

# The ins and outs of mitochondrial dysfunction in NASH

B Fromenty, MA Robin, A Igoudjil, A Mansouri, D Pessayre

## SUMMARY

Rich diet and lack of exercise are causing a surge in obesity, insulin resistance and steatosis, which can evolve into steatohepatitis. Steatosis and nonalcoholic steatohepatitis (NASH) can also be induced by drugs such as amiodarone, tamoxifen and some antiretroviral drugs. There is growing evidence that mitochondrial dysfunction, and more specifically respiratory chain deficiency, plays a role in the pathophysiology of NASH whatever its initial cause. In contrast, the  $\beta$ -oxidation of fatty acids can be either increased (as in insulin resistance-associated NASH) or decreased (as in drug-induced NASH). However, in both circumstances, the generation of reactive oxygen species (ROS) by the damaged respiratory chain is augmented, as components of this chain are over-reduced by electrons, which then abnormally react with oxygen to form increased amounts of ROS. Concomitantly, ROS oxidize fat deposits to release lipid peroxidation products that have detrimental effects on hepatocytes and other hepatic cells. In hepatocytes, ROS and lipid peroxidation products further impair the respiratory chain, either directly or indirectly through oxidative damage to the mitochondrial genome. This, in turn, leads to the generation of more ROS and a vicious cycle ensues. Mitochondrial dysfunction can also lead to apoptosis or necrosis depending on the energy status of the cell. ROS and lipid peroxidation products also activate stellate cells, thus resulting in fibrosis. Finally, ROS and lipid peroxidation increase the generation of several cytokines (TNF- $\alpha$ , TGF- $\beta$ , Fas ligand) that play sundry roles in the pathogenesis of NASH. Recent investigations have shown that some genetic polymorphisms can significantly increase the risk of steatohepatitis and that several drugs can prevent or even reverse NASH. For the next decade, reducing the incidence of NASH will be a major challenge for hepatologists.

**Key-words:** Steatosis · Steatohepatitis · Mitochondria · Reactive oxygen species · Lipid peroxidation · Cytokines.

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## RÉSUMÉ

### Les tenants et les aboutissants de la dysfonction mitochondriale au cours de la stéatohépatite non-alcoolique

Une alimentation trop riche et le manque d'exercice sont responsables d'une épidémie d'obésité, d'insulinorésistance et de stéatose, une lésion du foie pouvant évoluer en stéatohépatite. La stéatose et la stéatohépatite non-alcoolique (SHNA) peuvent être également induites par divers médicaments, tels que l'amiodarone, le tamoxifène et certains dérivés antirétroviraux. Il existe de plus en plus d'arguments expérimentaux et cliniques suggérant qu'un dysfonctionnement mitochondrial, et plus précisément un déficit de la chaîne respiratoire mitochondriale, joue un rôle physiopathologique important dans la SHNA, quelle qu'en soit sa cause. En revanche, l'oxydation mitochondriale des acides gras peut être soit augmentée (en cas de SHNA liées à une insulinorésistance), soit diminuée (dans le cas des SHNA induites par les médicaments). Cependant, dans les deux situations, il existe une augmentation de la production d'espèces réactives de l'oxygène (ERO) par la chaîne respiratoire endommagée. En effet, certains constituants de cette chaîne sont réduits en excès par les électrons qui réagissent alors anormalement avec l'oxygène pour former des ERO. Ces ERO peuvent alors oxyder les graisses accumulées dans l'hépatocyte, entraînant la génération de produits de la peroxydation lipidique qui ont des effets délétères sur les hépatocytes et les autres cellules du foie. Dans les hépatocytes, les ERO et les produits de la peroxydation lipidique endommagent de façon supplémentaire la chaîne respiratoire mitochondriale, soit directement, soit indirectement par l'intermédiaire de diverses altérations oxydatives du génome mitochondrial. Ceci a pour conséquence une production encore plus élevée d'ERO, ce qui enclenche un cercle vicieux. Le dysfonctionnement mitochondrial peut aussi entraîner une apoptose ou une nécrose, en fonction de l'état énergétique de la cellule. Les ERO et les produits de la peroxydation lipidique peuvent également activer les cellulaires étoilées du foie, favorisant la fibrose. Enfin, ces dérivés peuvent augmenter la production de plusieurs cytokines (TNF- $\alpha$ , TGF- $\beta$ , ligand de Fas) qui jouent des rôles divers dans la pathogenèse de la SHNA. Des études récentes ont montré que plusieurs polymorphismes génétiques peuvent augmenter de façon significative le risque d'apparition de la SHNA, et que certains médicaments sont susceptibles d'en limiter son évolution. Durant la prochaine décennie, la réduction de l'incidence de la SHNA sera un défi majeur pour les hépatologues.

**Mots-clés :** Stéatose · Stéatohépatite · Mitochondrie · Espèces réactive de l'oxygène · Peroxydation lipidique · Cytokines.

Address correspondence and reprint requests to:

B Fromenty, INSERM U481, Faculté de Médecine Xavier Bichat, 16, rue Henri Huchard, 75018 Paris, France.  
fromenty@bichat.inserm.fr

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Institut national de la Santé et de la Recherche médicale (INSERM) Unité 481, Faculté de Médecine Xavier Bichat, 750118 Paris, France.

**D**ue to a rich diet and lack of exercise, the incidence of obesity and insulin resistance is increasing rapidly in wealthy countries [1]. This thrifty trend is associated with a parallel surge in the prevalence of hepatic steatosis, which is characterized by the accumulation of fat droplets within the cytoplasm of hepatocytes [2-4]. In some patients, this hepatic steatosis remains isolated (without other liver lesions). In other patients, it triggers mild liver cell apoptosis and necrosis, a mild inflammatory cell infiltrate, and the slow development of hepatic fibrosis, which can progressively evolve into cirrhosis after several years or decades [2, 5]. The association of steatosis with these other liver lesions is called steatohepatitis [2, 5].

The term “nonalcoholic steatohepatitis” (NASH) is employed when steatohepatitis occurs in individuals whose alcohol consumption is nil or negligible (less than 20 g ethanol/day in women and less than 40 g in men) [6], while the term “nonalcoholic fatty liver disease” (NAFLD) is used to re-group cases of either steatosis or steatohepatitis in these patients.

The most frequent form of NASH is seen in patients with insulin resistance and diverse combinations of weight excess, hypertriglyceridemia and/or diabetes. This common form is sometimes termed “primary” NASH. With the increasing prevalence of obesity, NASH has become, by far, the most frequent cause of liver disease in the USA. In daily practice, therefore, the terms NASH or NAFLD are often implicitly employed to describe the particular form of liver disease that occurs in insulin-resistant patients.

However, insulin resistance is not the only cause of NASH. Steatohepatitis can also occur after the administration of certain drugs, or in patients with Wilson’s disease, total parenteral nutrition or a jejunio-ileal bypass [5]. In general, steatohepatitis tends to be more severe in patients with

these secondary forms of NASH or in patients with alcoholic steatohepatitis (ASH), than in patients with primary NASH [5].

Accumulating evidence suggests a major role of mitochondrial dysfunction in steatosis and steatohepatitis whatever their etiologies. Mitochondrial dysfunction not only impairs fat homeostasis in the liver but also leads to overproduction of reactive oxygen species (ROS) that trigger lipid peroxidation, cytokine overproduction and cell death.

In this review, we first quickly recall the role of mitochondria in fat metabolism and energy production. We then briefly discuss the mitochondrial alterations involved in drug-induced steatohepatitis. Subsequently, we review more extensively the metabolic and mitochondrial abnormalities of insulin-resistance associated NAFLD. Finally, we address the mechanisms whereby mitochondrial dysfunction can increase ROS generation and lipid peroxidation and how these effects can participate to cell death, inflammation and fibrosis.

