biogenesis adaptation to chronic injury.

\textbf{1. Introduction}

Nonalcoholic fatty liver disease (NAFLD), the most common liver pathology in the Western world \cite{1}, may clinically present with a heterogeneity of conditions ranging from benign steatosis (NAFL) to steatohepatitis (NASH) – the more progressive form of the disease \cite{2} – and to cirrhosis. The mechanisms underlying the transformation from non inflamed to inflamed fatty liver are not completely elucidated, even though lipid metabolism alterations, mitochondrial dysfunction, inflammation and oxidative stress are suggested to play a significant role \cite{3–6}.

Accumulating lipids in hepatocytes may be vulnerable to free radical-induced peroxidation. Lipid peroxides exert toxic effects on the mitochondrial DNA (mtDNA), RNA and proteins of the respiratory chain, leading to mitochondrial dysfunction \cite{7}.

Lipotoxicity refers to cellular injury caused by excess of free fatty acids (FFAs) and related-lipid metabolites \cite{8}. Excess cholesterol can lead to dysregulation of cholesterol metabolism, which is considered an underlying pathology in the development of many metabolic diseases \cite{9}. Loading mitochondria with free cholesterol sensitizes hepatic cells to inflammatory mediators such as Tumor Necrosis Factor (TNF) and cell-surface Fas receptor (Fas), which may precipitate steatohepatitis...
Free cholesterol accumulation in oxidative settings may promote its oxidization with final production of oxysterols that are involved in NAFLD damage [11,12]. Dietary fat and cholesterol induce the metabolic and hepatic features of NASH in mice only when acting synergistically but not when administered alone [13]. We have previously shown that the significant change in fatty acid and oxysterols profile induced by a dietary combination of high fat and high cholesterol accounts for liver injury, allowing the generation of interesting hypotheses on the role of interaction of lipid and cholesterol metabolites in the pathogenesis of NAFLD progression [14].

In the present study, after a lipidomic analysis of hepatic non-enzymatic oxysterols in animals fed high-fat or high-fat + high-cholesterol diet, we analyzed mitochondrial function, biogenesis and mitochondrial respiratory chain proteins, and we demonstrated a significant impairment in the respiratory chain and energy homeostasis secondary to depletion of specific respiratory chain complexes. The analysis of mitochondrial biogenesis signaling revealed that FFAs and non-enzymatic oxysterols synergistically interact and limit the adaptive response of liver cells to chronic lipid accumulation, promoting transition from steatosis to steatohepatitis. The observations were then confirmed in human NASH.

2. Materials and methods

Further details are provided in the Supporting Information.
2.1.3. Rat hepatocytes isolation, culture and treatment

Apoptosis was analyzed using the annexin V-ported [14].

A concentration of 10^5 7-OHC; 5,6-ve OXPHOS complexes simultaneously a mix of oxysterols generated a free radical mediated mechanism (7-β-epoxy; 5,6,5,6-ve triol; triol; 7-ketocholesterol, 7KC; 6-oxo-cholestan-3-β,5a-diol, 6-oxo) by mass spectrometry with isotope-dilution methods, as previously reported [14].

2.1.4. Oxographic measurements

Respiratory rates were measured in isolated hepatocytes as described [18]. Freshly prepared liver mitochondria were assayed for oxygen consumption as previously reported [19]. Mitochondrial membrane potential (Δψ) and proton leak analysis were measured as previously reported [19].

2.1.5. Evaluation of F0F1ATPase activity and tissue ATP content

F0F1ATPase activity was measured following ATP hydrolysis with an ATP-regenerating system coupled to NADPH oxidation [20]. The hepatic ATP concentration was assessed by bioluminescence (Enliten ATP assay kit - Promega Corporation, Madison, WI, USA) according to the method of Yang [21].

2.1.6. Measurement of mitochondrial H2O2 production

The rate of peroxide production was determined in isolated liver mitochondria following the oxidation of Amplex Red by horseradish peroxidase as previously reported [19].