## Normal role of mitochondria in hepatic fat metabolism and energy production

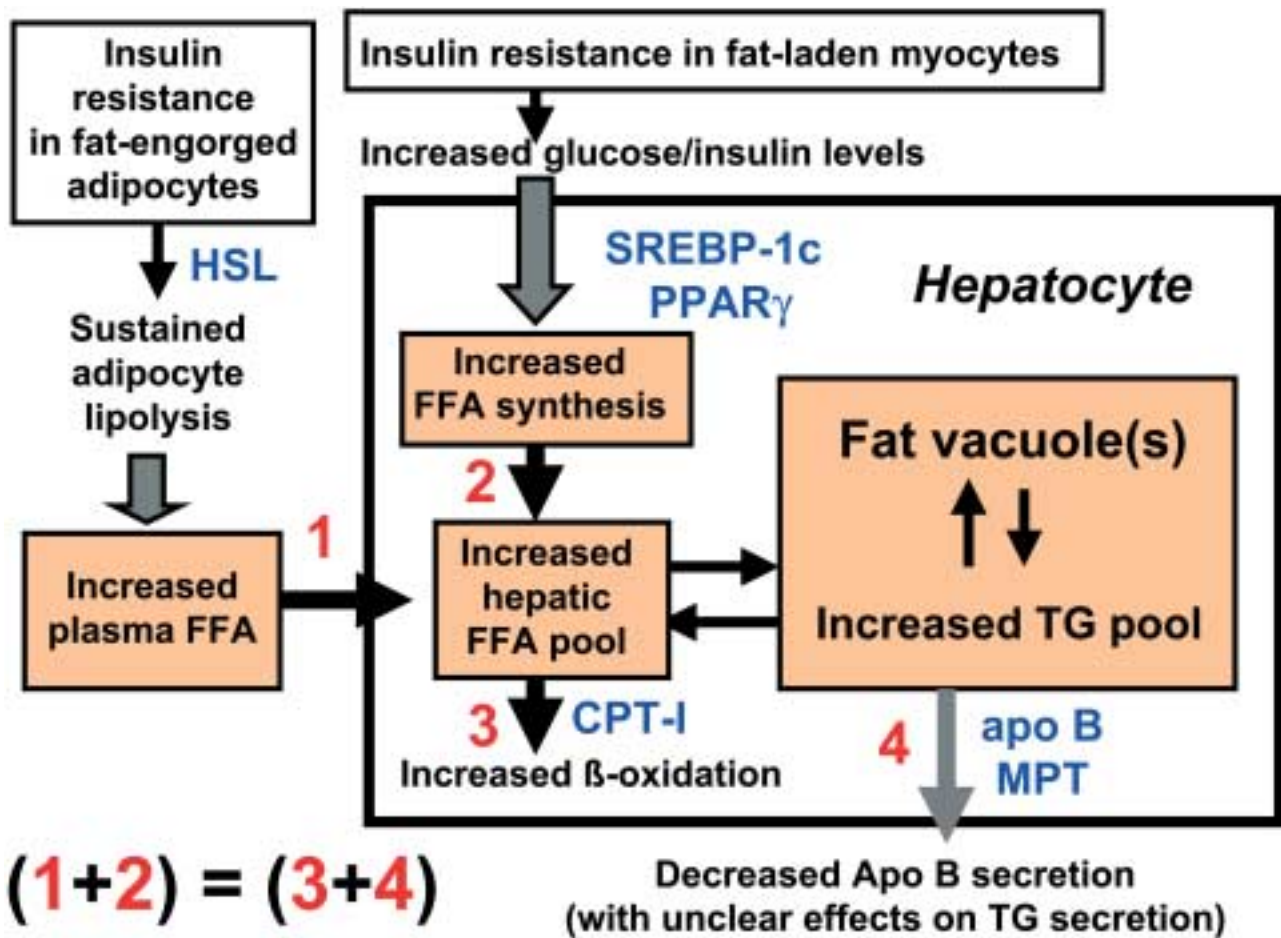
### Hepatic fat metabolism

Hepatic free fatty acids (FFAs) can have different sources (*Fig 1*). They can be taken up by the liver from the plasma FFAs that are released by adipose tissue. They can be generated in the liver from the hydrolysis of chylomicrons coming from the intestine. Finally, they can be directly synthesized *de novo* within the hepatocytes [5]. Depending on the nutritional/hormonal status, hepatic FFAs either enter the mitochondria to undergo mitochondrial  $\beta$ -oxidation, or are esterified into triglycerides (a storage form of 3 FFA molecules bound to glycerol by ester bonds).

Hepatic triglycerides, in turn, either accumulate within the cytoplasm of hepatocytes as fat droplets (probably surrounded by a layer of phospholipids and proteins such as the adipose differentiation-related protein), or are secreted as very low density lipoproteins (VLDL) [7, 8]. Plasma VLDL particles consist in a droplet of triglycerides (and cholesterol esters) surrounded by phospholipids and a large protein termed apolipoprotein B (apo B). Apo B is cotranslationally lipidated in the endoplasmic reticulum lumen by microsomal triglyceride transfer protein (MTP) and is further lipidated in the Golgi apparatus [9]. The extent of lipidation directs the fate of apo B molecules. Fully lipidated apo B molecules quickly follow vesicular flow, to be secreted into the plasma. In contrast, incompletely lipidated apo B molecules fail to completely translocate into the endoplasmic reticulum lumen and/or undergo retrotranslocation to the cytosol, to be ubiquitinated, and to be finally digested by the proteasome [10].

#### Abbreviations

<b>Apo B :</b>	apolipoprotein B
<b>ASH :</b>	alcoholic steatohepatitis
<b>AZT :</b>	zidovudine
<b>Bid :</b>	BH3 interacting domain death agonist
<b>CPT-I :</b>	carnitine palmitoyltransferase I
<b>CYP :</b>	cytochrome P450
<b>DdI :</b>	didanosine
<b>d4T :</b>	stavudine
<b>FFA :</b>	free fatty acid
<b>4-HNE :</b>	4-hydroxynonenal
<b>IL-8 :</b>	interleukin-8
<b>MDA :</b>	malondialdehyde
<b>MnSOD :</b>	manganese superoxide dismutase
<b>MtDNA :</b>	mitochondrial DNA
<b>MTP :</b>	microsomal triglyceride transfer protein
<b>NASH :</b>	non-alcoholic steatohepatitis
<b>8-OH-dG :</b>	8-hydroxydeoxyguanosine
<b>ROS :</b>	reactive oxygen species
<b>TNF-<math>\alpha</math> :</b>	tumor necrosis factor- $\alpha$
<b>TGF-<math>\beta</math> :</b>	transforming growth factor- $\beta$
<b>UCP2 :</b>	uncoupling protein 2
<b>VLDL :</b>	very low density lipoprotein



**Figure 1**

Mechanisms of fat accretion in the liver during insulin resistance-associated NASH. Insulin resistance in fat-engorged adipocytes hampers the inhibitory action of insulin on hormone-sensitive lipase (HSL), thus increasing the hydrolysis of triglycerides (TG) and the release of free fatty acids (FFA) from adipose tissue. Increased plasma FFA levels increase the hepatic uptake of FFA (arrow 1). Concomitantly, insulin resistance in fat-laden myocytes can increase glucose and/or insulin levels, which may increase hepatic fatty acid synthesis (arrow 2) through the up-regulation of sterol regulatory element-binding protein 1c (SREBP-1c) and peroxisome proliferator-activated receptor  $\gamma$  (PPAR $\gamma$ ). Increased plasma FFA uptake and increased hepatic FFA synthesis cause the expansion of the FFA pool, which is in equilibrium with an expanded pool of triglycerides. Because fat cannot accrue indefinitely within hepatocytes, a new steady state is reached, whereby increased input pathways are finally compensated for by increased output pathways. A major compensatory pathway is the enhancement of mitochondrial FFA  $\beta$ -oxidation (arrow 3) due the increased resistance of carnitine palmitoyltransferase I (CPT-I) to the inhibitory effects of its endogenous inhibitor, malonyl-CoA. VLDL secretion involves the intrahepatic lipidation of apolipoprotein B (apo B) by microsomal triglyceride transfer protein (MTP). Although apo B secretion seems to be reduced during insulin resistance, it is unclear yet whether this also affects hepatic TG secretion.

### Mitochondrial fatty acid oxidation

The entry of long-chain FFAs into the mitochondria is critically dependent on carnitine palmitoyl transferase I (CPT-I), an outer membrane enzyme whose activity is inhibited by malonyl-CoA [11]. Malonyl-CoA is formed by acetyl-CoA carboxylase and is the first step in the synthesis of fatty acids from acetyl-CoA [11]. Unlike long-chain FFAs, short-chain and medium-chain FFAs freely enter the mitochondria, without requiring CPT-I and other proteins involved in the carnitine shuttle [12].

After a carbohydrate meal, high glucose and insulin levels cause brisk hepatic fatty acid synthesis [11, 13]. Indeed, glycolysis generates pyruvate, which is transformed by mito-

chondria into acetyl-CoA and then citrate, which leaves the organelle through the tricarboxylate carrier. In the cytosol, citrate regenerates acetyl-CoA, which is used for malonyl-CoA and then fatty acid synthesis. High malonyl-CoA levels inhibit CPT-I and decrease fatty acid  $\beta$ -oxidation [11]. Therefore, FFAs are not degraded, but are instead directed towards the formation of triglycerides, which are secreted as VLDL [11].

In contrast, in the fasting state, low insulin levels favor the release of FFAs by adipose tissue and their hepatic  $\beta$ -oxidation into ketone bodies [13]. Indeed, during fasting, hepatic malonyl-CoA levels are low, allowing extensive mitochondrial import of long-chain FFAs and thus active

$\beta$ -oxidation. During their  $\beta$ -oxidation in mitochondria, free fatty acids undergo a first dehydrogenation, then hydration, then a second dehydrogenation, and finally thiolysis, releasing one acetyl-CoA molecule and a shortened fatty acid. The cycle is repeated to split the fatty acid into acetyl-CoA subunits. Under fasting conditions, acetyl-CoA moieties mostly condense into ketone bodies (acetoacetate and  $\beta$ -hydroxybutyrate). Ketone bodies are then secreted by the liver to be oxidized in muscles and other peripheral tissues by the tricarboxylic acid cycle [11, 12].