2.1.7. q-PCR array of mitochondrial energy metabolism – related genes

20 ng cDNA was loaded into each well in RT2 Profiler 96-well PCR array plates (PARN-008Z, QIAGEN, Valencia CA). The median cycle threshold value (CT) was uploaded onto the SABiosciences website (http://pcrdataanalysis.sabiosciences.com/pcr/arrayanalysis.php) and the fold change of each gene expression was calculated using the provided software according to manufacturer’s instruction.

2.1.8. Gene expression analysis by real-time RT-PCR

Real-time RT-PCR was performed on RNA extracted from human liver tissue or rat primary hepatocytes, using SYBR Green I assay in Bio-Rad iCycler detection system as previously reported [19]. A PCR master mix containing the specific primers shown in Supplementary Table 1 was used. The threshold cycle (CT) was determined, and the relative gene expression subsequently was calculated as follows: fold change = 2^−ΔΔCT, where ΔΔCT = ΔCT − CT target housekeeping and Δ(ΔCT) = ΔCT − ΔCT control treatment.

2.1.9. Blue Native bidimensional polyacrylamide gel electrophoresis (BN-PAGE)

BN-PAGE was performed on human liver mitochondria proteins as described [22]. First, solubilized samples were stained with a charged (Coomassie) dye. The intact mitochondrial complexes were then separated by electrophoresis based upon how much dye was bound, which is proportional to their size. After this first dimension gel, which was run in a 5–12% acrylamide gradient, a lane was cut out and placed on a glass plate for incubation with lysis buffer at room temperature. The protein components of the resolved complexes were separated in a second dimension after soaking the gel in denaturing SDS buffer. For the detection of all five OXPHOS complexes simultaneously a mix of monoclonal antibodies was used (MitoScience MS603 kit, AbCam, Oregon, USA).

2.1.10. Statistical analysis

The data were normally distributed and were expressed as mean ± standard deviation of the mean (SDM). Differences between the groups were determined by one-way analysis of variance (ANOVA) with Tukey-Kramer as post hoc test. Statistical significance was accepted when P < 0.05. The GraphPad Prism 6.0 Software was used to perform the analysis.

3. Results

3.1. Combination diet (HF + HCh) increases non-enzymatic oxysterol level in NASH liver

Rats fed high-fat diet (HF) for 6 weeks showed severe liver steatosis...
but mild lobular inflammation; on the other hand, animals fed the high-fat + high-cholesterol diet (HF+HCh) exhibited a severe liver damage characterized by macrovesicular steatosis, hepatocytes ballooning and diffused lobular infiltration, as well as increased serum amino-transferase levels, suggestive of NASH (Fig. 1A and B, Table 1).

The lipidomic analysis of non-enzymatic oxysterols in rat liver revealed a significant increase of 7β-hydroxycholesterol (7β-OHC), 7-ketocholesterol (7-KC) and 5α-cholestan-3β,5,6β-triol (Triol) in the HF + HCh livers, as compared to the CTRL and HF groups (Fig. 1C).

### 3.2. Mitochondrial function, ATP homeostasis and biogenesis are severely impaired in livers feeding HF and HC combination diet

Freshly isolated mitochondria were analyzed in terms of oxygen consumption rate, mitochondrial membrane potential (MMP), hydrogen peroxide production rate, ATP homeostasis and proton leak kinetics.

Oxygen uptake was impaired by the mitochondrial Complex I (while it was enhanced by the Complex II) in the HF + HCh group as compared to CTRL rats; however, MMP was reduced both in the HF and in the HF + HCh group (Table 2).

We then analyzed the hepatic ATP homeostasis and observed that both diets induced a reduction in the liver ATP content when compared to CTRL (Fig. 2A). To determine whether the reduction of ATP was dependent on an impairment in mitochondrial ATP synthesis, the enzymatic activity of Complex V (ATP-synthase) was measured and we observed a severe reduction in animals fed the HF+HCh but not the HF diet (Fig. 2B).

The dissipation of MMP through the mitochondrial membranes (proton leak) is a well-established hallmark of mitochondrial dysfunction in NALFD [23]. Mitochondria proton leak was observed in both the HF and the HF+HCh livers as compared to CTRL (Fig. 3A), and was associated with increased Uncoupling Protein 2 (UCP2) expression (Fig. 3B-D).

Impaired mitochondria produce free radicals, which were quantified by measuring the hydrogen peroxide (H$_2$O$_2$) production rate using pyruvate/malate or succinate as oxidative substrates. The rate of H$_2$O$_2$ synthesis was not changed in the HF group with respect to CTRL.
on the contrast, the HF+HCh group showed a higher rate of mitochondrial ROS production (Fig. 2C).

Taken together, these data show that the addition of cholesterol to a high fat diet induces a significant change in the non-enzymatic oxysterol profile that in turn induces a severe hepatic impairment of mitochondrial function.

A Mitochondrial Energy Metabolism PCR Array was used to determine the expression profile of 84 key genes involved in mitochondrial respiration, including those encoding components of the electron transport chain and oxidative phosphorylation complexes.

As shown in Table 3A, the HF group presented the up-regulation of Cox15, Bcs1L and Atp4a genes as compared to CTRL. On the contrast, several genes encoding for subunits of mitochondrial Complex I, IV and V were down-regulated in the HF+HCh (Table 3B), suggesting a severe reduction of respiratory chain proteins.

3.3. Non-enzymatic oxysterols synergistically interact with FFAs and impair mitochondrial function and biogenesis in vitro

To study a possible synergistic interaction between specific oxysterols and fatty acids in the induction of liver injury, different combinations of free fatty acids with 7β-OHC, 7-KC or triol were tested in vitro. Rat primary hepatocytes were incubated with 50 µM palmitic acid (PA) as saturated fatty acid, and 2 mM oleic acid (OA) as unsaturated fatty acid for 24 h. The addition of Triol to PA/OA significantly reduced the viability of the primary hepatocytes at concentrations of 10 or 100 µM (Fig. 4A); however, the same concentration of 7β-OHC or 7-KC did not induce any significant effect (supplementary material, Fig. S3A and B). By flow cytometry we verified that the combination of the lowest concentration of Triol (10 µM) + PA/OA induced hepatocellular necrosis (7-AAD positive cells) rather than apoptosis (annexin V-stained cells), while minimal effects were observed using Triol or PA/OA alone (Fig. 4B).

The effects of 7β-OHC, 7-KC and triol were also tested on the
mitochondrial bioenergetics; to this, the oxygen consumption was measured on both isolated liver mitochondria and intact primary hepatocytes. No toxic effect was observed with 7β-OHC or 7-KC (supplementary material, Fig. S4); on the contrary, a dose-dependent reduction of mitochondrial respiration was reported with Triol (Fig. 5A-C). These data suggest that the combination of Triol + PA/OA induced hepatocyte toxicity via mitochondrial bioenergetics impairment.

Peroxisome proliferator-activated receptor-γ coactivator 1α (PGC1α) and its downstream targets, mitochondrial transcription factor A (TFAM) and nuclear respiratory factor 1 (NRF1) are the master regulators of mitochondrial biogenesis. Interestingly, we observed that incubation of primary hepatocytes with PA + OA in the presence of triol – but not of 7β-OHC or 7-KC – significantly induced down-regulation of PGC1α, TFAM and NRF1, suggesting a causative role for the combination of free fatty acids and Triol in the mitochondrial biogenesis failure and in mitochondrial dysfunction (Fig. 5D).

3.4. Mitochondrial biogenesis is impaired in NASH patients as compared to simple steatosis and healthy livers

The characteristics of studied population are summarized in the Table S2. NASH showed higher fasting serum triglycerides and cholesterol as compared to healthy and steatosis subjects. Freshly isolated mitochondria from human liver specimens were analyzed by 2D electrophoresis and a reduction of proteins related to Complex I, Complex IV and Complex V was observed only in NASH (Fig. 6A) but not in simple steatosis. We then measured the mRNA expression of master regulators of mitochondrial biogenesis and, very interestingly, we observed a 50% reduction in the expression of PGC-1α in NASH faces with the difficulty to identify patients progressing from simple steatosis to steatohepatitis, who present with a worse prognosis [24]. One of the burning questions in NAFLD remains which factors act as the driving forces toward a progressive inflammatory disease phenotype. Mitochondrial dysfunction has been suggested to play a significant role in the progression of NAFLD [23]. The present study provides a new insight on the mechanism by which free fatty acids and oxysterols interact and induce NAFLD development and progression through the impairment of mitochondrial function and biogenesis response to chronic liver injury.