### Production of energy and ROS by the respiratory chain

FFA oxidation in mitochondria, and the oxidative degradation of other fuels in mitochondria and elsewhere are associated with conversion of oxidized cofactors ( $\text{NAD}^+$  and FAD) into reduced cofactors (NADH and  $\text{FADH}_2$ ) [12]. These reduced cofactors are then re-oxidized by the mitochondrial respiratory chain, which is embedded in the mitochondrial inner membrane. This re-oxidation regenerates the  $\text{NAD}^+$  and FAD necessary for other cycles of fuel oxidation [12].

During their oxidation, NADH and  $\text{FADH}_2$  transfer their electrons to the first complexes of the respiratory chain. Electrons then migrate along the respiratory chain, and this flow of electrons is coupled with the extrusion of protons from the mitochondrial matrix into the mitochondrial intermembrane space. This extrusion creates a large electrochemical potential across the inner membrane, thus creating a reservoir of latent, potential energy.

When energy is needed, protons re-enter the matrix through the  $F_0$  portion of ATP synthase, causing the rotation of a molecular rotor in the  $F_1$  portion of ATP synthase and the conversion of ADP into ATP. The adenine nucleotide translocator (ANT) then extrudes ATP from mitochondria, in exchange for cytosolic ADP [5]. Cytosolic ATP is then used to power all the cell processes that require energy. The whole biochemical process which couples the oxidation of reduced cofactors (NADH and  $\text{FADH}_2$ ) to the phosphorylation of ADP into ATP is referred to as oxidative phosphorylation (OXPHOS).

Most of the electrons, which are donated to the respiratory chain, migrate all the way along the respiratory chain, to finally reach cytochrome *c* oxidase, where they safely combine with oxygen and protons to form water [5, 12]. However, at several upstream sites of the respiratory chain, a fraction of these electrons can directly react with oxygen, to form the superoxide anion radical. This radical is then dismutated by mitochondrial manganese superoxide dismutase (MnSOD) into hydrogen peroxide ( $\text{H}_2\text{O}_2$ ), which is detoxified into water by mitochondrial glutathione peroxidase [5].

ROS are thus continuously generated by the respiratory chain in healthy mitochondria. Fortunately, in the physiological state, most mitochondrial ROS are detoxified into

water and only a small amount of residual ROS persists. It is currently unknown whether residual ROS have physiological roles, but recent data suggest that  $\text{H}_2\text{O}_2$  is able to function as a signaling molecule, allowing communication between the mitochondria and the cytosol [14]. In contrast to healthy organelles, damaged mitochondria produce larger amount of ROS that can damage mitochondria and other cellular components as described further on (Fig 2).

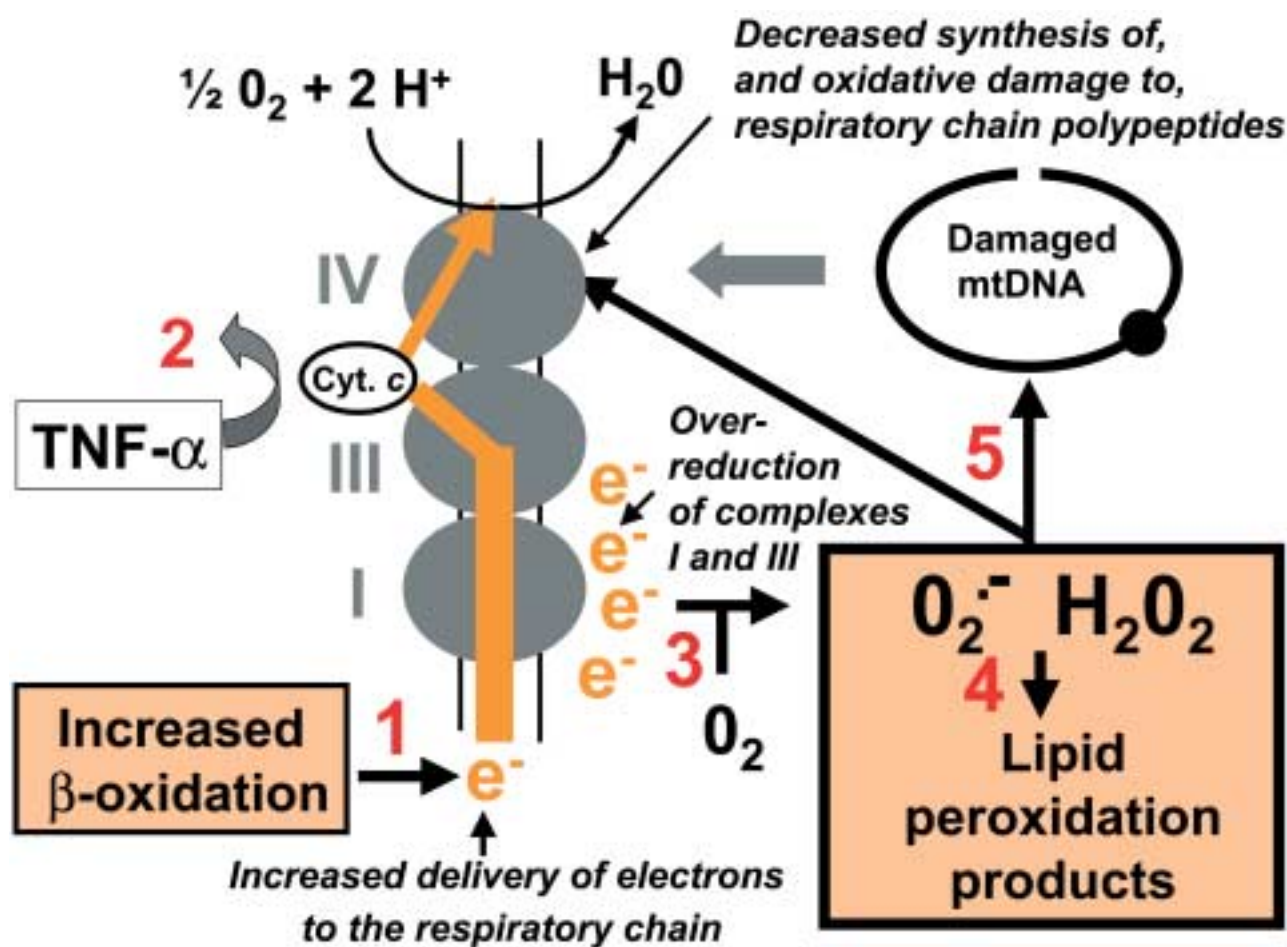
### Mitochondrial genome

Although most respiratory chain polypeptides are encoded by nuclear DNA, 13 respiratory chain polypeptides are encoded by mitochondrial DNA (mtDNA), which also encodes the two mitochondrial ribosomal RNAs and all the mitochondrial transfer RNAs that are necessary for the synthesis of mtDNA-encoded polypeptides in the mitochondrial matrix.

mtDNA is a circular, double stranded molecule, located in the mitochondrial matrix. Each cell contains many copies of this mitochondrial genome, as there are several mtDNA copies in a single mitochondrion and many mitochondria per cell [12, 15]. mtDNA is extremely sensitive to oxidative damage due to its proximity to the inner membrane (the main cellular source of ROS), the absence of protective histones and incomplete repair mechanisms in mitochondria [12, 16]. Although mitochondria possess a base excision repair (BER) system, the nucleotide excision repair (NER) pathway is absent, or not fully operational, in mitochondria [17]. The presence of a BER system allows mitochondria to efficiently repair oxidized DNA bases (such as 8-hydroxydeoxyguanosine) and abasic sites (i.e., DNA sites lacking a purine or pyrimidine base). In contrast, lack of an efficient NER system impairs the repair of bulky mtDNA adducts [17]. To rid themselves of such lesions, mitochondria must instead resort to nuclease-mediated degradation of heavily damaged mtDNA molecules, a process which may cause mtDNA depletion [17, 18].

### Drug-induced steatohepatitis

Several drugs can induce steatosis and steatohepatitis in patients who do not drink alcohol. These drugs include the anti-arrhythmic and anti-anginal drugs, diethylaminoethoxyhexestrol, perhexiline and amiodarone [12, 19, 20], the anti-estrogen and anti-neoplastic drug, tamoxifen [20, 21], and some antiretroviral nucleoside analogs, such as stavudine (d4T), zidovudine (AZT) and didanosine (ddI) [22-24]. Although these drugs initially impair mitochondrial function through quite different mechanisms, they all eventually impair mitochondrial  $\beta$ -oxidation, as explained further on. Although mitochondrial dysfunction is thought to play a key role in the pathogenesis of drug-induced steatosis and steatohepatitis [5, 12, 20], other mechanisms can be involved with some drugs, such as inhibition of MTP activity [25] or hyper-



**Figure 2**

Electron overproduction,  $TNF-\alpha$ , reactive oxygen species (ROS), lipid peroxidation and impairment of the flow of electrons in the respiratory chain. In the normal liver, the electrons, which are given to the respiratory chain, flow along this chain, up to complex IV (cytochrome *c* oxidase), where they safely combine with oxygen and protons to form water. 1. However, in the fatty liver of insulin-resistant patients, high  $\beta$ -oxidation rates increase the delivery of electrons to the respiratory chain. 2. The adipocytes of obese persons release large amounts of  $TNF-\alpha$ , which acts on its hepatocyte receptor to permeabilize mitochondria, and partially release cytochrome *c* from the mitochondrial intermembrane space. 3. The imbalance between a high input and a restricted flow of electrons causes over-reduction of complexes I and III of the respiratory chain. These overly reduced complexes react with oxygen to form the superoxide anion radical, which is dismutated into  $H_2O_2$  by MnSOD. 4. ROS oxidize the unsaturated lipids of fat deposits to trigger lipid peroxidation. 5. Both ROS and reactive lipid peroxidation products directly damage respiratory chain polypeptides and also damage mtDNA. These effects may both decrease the synthesis of, and cause damage to, respiratory chain polypeptides. This further impairs the flow of electrons in the respiratory chain, and further increases mitochondrial ROS production, thus generating a vicious cycle. Adapted from a previous figure [4].

triglyceridemia [20]. Finally, ethanol-induced mitochondrial dysfunction is a major cause of fatty liver [3, 12, 26], but the effects of alcohol are briefly mentioned here, since this review mainly focuses on NASH.

### Inhibition of mtDNA replication

Anti-HIV nucleoside reverse transcriptase inhibitors (NRTIs) such as AZT, d4T or ddI can be incorporated into the growing strand of replicating mtDNA by polymerase  $\gamma$ , the only DNA polymerase involved in mtDNA replication. However, these dideoxynucleoside analogs do not have a 3'-hydroxyl end on their sugar moiety. Once a single molecule of the analog has been incorporated, the DNA lacks a

3'-OH group, and polymerase  $\gamma$  cannot add any other nucleotide into the nascent DNA strand. This blocks mtDNA replication and can cause mtDNA depletion [12, 27, 28]. In mice treated for 2 weeks with five different analogs, we found that NRTI-induced mtDNA depletion was more frequently observed in the liver than in other tissues (brain, muscles, heart and white adipose tissue) [29]. Along with clinical data [12], these results suggest that hepatic mtDNA could be a prime target of NRTI toxicity.

Severe mtDNA depletion can decrease the synthesis of the mtDNA-encoded polypeptides and impair the respiratory chain. This, in turn, can hamper the re-oxidation of NADH and  $FADH_2$  into  $NAD^+$  and FAD, thus leading to

the inhibition of mitochondrial  $\beta$ -oxidation and the tricarboxylic acid cycle, which both require  $\text{NAD}^+$  and FAD. However, it is noteworthy that recent data from our laboratory suggest that some NRTIs may decrease the flux of fatty acid oxidation in the liver by mechanism(s) not involving OXPHOS impairment as discussed further on.

### Oxidative damage to mtDNA

In addition to mtDNA depletion, NRTIs can induce oxidative damage to mtDNA. For instance, AZT administration to mice causes the accumulation of the oxidized base, 8-hydroxydeoxyguanosine (8-OH-dG) in muscle and liver mtDNA [30, 31]. AZT administration also increases the urinary excretion of 8-OH-dG in humans [31]. Increased levels of 8-OH-dG suggest the presence of several other oxidative DNA lesions [32]. Interestingly, unrepaired oxidative bases can be misread by DNA polymerase  $\gamma$  during the process of mtDNA replication, thus leading to the introduction of point mutations [33]. This may explain, at least in part, the accumulation of diverse heteroplasmic mtDNA point mutations observed in the peripheral blood cells of patients treated with NRTIs [34].

mtDNA deletions have also been reported in several patients receiving NRTIs [35]. Interestingly, mtDNA deletions could also result from the harmful effect of oxidative stress on mtDNA. Indeed, the presence of some oxidative lesions and strand breaks in mtDNA seems to favor "slipped mispairing" of repeated and remote sequences during replication that eventually leads to the loss of thousands of base pairs [26, 36]. Thus, oxidized bases, mtDNA point mutations and deletions may all have a common origin, namely oxidative damage to mtDNA. Finally, oxidative lesions and mtDNA sequence changes might explain why steatosis and lactic acidosis can occur in some patients despite unchanged mtDNA levels.

### Inhibition of respiratory chain and OXPHOS impairment

Beside their effects on mtDNA abundance and sequence, some NRTIs seem to have other metabolic and mitochondrial effects [28, 37]. For instance, relatively low concentrations of AZT have been shown to inhibit mitochondrial respiration in the presence of ADP [38], an effect which could be related to the inhibition of ADP/ATP translocase (also called adenine nucleotide translocator, ANT), a key OXPHOS enzyme allowing ADP entry within mitochondria in exchange for ATP [39]. Whether other NRTIs also present such inhibitory effects on OXPHOS is currently unknown.

The cationic amphiphilic drugs amiodarone and perhexiline have dual effects on mitochondrial respiration *in vitro*, with a transient uncoupling effect (which increases respiration) followed by inhibition of electron transfer at the level of complexes I and II of the respiratory chain [40, 41]. Amiodarone and perhexiline are protonated in the acidic inter-

membrane space to be electrophoretically transported in the mitochondrial matrix, where they release one proton in the more alkaline matrix. This re-entry of protons decreases the mitochondrial membrane potential, and unleashes the flow of electrons in the respiratory chain to increase respiration, while the swift accumulation of these drugs within mitochondria inhibits both the respiratory chain and  $\beta$ -oxidation [40-43]. These mitochondrial effects are responsible for a marked impairment of fatty acid oxidation and decreased ATP formation [40, 42, 44]. Interestingly, tamoxifen is, like amiodarone, a cationic amphiphilic drug, which can also act as an OXPHOS uncoupler and an inhibitor of the respiratory chain [45, 46].

### Inhibition of mitochondrial $\beta$ -oxidation

NRTI-induced mtDNA depletion and respiratory chain impairment can lead to an inhibition of mitochondrial  $\beta$ -oxidation of fatty acids as discussed above. However, ongoing experiments in our laboratory suggest that some NRTIs can impair hepatic mitochondrial  $\beta$ -oxidation without any inhibition of the respiratory chain. Indeed, high doses of d4T in mice decreased the hepatic expression of PPAR- $\alpha$  and decreased fatty acid oxidation *in vivo*, as shown by decreased plasma ketone bodies and by decreased [ $^{14}\text{C}$ ]CO $_2$  exhalation after administration of labeled palmitic acid, whereas the activity of complexes I and IV of the hepatic respiratory chain were unaffected [47]. Because the nuclear receptor PPAR- $\alpha$  is known to increase the expression of several enzymes involved in fatty acid oxidation in mitochondria and peroxisomes [48], decreased hepatic PPAR- $\alpha$  expression in the setting of NRTI therapy may play a significant role in the development of steatosis. Interestingly, treatment with L-carnitine prevented all the metabolic abnormalities observed in d4T-treated mice [47]. It is noteworthy that some NRTIs seem to interfere with L-carnitine metabolism. It has been shown recently that AZT is a non-competitive inhibitor of L-carnitine transport in myoblastic cells [49]. Ongoing experiments in our laboratory suggest that AZT can decrease L-carnitine levels in mouse liver, while other investigations suggest that AZT-induced L-carnitine depletion can also occur in muscle [50].

Amiodarone and perhexiline were shown to inhibit mitochondrial  $\beta$ -oxidation *in vitro* independently of their inhibitory effect on the mitochondrial respiratory chain [40, 42]. However, the exact mechanism whereby these drugs inhibit  $\beta$ -oxidation enzymes has not been determined.

## Insulin resistance-associated NAFLD

### Insulin resistance

After a meal in lean persons, a mild increase in glucose causes a mild increase in the release of insulin by pancreatic  $\beta$ -cells. Insulin acts on its receptor on the surface of adipocytes and myocytes to trigger the phosphorylation of insulin

receptor substrates (IRS), which activate phosphatidylinositol 3-kinase and Akt/protein kinase B, to eventually cause the translocation of GLUT-4 glucose transporters from intracellular storage vesicles to the plasma membrane of adipocytes and myocytes [51]. Abundant expression of GLUT-4 transporter on the plasma membrane of myocytes and adipocytes causes efficient glucose uptake, which limits the increase in glucose and insulin.