The accumulation of toxic lipids causes inflammation in the setting of liver steatosis [25]. Several studies focused on triglyceride

Table 3
List of the genes up- or down-regulated (> 2 fold) in the HF (A) and HF + HC (B) groups with respect to CTRL. Changes in gene expression between groups were evaluated using RT2 Profiler 96-well PCR array plates. Data analysis was done by the 2-DDCt method on the manufacturer's Web portal (http://www.SABiosciences.com/prcrarraydataanalysis.php), QIAGEN, CA, USA.

| RefSeq   | Symbol | Description                                                                 | Fold Change |
|----------|--------|-----------------------------------------------------------------------------|-------------|
| A        |        |                                                                             |             |
| NM_01030699 | Cox15  | Cytochrome c oxidase assembly protein (yeast)                                | 2,1936      |
| NM_0107666 | Bcs1l  | BCS1-like (yeast)                                                          | 4           |
| NM_012509  | Atp4a  | ATPase, H+/K+ transporting, alpha polypeptide                               | 2,1936      |
| B        |        |                                                                             |             |
| NM_133517  | Atp12a | ATPase, H+/K+ transporting, alpha polypeptide                               | −3,4983     |
| NM_012510  | Atp4b  | ATPase, H+/K+ exchanging, beta polypeptide                                  | −3,4983     |
| NM_023093  | Atp5a1 | ATP synthase, mitochondrial F1 complex, alpha subunit 1                    | −2,0093     |
| NM_134364  | Atp5b  | ATP synthase, mitochondrial F1 complex, beta polypeptide                    | −2,4172     |
| NM_053825  | Atp5i  | ATP synthase, mitochondrial F0 complex, subunit e                            | −2,1535     |
| NM_080481  | Atp5j  | ATP synthase, mitochondrial F0 complex, subunit e                            | −3,4983      |
| NM_053602  | Atp5k  | ATP synthase, mitochondrial F0 complex, subunit F6                          | −2,1043     |
| NM_212516  | Atp5l  | ATP synthase, mitochondrial F0 complex, subunit G                           | −2,0093     |
| NM_033718  | Atp6a1p1 | ATPase, H+ transporting, lysosomal accessory protein 1                      | 2,1535      |
| NM_001014199 | Atp6v1c2 | ATPase, lysosomal V1 subunit C2                                             | −3,4983     |
| NM_001108979 | Atp6v1e2 | ATPase, lysosomal V1 subunit E2                                             | 2,1043      |
| NM_0107666 | Bcs1l  | BCS1-like (yeast)                                                          | −2,0093     |
| NM_017202  | Cox41  | Cytochrome c oxidase subunit IV isoform 1                                   | −2,4172     |
| NM_145783  | Cox5a  | Cytochrome c oxidase, subunit Va                                            | −2,1043     |
| NM_012812  | Cox6f2 | Cytochrome c oxidase subunit VII polypeptide 2                              | −3,4184     |
| NM_019360  | Cox6c  | Cytochrome c oxidase, subunit Vic                                           | −3,1895     |
| NM_022503  | Cox7a2 | Cytochrome c oxidase subunit VIIa polypeptide 2                             | −2,2038     |
| NM_182819  | Cox7b  | Cytochrome c oxidase subunit VIIb                                          | −2,1535     |
| NM_183055  | Cox8c  | Cytochrome c oxidase, subunit VIII                                          | −3,4983     |
| NM_001009706 | Lhpp  | Phosphoryls phosphohistidinotransferase inorganic pyrophosp phosphatase    | 4,9474      |
| NM_001108813 | Ndufa1 | NADH dehydrogenase 1 alpha subcomplex, 1                                   | 3,0455      |
| NM_212517  | Ndufa11 | NADH dehydrogenase 1 alpha subcomplex 11                                  | 2,0562      |
| NM_001106153 | Ndufa2 | NADH dehydrogenase 1 alpha subcomplex, 2                                  | −2,0093     |
| NM_001106646 | Ndufa6 | NADH dehydrogenase 1 beta subcomplex, 6                                   | 2,2553      |
| NM_001108442 | Ndufa7 | NADH dehydrogenase 1 beta subcomplex, 7                                   | 2,9097      |
| NM_001127294 | Ndufa9 | NADH dehydrogenase 1 beta subcomplex, 9                                   | −3,1895     |
| NM_001109107 | Ndufs2 | NADH dehydrogenase Fe-S protein 2                                          | 2,2038      |
| NM_001106489 | Ndufs3 | NADH dehydrogenase Fe-S protein 3                                          | −2,2553      |
| NM_001005359 | Sdhb  | Succinate dehydrogenase complex, subunit B, iron sulfur                    | 2,2038      |
| NM_198788  | Sdhb   | Succinate dehydrogenase complex, subunit D                                  | 2,2553      |
| NM_133418  | Scle2a5a10 | Solute carrier family 25, member 10                                      | −3,3404     |
| NM_001047880 | Scle2a5a15 | Solute carrier family 25, member 15                                       | −2,2553     |
| NM_012682  | Ucp1   | Uncoupling protein 1                                                       | −3,4983     |
| NM_013167  | Ucp3   | Uncoupling protein 3                                                       | −3,4983     |
| NM_001025134 | Ucspq | Ubiquinol-cytochrome c reductase, complex III subunit VII                  | 2,3983      |
accumulation as pathogenic factor [26,27]. Nevertheless, triglyceride storage may protect liver cells from fatty acid-induced apoptotic pathways [28], and blocking triglyceride synthesis leads to less steatosis but more inflammation, oxidative stress and fibrosis [29]. Even though the type of hepatotoxic lipid has not been identified yet, both free fatty acids and cholesterol, especially when accumulated in mitochondria, could lead to liver injury and hepatocellular damage [10,30]. It has been recently demonstrated that administration of peroxidized fatty acids produces a dramatic inflammatory increase into the liver [31]. However, a lipidomic analysis on humans demonstrated that – more than free fatty acids – free cholesterol accumulates in NAFLD [32].

Cholesterol is essential for proper cell function; however, free cholesterol accumulation in an oxidative milieu may promote its oxidation with final production of oxysterols. Cholesterol oxidation involves several mono-oxygenation reactions, which are catalyzed by cytochromes P450 (CYPs). During normal hepatic cholesterol degradation, it can be enzymatically converted to 7α-hydroxycholesterol by 7α-hydroxylase. Other CYPs such as 27-hydroxylase and 4β-hydroxylase catalyze the formation of the mayor oxysterol species 27-hydroxycholesterol and 4β-hydroxycholesterol, respectively. Alternatively, cholesterol can be metabolized by non-enzymatic autoxidation, which leads to the formation of 7-ketocholesterol, 7α-hydroxycholesterol, 7β-hydroxycholesterol, 5,6α-epoxycholesterol and 5,6β-epoxycholesterol. There is increasing interest in the biological activity of oxysterols, not
Fig. 5. Mitochondrial function and biogenesis impairment in primary hepatocytes after co-treatment with fatty acids and the oxysterol triol. (A) Oxygen consumption rate of isolated liver mitochondria after incubation with increased concentrations of 5α-cholestane-3β,5,6β-triol (Triol) for 24 h. Mitochondrial respiration was started by pyruvate + malate and glutamate (Complex I) or succinate in the presence of rotenone (Complex II). Data are expressed as mean ± SDM of three consecutive experiments. Statistical differences were assessed by one-way ANOVA and Tukey-Kramer as post-hoc test (* = p < 0.05 vs CTRL, ** = p < 0.01 vs CTRL). (B) Representative oxygraphic measurement of intact hepatocytes respiration after 24 h incubation with palmitic acid (PA) + oleic acid (OA) ± Triol. (C) Mitochondrial/cellular respiratory rate measured in intact rat primary hepatocytes after 24 h incubation with PA+OA ± Triol. (D) Gene expression of peroxisome proliferator-activated receptor-γ coactivator 1 α (PGC1α), mitochondrial transcription factor A (TFAM) and nuclear respiratory factor 1 (NRF1) measured by real time rt-PCR in the rat primary hepatocytes after 24 h incubation with PA+OA ± Triol. Data are expressed as mean ± SDM of three consecutive experiments. Statistical differences were assessed by one-way ANOVA and Tukey-Kramer as post-hoc test.
only because they are non-invasive markers of oxidative stress in vivo [33], but also because they may be part of the fine signaling controlling hepatocyte survival in several conditions [34].