In obese people, however, adipocytes may produce less GLUT-4 transporter [51]. More importantly, both fat-engorged adipocytes and fat-laden myocytes are resistant to the signaling effects of the insulin receptor [51]. It is suggested that acyl-CoA or other derivatives of FFAs may limit the activation of IRS and phosphatidylinositol 3-kinase [51]. The mechanism could involve the activation of Jun N-terminal kinase and, hence, the serine phosphorylation and thus inactivation of IRS [52]. Whatever the mechanism, insufficient translocation of GLUT 4 transporters to the plasma membrane limits glucose uptake by adipocytes and myocytes [51]. This insufficient uptake tends to increase blood glucose, thus causing a compensatory increase in the release of insulin by pancreatic  $\beta$  cells [53]. In some subjects, however, this compensatory insulin increase is not enough, or secondarily fails, and frank diabetes develops. Therefore, insulin resistance in adipocytes and muscles tends to increase glucose and/or insulin levels.

### Insulin resistance and hepatic steatosis

Both insulin resistance and high levels of insulin are thought to play a key role in the pathogenesis of hepatic steatosis in patients with various combinations of weight excess, dyslipidemia and/or type 2 diabetes. As explained above, excessive accumulation of fat in adipocytes and muscles causes insulin resistance in these organs, and contributes to fat accretion in the liver, as explained further on. The presence of fat in the liver in turn causes insulin resistance in this organ as well. However, in the liver, insulin resistance may only affect some, but not all, insulin-sensitive metabolic pathways [6]. Thus, paradoxically, some hepatic metabolic pathways (e.g., fatty acid synthesis) might be over-activated as a consequence of high insulin levels, while other metabolic pathways (e.g., fatty acid oxidation or gluconeogenesis) might be instead over-activated as a consequence of hepatic insulin resistance [54].

### Mechanisms for hepatic steatosis

Although the mechanisms for fatty liver in primary NASH are not yet fully elucidated, they seem to involve an increased delivery of FFAs to the liver, and an increased hepatic fatty acid synthesis (*Fig 1*). In addition, a decreased hepatic apo B secretion has been observed in NASH, although it is not clear whether hepatic triglyceride secretion is increased, or can be sometimes decreased, in these patients.

Increased fatty acid delivery to the liver plays a key role in the development of steatosis in patients with type 2 diabetes (*Fig 1*). Normally, insulin blocks adipose tissue lipolysis due to the inhibition of hormone-sensitive lipase (HSL) [13]. HSL hydrolyzes triacylglycerol into 2 molecules of fatty acids and monoacylglycerol, which is then hydrolyzed by another lipase to glycerol and one fatty acid [13]. Normally, the adipocytes of lean, insulin-sensitive people, store fat after meals and then release fat during fasting. In contrast, the fat-engorged, insulin-resistant adipocytes of obese persons keep releasing large amounts of glycerol and fatty acids in the circulation, a situation that normally occurs only during the fasting state [55]. Because fatty acids are readily taken up by the hepatocytes, insulin resistance greatly increases the amount of fatty acids delivered to the liver.

Not only are FFAs taken up in larger quantities from the plasma, but they are also synthesized more actively in the liver (*Fig 1*). Indeed, increased levels of insulin and glucose tend to increase fatty acid and triacylglycerol synthesis. It is likely that this effect is, at least in part, due to increased levels of sterol regulatory element-binding protein 1c (SREBP-1c) and peroxisome proliferator-activated receptor  $\gamma$  (PPAR  $\gamma$ ), two transcriptional factors which activate the expression of key enzymes involved in lipogenesis [56, 57]. Indeed, SREBP-1c and PPAR  $\gamma$  increase the expression of citrate lyase, acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS), which are all involved in fatty acid synthesis, and also increase the expression of glycerol-3-phosphate acyltransferase (GPAT) and stearoyl-CoA desaturase 1 (SCD1), which promote triacylglycerol formation [56]. Studies in genetically obese *ob/ob* mice have shown a 4-fold increase in the amount of transcriptionally active SREBP-1c and a 6-fold increase in *de novo* fatty acid synthesis in the liver, thus suggesting that enhanced lipogenesis is a key event leading to massive steatosis in these mice [56].

NASH also seems to be characterized, at least in some individuals, by decreased egress of apo B from the liver (*Fig 1*). Indeed, recent evidence suggests that hepatic apolipoprotein B secretion into the plasma can be disturbed in primary NASH [58, 59]. Interestingly, decreased hepatic apo B mRNA has been shown in several patients with NASH [60], suggesting that lower apo B synthesis in NASH could be due, at least in part, to decreased transcription and/or decreased apo B mRNA stability. It is possible that hyperinsulinemia could play a role, as insulin lowers apo B synthesis and stability [58, 61]. Importantly, decreased hepatic apo B synthesis and/or secretion in NASH can be associated with either reduced or increased plasma apo B concentration and high levels of plasma triglycerides [58, 59]. Thus, lipoprotein metabolism is affected in a complex way in the liver but also in extra-hepatic tissues.

Polymorphisms or mutations affecting either apo B or MTP could increase the risk of NASH, as discussed further on. Finally, it is noteworthy that MTP activity can be im-

paired by the hepatitis C virus core protein [62], certain drugs [25] and ethanol [63], which may further increase hepatic lipid deposition in insulin-resistant patients with these added causes of steatosis.

### Compensatory increase in mitochondrial $\beta$ -oxidation and ketogenesis

In patients with primary NASH, the liver is laden with fat, but does not enlarge indefinitely. Obviously, a new steady state must be achieved, whereby the increased hepatic uptake and increased hepatic synthesis of FFAs are now compensated by an increased hepatic removal of fatty acids. This is achieved by increased mitochondrial  $\beta$ -oxidation of fatty acids and increased ketogenesis in patients with NASH [64, 65] (*Fig 1*). Increased mitochondrial  $\beta$ -oxidation was also found in the liver of genetically obese-diabetic (ob/ob) mice that also present massive steatosis [66, 67].

The mechanisms responsible for the increased mitochondrial  $\beta$ -oxidation in insulin resistance-associated NASH are poorly understood. A first mechanism may be the increased pool size of FFAs, due to the increased hepatic uptake of FFAs and increased FFA synthesis in the liver.

A second mechanism may be enhanced CPT-I activity, thus further increasing the entry of long-chain fatty acids into mitochondria (*Fig 1*). Indeed, CPT-I (and probably CPT-II) is enhanced in rodent models of either type 1 or type 2 diabetes, and, furthermore, the affinity of CPT-I for its physiological inhibitor, malonyl-CoA is profoundly decreased [66, 68]. The loss of CPT-I inhibition by malonyl-CoA may explain why  $\beta$ -oxidation is increased in type 2 diabetic states, despite high insulin and malonyl-CoA levels. The nuclear receptor PPAR- $\alpha$  regulates the expression of many enzymes involved in fat catabolism, such as mitochondrial and peroxisomal  $\beta$ -oxidation enzymes [69]. Hypothetically, hepatic PPAR- $\alpha$  activation could be responsible, at least in part, for the increased expression and/or activity of CPT-I and the uncoupling protein-2 (UCP-2) in fatty livers, since both proteins are PPAR- $\alpha$  targets [48, 70]. Apart from data on CPT-I, however, little is known about other enzymes involved in  $\beta$ -oxidation and ketogenesis in NASH. It is possible that the synthesis of acetoacetate from acetyl-CoA by the mitochondrial HMG-CoA synthase (i.e. ketogenesis *per se*) may be increased in NASH [71], although direct evidence is lacking. The mRNAs of several enzymes involved in fatty acid oxidation were found to be decreased in patients with cirrhotic NASH [60], although the functional significance of this observation is unclear.

Another mechanism increasing fatty acid oxidation in NASH may involve peroxisomes. Indeed, proliferation and enlargement of hepatic peroxisomes have been observed in patients with fatty liver [72, 73].

A last mechanism permitting increased mitochondrial  $\beta$ -oxidation rates may involve several adaptations allowing sufficient re-oxidation of oxidized co-factors despite the dys-

functional respiratory chain. As discussed further on, the respiratory chain is deficient in patient with NASH. Severe impairment of the respiratory chain can hamper the re-oxidation of NADH and FADH<sub>2</sub> into NAD<sup>+</sup> and FAD, respectively. Both the mitochondrial  $\beta$ -oxidation cycle and the mitochondrial tricarboxylic acid cycle rely on several dehydrogenases, whose activity requires the presence of NAD<sup>+</sup> and FAD. Therefore, severe impairment of the respiratory chain can secondarily inhibit fatty acid oxidation [12, 40]. It may thus seem paradoxical that the respiratory chain and OXPHOS are dysfunctional in NASH and yet, the  $\beta$ -oxidation of fatty acids is augmented. Although the reasons for this apparent paradox are unknown, several hypotheses can be put forward. Firstly, the impairment of the respiratory chain may be mild enough to maintain a pool of oxidized factors, which is sufficient to allow fatty acid oxidation. Secondly, because ketone body production is increased in NASH [64, 65], significant NADH re-oxidation into NAD<sup>+</sup> could occur during the reduction of acetoacetate into  $\beta$ -hydroxybutyrate by D- $\beta$ -hydroxybutyrate dehydrogenase. Indeed, it has been shown that the inhibition of fatty acid oxidation caused by rotenone (a specific and potent inhibitor of complex I of the respiratory chain) in isolated mouse liver mitochondria can be relieved by the addition of acetoacetate, which consumes NADH and thus regenerates the NAD<sup>+</sup> required for  $\beta$ -oxidation [40]. It is noteworthy that insulin and hyperlipidemic states enhance D- $\beta$ -hydroxybutyrate dehydrogenase expression and activity in rat liver [74]. Accordingly, increased conversion of acetoacetate to  $\beta$ -hydroxybutyrate in NASH could be one mechanism sustaining fatty acid oxidation despite the dysfunctional respiratory chain. Finally, the increased expression of UCP-2 may increase the re-oxidation of NADH and FADH<sub>2</sub> into NAD<sup>+</sup> and FAD, as discussed in the next paragraph.

### Increased hepatic expression of UCP2 in animal models of NAFLD

Increased hepatic expression of UCP2 has been demonstrated in genetically obese, ob/ob mice [75]. UCPs are mitochondrial inner-membrane proteins, whose main function is to mediate proton leak across the inner membrane and thus to uncouple substrate oxidation from ATP synthesis. Thus, increased expression of liver UCP2 in NASH may compromise ATP synthesis and increase the vulnerability of the fatty liver to some types of injury [76]. On the other hand, by increasing respiration, UCP2 may increase the re-oxidation of NADH into NAD<sup>+</sup>, which is required for both  $\beta$ -oxidation and the tricarboxylic acid cycle [77, 78]. Accordingly, increased UCP2 expression may be a way to increase the hepatic oxidation of fatty acids and limit steatosis [78]. Another beneficial effect of UCP2 might be to unleash the flow of electrons in the respiratory chain, thereby preventing the over-reduction of respiratory complexes and excessive mito-

chondrial ROS formation [79]. Thus the increased expression of UCP2 has both detrimental and benefic effects in fatty liver. Possibly due to these mitigated effects, lack of UCP2 expression in the liver of ob/ob mice did not seem to significantly affect the severity of fatty liver disease in these animals [80].

### Mitochondrial dysfunction in patients with NAFLD

Obese or diabetic patients with severe hepatic steatosis have reduced exhalation of [ $^{13}\text{C}$ ]CO $_2$  after  $^{13}\text{C}$ -methionine administration, compared to healthy subjects [81]. The mitochondrial degradation of methionine involves both decarboxylation and dehydrogenation steps, which generate reducing equivalents that feed the respiratory chain [82]. Although it is not yet clear which of these reactions are impaired in patients with NAFLD, this study suggests that mitochondrial function can be impaired in patients with severe steatosis, but without steatohepatitis.

In patients with NASH, ultrastructural abnormalities of liver mitochondria, with para-cristalline inclusions in megamitochondria, have been demonstrated [64, 83]. Although it is not known what are the biochemical changes which trigger these morphological lesions, these observations again suggest mitochondrial damage.

Indeed, a recent abstract indicates that mtDNA is severely depleted in patients with NASH [84]. In other conditions, profound mtDNA depletion can severely affect mitochondrial function [12, 18] and induce steatosis and other liver lesions [85-87]. Interestingly, decreased expression of several mtDNA-encoded polypeptides [84, 88] and lower activity of complexes I, III, IV and V [89] have been found in patients with NASH, thus suggesting that mtDNA depletion could play a role in the mitochondrial dysfunction of NASH. However, the activity of complex II (that does not include mtDNA-encoded polypeptides) was also decreased in NASH [89], thus indicating that other mechanisms, such as oxidative stress, can directly damage respiratory chain polypeptides and contribute to the impaired activity of respiratory complexes [90]. Oxidative stress in type 1 and 2 diabetes could be due to several concomitant factors, including glucose autoxidation, the glycation and inhibition of some antioxidant enzymes, and, lastly, mitochondrial dysfunction [91, 92].

### Mitochondria as a source of ROS in NASH

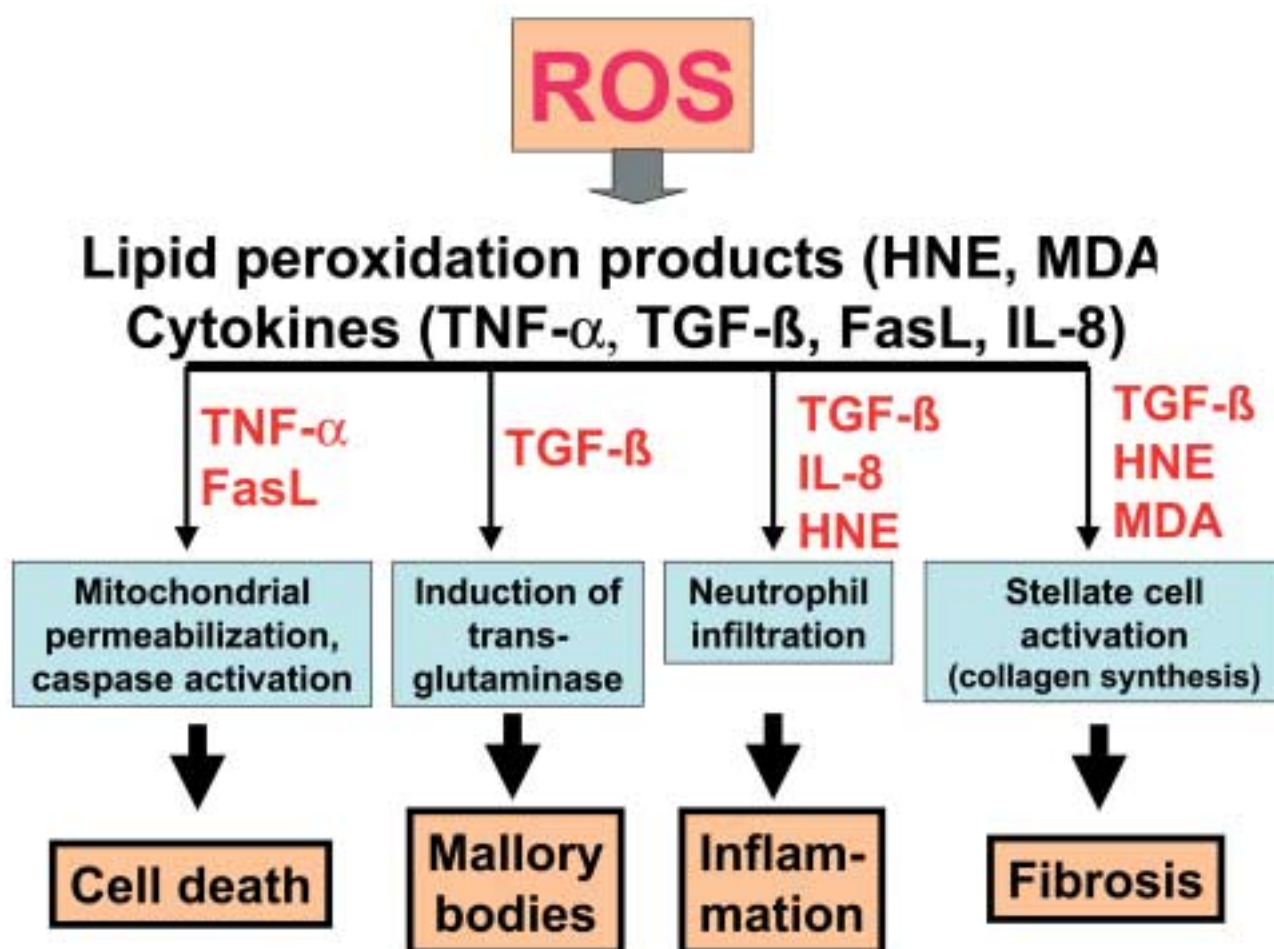
There is now some evidence that liver mitochondria can be a significant source of ROS in NASH. Although different studies point to the damaged respiratory chain as a main source of mitochondrial ROS in NASH (*Fig 2*), recent data suggest that ectopic expression of cytochrome P450 2E1 (CYP2E1) in mitochondria could also play a role in ROS overproduction.