We have previously demonstrated that steatohepatitis induced by dietary excess of both fatty acids and cholesterol is associated with a definite oxysterol pattern [14]. In the present study, we have focused on the non-enzymatic oxysterols since the hepatic expression of several enzymes involved in oxysterol generation is reduced in human NASH.

Fig. 6. Reduction of mitochondrial respiratory proteins and master regulators of biogenesis in human NASH. (A) Representative second dimension of a BN-PAGE performed on mitochondrial proteins isolated by the liver of healthy subjects (HEALTHY) and patients affected by simple steatosis (NAFL) or steatohepatitis (NASH). Bands characteristic of individualOXPHOS complexes are recognizable in all three experimental groups. Protein extracts were prepared for each patient, and each individual was assessed separately. Each lane contained 15 μg of mitochondrial protein extract. (B) Gene expression of peroxisome proliferator-activated receptor-γ coactivator 1 α (PGC1α), mitochondrial transcription factor A (TFAM) and nuclear respiratory factor 1 (NRF1) measured by real time rt-PCR in the liver of all patient groups studied. Data are expressed as mean ± SDM of 8 + 7 + 10 experiments. Statistical differences were assessed by one-way ANOVA and Tukey-Kramer as post-hoc test.
able to induce mitochondrial dysfunction and mitochondria-dependent apoptosis [40–42]. We observed that such oxysterols do not affect cell viability because hepatocytes rapidly metabolize 7KC, the major dietary cholesterol (7KC) and triol. This model of liver disease associates non-enzymatic oxysterols such as 7-ketocholesterol (7KC), triol, and some of enzymatic oxysterols may be even protective, by countering the hepatic lipid accumulation and inflammation [36–38]. However, triol significantly impaired mitochondrial respiration. Very interestingly, the addition of triol to free fatty acids reduces hepatocytes viability by impairing mitochondrial respiration. Our data show, for the first time, that NASH induced by dietary fatty acid and cholesterol excess associates with mitochondrial dysfunction. Furthermore, when hepatocytes are cultured with a mixture of fatty acids and oxysterols, both mitochondrial respiration and cell viability decrease, suggesting a possible synergic interaction between different lipid species in the pathogenesis of liver cell damage.

A large body of evidence indicates that high-fat diets induce adaptive mechanisms in mitochondrial bioenergetics, such as increased electron transport chain performance and uncoupling [23,45]. On the other side, high-cholesterol diet increases liver mitochondria respiration, uncoupling and induces expression of genes involved in mitochondrial biogenesis [46].

Our data show that combination of HF and HCh diets induce mitochondrial dysfunction, alters energy homeostasis and limits the adaptive response to energy demand by impairing mitochondrial biogenesis. These finding are strengthened by the bi-dimensional analysis of mitochondrial respiration chain content in human liver, demonstrating that proteins of the Complex I, IV and V are reduced in non-alcoholic steatohepatitis but not in simple steatosis. Moreover, mitochondrial biogenesis response is impaired at the level of PGC-1α, NRF1, and TFAM, which are involved in mtDNA transcription, maintenance, replication and repair [47] in good agreement with previous observations reporting that altered mitochondrial function in human NASH is dependent by defective mitochondrial biogenesis [48].

In conclusion, high-fat diet causes simple steatosis and mild liver injury that do not overwhelm defense response of the liver; on the contrast, the combination of FFAs and cholesterol induces severe inflammation and steatohepatitis and, at cellular level, impairment of mitochondrial function and biogenesis. The pathogenic contribution of cholesterol depends on oxysterols production that are able to disrupt mitochondrial homeostasis, reduce mitochondrial function and energy production and impair the capacity of repair by inhibiting mitochondrial biogenesis. Oxysterols may represent a potential target of novel therapeutic strategy in NAFLD progression.

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Competing interests

the Authors declare no competing interests.

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.redox.2017.11.016.

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