### ROS overproduction by the damaged respiratory chain

As indicated in the previous sections, impairment of the respiratory chain appears to be a common feature in both drug-induced and primary NASH, although this is associated with decreased fatty acid oxidation in drug-induced NASH, but increased oxidation in insulin resistance-associated NASH. Several studies have consistently found that hampering the flow of electrons within the respiratory chain causes over-reduction of some respiratory chain components, which then react abnormally with oxygen to form the superoxide anion radical, thus increasing mitochondrial ROS formation [4] (*Fig 2*). Complexes I and III of the respiratory chain have been found repeatedly to be involved in ROS generation [93, 94]. For instance, cytochrome *c*-depleted mitochondria incubated with substrates generating NADH (e.g. glutamate and malate, that give their electrons to complex I) produce 3 times as much hydrogen peroxide at the level of complex I as control mitochondria [94]. Accordingly, in insulin resistance-associated NASH, the concomitant increase in  $\beta$ -oxidation flux, which enhances NADH generation and thus the delivery of electrons to the respiratory chain, and respiratory chain impairment, which partially blocks the flow of electrons within the respiratory chain, is likely to produce large amount of ROS (*Fig 2*). Interestingly,  $\beta$ -oxidation of fatty acid *per se* [95, 96] or glucose oxidation *per se* [97] can lead to significant mitochondrial ROS generation, probably by increasing the amount of reducing equivalents and electron transfer within the respiratory chain. Increased mitochondrial ROS formation has been demonstrated in genetically obese-diabetic ob/ob mice [98], though the precise site of ROS generation has not been determined in these investigations. Finally, increased mitochondrial ROS formation has been also documented in rats fed a choline-deficient diet [99], a rodent model of steatosis and steatohepatitis [100, 101].

### Possible involvement of mitochondrial CYP2E1

Several recent investigations have reported increased hepatic expression and activity of cytochrome P450 2E1 (CYP2E1) in patients with NASH [65, 102]. Although the exact mechanism of increased CYP2E1 is unknown, increased CYP2E1 could be an important source of ROS in hepatocytes [103]. Interestingly, in addition to its localization in the endoplasmic reticulum, CYP2E1 is also found in significant amounts within mitochondria [104, 105]. Mitochondrial CYP2E1 can produce ROS and induce lipid peroxidation [106]. Mitochondrial CYP2E1 protein and activity have been found to be increased in the liver and other tissues of streptozotocin-induced diabetic rats [106]. Although a recent study showed a trend toward lower microsomal CYP2E1 in genetically obese/diabetic ob/ob mice [107], it is tempting to speculate that CYP2E1 could be redistributed from microsomes to mitochondria in the ob/ob liver. Taken

**Figure 3**

Relationship between reactive oxygen species (ROS) overproduction, lipid peroxidation and the development of liver lesions in NASH. Increased ROS generation (in particular by the damaged respiratory chain) in a cell engorged with fat enhances lipid peroxidation and the formation of chemically reactive, and biologically active, lipid peroxidation products, such as 4-hydroxynonenal (HNE) and malondialdehyde (MDA). ROS overproduction also increases the expression of several cytokines, such as tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), transforming growth factor- $\beta$  (TGF- $\beta$ ), Fas ligand (FasL) and interleukin-8 (IL-8). Lipid peroxidation products and cytokines are involved in the development of different liver lesions via different mechanisms. TNF- $\alpha$  and FasL bind to their cognate receptors, to induce mitochondrial membrane permeabilization and caspase activation, leading to cell death by apoptosis or necrosis, depending on cell ATP levels. TGF- $\beta$  induces transglutaminase, which could favor the polymerization of cytokeratins to generate Mallory bodies. TGF- $\beta$ , IL-8 and HNE are chemoattractants for human neutrophils, which may account, at least in part, for neutrophil infiltration and inflammation. Finally, TGF- $\beta$ , HNE and MDA can activate hepatic stellate cells into collagen-producing myofibroblastic cells, thus favoring fibrosis.

together, these data may suggest that the mitochondrial expression of CYP2E1 could play a role in mitochondrial ROS generation.

### Harmful effects of fat and ROS in the liver

Although the reasons for the deleterious effects of steatosis are still incompletely understood, there is growing evidence that, in the presence of oxidizable fat in the liver, ROS can trigger lipid peroxidation, which generates several reactive aldehydes (*Fig 3*). ROS and reactive aldehydes, in turn, further impair mitochondrial function, to further increase mitochondrial ROS. A ROS-dependent vicious circle may thus ensue (*Fig 2*).

### Lipid peroxidation

Even in the basal (fat-free) state, hepatocytes produce ROS. These ROS are formed mainly in mitochondria, but also at other sites, including microsomal CYP. Yet another potential source of ROS in liver is the NADPH oxidase of Kupffer cells [108]. In liver with NASH, the mitochondrial respiratory chain and CYP2E1 are producing more ROS as indicated in a previous section. Moreover, the expression of the CD14 endotoxin receptors on Kupffer cells is increased in animals with either obesity-mediated or alcohol-mediated hepatic steatosis [109, 110]. Increased sensitivity of Kupffer cells to bacterial endotoxin may thus increase ROS formation by these cells. This abundant formation of endogenous

(i.e. inside the hepatocyte) and exogenous ROS may start to oxidize the unsaturated lipids of fat deposits within the hepatocytes to cause extensive lipid peroxidation [5].

Indeed, eleven different treatments causing acute or chronic steatosis in mice always increased hepatic thiobarbituric acid reactants (that represent end-products of lipid peroxidation) and ethane exhalation, an *in vivo* index of lipid peroxidation [111]. After a single dose of tetracycline or ethanol, there was a parallel time course in the rise and fall of hepatic triglycerides, and the rise and fall of lipid peroxidation products, suggesting a cause-and-effect relationship between the presence of oxidizable fat in the liver and peroxidation [111]. Extensive lipid peroxidation also occurs in animals with hepatic steatosis due to a choline/methionine-deficient diet [112], in genetically obese, leptin-deficient, ob/ob mice (personal unpublished results), and in patients with NASH [64]. This extensive lipid peroxidation releases several reactive aldehydes that can damage mitochondria (Fig 2).

#### Lipid peroxidation-induced mitochondrial dysfunction

The peroxidation of hepatic triglycerides releases reactive aldehydes, such as 4-hydroxynonenal (4-HNE) and malondialdehyde (MDA) which can damage several key mitochondrial components. Firstly, lipid peroxidation products can damage the mitochondrial genome [18, 113, 114]. Because mtDNA encodes 13 of the respiratory chain polypeptides, mtDNA damage is expected to impair the flow of electrons in the respiratory chain as indicated previously. Secondly, lipid peroxidation products, including 4-HNE and MDA, also directly attack and inactivate respiratory chain components, including cytochrome *c* oxidase, the terminal oxidase of the respiratory chain [115, 116]. These two effects of lipid peroxidation products could thus partially hamper the flow of electrons in the respiratory chain [3] (Fig 2). ROS and lipid peroxidation can also lead to ultrastructural mitochondrial alterations and megamitochondria [117, 118]. As indicated above, patients with NASH exhibit ultrastructural mitochondrial lesions, with the presence of crystalline inclusions in megamitochondria [64]. Finally, it is noteworthy that some lipid peroxidation products are potent inducers of the mitochondrial permeability transition (MPT) that can lead to cell death [119].

#### Lipid peroxidation and ROS-dependent vicious cycles

Lipid peroxidation-induced mitochondrial dysfunction leads to the generation of more mitochondrial ROS by the damaged respiratory chain. Increased mitochondrial ROS formation may trigger several vicious cycles, since ROS further damage mitochondria to further increase mitochondrial ROS formation (Fig 2).

1. ROS directly damage mtDNA, respiratory chain polypeptides and mitochondrial cardiolipin [3, 18]. The mitochondrial genome is particularly prone to oxidative damage, which causes oxidation and loss of bases, strand breaks, multiple mtDNA deletions, and mtDNA depletion [12, 18].

Hence, hepatic mtDNA depletion in NASH may be, at least in part, the consequence of increased oxidative stress. Interestingly, oxidized bases such as 8-hydroxydeoxyguanosine, multiple mtDNA deletions and mtDNA depletion have also been detected in the extra-hepatic tissues of diabetic animals and humans [120-123], thus suggesting that oxidative stress-induced mtDNA lesions may not be restricted to the liver in patients with NASH.

2. ROS cause NF- $\kappa$ B activation, which induces the synthesis of tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) [124]. TNF- $\alpha$  is also synthesized by fat-engorged adipocytes and, furthermore, is released by Kupffer cells stimulated by endotoxin. TNF- $\alpha$  damages mitochondria and increases mitochondrial ROS formation, as discussed further on.

3. ROS may deplete some antioxidants to further aggravate ROS-induced damages. Glutathione levels have been found significantly lowered in the fatty liver of obese ob/ob mice [125], though a more recent study showed a significant increase in glutathione content in ob/ob liver mitochondria [98]. Low serum vitamin E levels are found in some obese children with steatohepatitis [126], and supplementation with vitamin E can decrease transaminases in obese children [127].

4. Finally, increased mitochondrial ROS formation further increases lipid peroxidation to release more reactive aldehydes that further damage mtDNA and respiratory chain polypeptides [3].

Thus several vicious cycles can damage mitochondria and enhance ROS formation in patients with NASH.

### Potentiating effects of aging on oxidative stress and mitochondrial dysfunction

Although NASH can affect individuals of any age, including children [128], older age seems to increase the risk of NASH [103, 129]. Aging is associated with mitochondrial dysfunction and decreased mitochondrial antioxidant defenses in the liver. Indeed, hepatic mitochondria of old individuals present oxidative damages to the mitochondrial genome, proteins and lipids (including cardiolipin, a phospholipid that plays a key role in OXPHOS), thus leading to an impairment of both electron flow in the respiratory chain and ATP synthesis [130-132]. Moreover, aged hepatic mitochondria present a marked increase in the GSSG/GSH ratio that is correlated with the accumulation of oxidized bases within the mitochondrial genome [133]. Accordingly, aging-related mitochondrial dysfunction and ROS overproduction might further exacerbate the mitochondrial abnormalities of NASH.

### Potentiating effects of ethanol consumption on oxidative stress and mitochondrial dysfunction

Ethanol consumption (over 20 and 40 g/d, respectively in women and men) increases the risk of steatosis and steatohepatitis in obese individuals [2, 134]. Ethanol intoxication is

known to increase ROS generation and lipid peroxidation and induce oxidative damage to mitochondrial components, including mtDNA, proteins and lipids [12, 18, 135, 136], which led us to propose that alcohol can induce premature oxidative ageing of liver mitochondria [137]. Thus, like ageing, ethanol-induced ROS generation and mitochondrial dysfunction may aggravate the hepatic mitochondrial abnormalities of primary NASH. Several reviews on ethanol-induced mitochondrial dysfunction have been published recently [26, 138].

### From ROS formation to steatohepatitis lesions

As indicated in the previous section, ROS cause lipid peroxidation, which releases reactive aldehydes such as MDA and 4-HNE [3, 139]. ROS also increase the expression of several cytokines, including transforming growth factor- $\beta$  (TGF- $\beta$ ), interleukin-8 (IL-8), TNF- $\alpha$  and Fas ligand [3, 139].

Both lipid peroxidation products and cytokines seem to be involved in steatohepatitis liver lesions [3, 139] (*Fig 3*).

#### Inflammation and Mallory bodies

TGF- $\beta$ , HNE and IL-8 are chemoattractants for human neutrophils, which may account, in part, for the neutrophil infiltrate [3]. TGF- $\beta$  also induces tissue transglutaminase [3]. This enzyme is associated with the cytoskeleton, including intermediary filaments. Transglutaminase catalyses the formation of  $\epsilon$ -lysine- $\gamma$ -glutamyl cross-links between a lysine on one polypeptide chain and a glutamine on another polypeptide chain. The induction of tissue transglutaminase by TGF- $\beta$  could polymerize cytokeratins to generate dense eosinophilic material in the liver cell, the Mallory bodies [3] (*Fig 3*).

#### Cell death

Normally, hepatocytes express Fas (a membrane receptor), but not Fas ligand, preventing them from killing their neighbors [140]. However, several conditions leading to increased ROS formation, such as drugs or alcohol abuse, cause Fas ligand expression by hepatocytes, so that Fas ligand on one hepatocyte can now interact with Fas on another hepatocyte, to cause fratricidal apoptosis [140]. ROS also increase the synthesis of TNF- $\alpha$ , and patients with NASH have high hepatic TNF- $\alpha$  mRNA levels [141]. Interestingly, TNF- $\alpha$  is also synthesized by fat-engorged adipocytes in obese people, and may be released in excess by Kupffer cells stimulated by bacterial endotoxin, due to the overexpression of endotoxin receptors on these cells.

The interaction of Fas ligand with Fas, or the interaction of TNF- $\alpha$  with TNF- $\alpha$  receptor 1 (TNFR1), activates procaspase 8 into caspase 8, which cuts BH3 interacting domain death agonist (Bid) [140] (*Fig 3*). Truncated Bid enters the outer mitochondrial membrane to permeabilize this membrane. Bid also induces a conformational change in Bcl-2-associated x protein (Bax) and its analogue Bak, which trans-

locate to mitochondria to form channels in the outer mitochondrial membrane.

Increased permeability of the outer mitochondrial membrane may release cytochrome *c* from the intermembrane space of some mitochondria, thus blocking the flow of electrons into the respiratory chain and increasing mitochondrial ROS formation (*Fig 2*). ROS could then act on the same or other mitochondria to open an inner membrane pore, whose opening causes matrix expansion and outer membrane rupture [142].

Due to increased permeability and rupture of the outer membrane, cytochrome *c* and other proapoptotic factors leave the mitochondrial intermembrane space to activate caspase 9 in the cytosol. Caspase 9 activates effector caspases, which trigger apoptosis [142]. Indeed, apoptosis seems to play an important role in both ASH and NASH [143, 144]. Finally, it is noteworthy that increased permeability of the mitochondrial membranes can also lead to necrosis. Indeed, if this permeability impairs ATP synthesis in a majority of mitochondria, ATP levels drop, thus leading to plasma membrane failure and necrotic cell death [145].

#### Fibrosis

Hepatic fibrosis results from the activation of Kupffer cells and stellate cells. Activated Kupffer cells release TGF- $\beta$ , which activates hepatic stellate cells into collagen-producing myofibroblastic cells (*Fig 3*). There is now strong evidence that ROS and lipid peroxidation play a key role in the initiation and progression of fibrosis [146]. Lipid peroxidation products probably participate in the fibrogenesis process of steatohepatitis in two ways [3]. First, lipid peroxidation products enhance the production by macrophages of TGF- $\beta$  [147]. Second, lipid peroxidation products directly enhance collagen production by activated stellate cells [148]. Consistent with a role of lipid peroxidation in steatosis-triggered fibrosis, vitamin E supplementation inhibited fibrosis in mice fed a methionine/choline-deficient diet, an animal model of NASH [149]. In a pilot human study, vitamin E supplementation decreased plasma TGF- $\beta$  and tended to decrease hepatic fibrosis in patients with NASH [150].

There is now evidence that leptin has a permissive effect for fibrogenesis [151, 152]. Leptin acts on Kupffer cells and hepatic sinusoidal endothelial cells to release TGF- $\beta$  [153], and leptin also enhances the effectiveness of TGF- $\beta$  action on fibrogenesis by increasing the expression of TGF- $\beta$  receptors in stellate cells [152]. Leptin may also directly increase collagen formation by stellate cells [154]. Activated stellate cells express the active, long form of the leptin receptor (Ob-RL) and also release leptin, which may thus act in an autocrine loop in these cells [154]. Because plasma levels of leptin are increased in obese patients [155], the profibrogenic action of leptin could have a significant role in the pathogenesis of NASH in obese subjects. However, a recent study in patients with NASH failed to detect a correlation

between hyperleptinemia and fibrosis or inflammation [156]. Thus, further studies are needed in order to determine the exact role of leptin in fibrogenesis during NASH.

### Genetic susceptibility to NASH

For the same amount of weight excess, or for the same degree of insulin resistance, some subjects only have steatosis, while others develop steatohepatitis and cirrhosis. Genetic polymorphisms could explain, at least in some patients, a faster progression of the liver disease.

Indeed, recent studies have suggested that polymorphisms in TNF- $\alpha$  promoter [157] and MTP gene [158, 159] may have a significant role in steatosis and NASH. Regarding TNF- $\alpha$  promoter polymorphism, one allele (the so-called TNFA allele) seems to increase the risk of insulin resistance and nonalcoholic fatty liver disease, most probably by increasing the release of TNF- $\alpha$  by Kupffer cells [157]. TNF- $\alpha$  can disturb insulin signaling in different tissues [160] and can increase fatty acid synthesis in liver [161]. Moreover, a recent study in cultured myocytes suggests that TNF- $\alpha$  induces mtDNA depletion via a ROS-dependent mechanism [162]. If such a mechanism operates also in liver, this could explain, at least in part, the mtDNA depletion observed in some patients with NASH (see above). Finally, it is noteworthy that, besides increased TNF- $\alpha$  expression, NASH seems to be associated, at least in some individuals, with increased expression of the mRNA of the p55 TNF- $\alpha$  receptor I [141].

An MTP polymorphism, which decreases MTP activity, may cause less hepatic VLDL secretion, and thus more fat accretion and more liver lesions [158, 159]. Patients heterozygous for mutations in the apo B gene may also have an increased risk for NASH [103, 163].

At least in some studies, patients heterozygous for HFE gene (i.e. the gene whose defect leads to hemochromatosis) mutations may be more susceptible to NASH [164, 165]. Carriers of a HFE mutation may have increased iron in the liver and a slightly increased risk of fibrosis [164]. This may be related to iron-mediated oxidative stress and lipid peroxidation [164, 165].

Finally, as some polymorphisms seem to increase the risk of NASH, it is conceivable the same polymorphisms could also favor NASH. These polymorphisms include a polymorphism in the CD14 endotoxin receptor [166] and a dimorphism in the mitochondrial targeting sequence of MnSOD [167, 168].

### Implications in clinical management

Beside genetic factors, life style and dietary habits may play a significant role in the pathogenesis of NASH. For instance, it has been shown recently that individuals whose dietary intake is rich in saturated fat and poor in polyunsaturated fat have an increased risk of NASH [59].

Weight loss in obese individuals and especially in children seems to be efficient in reducing fat accumulation in liver and the risk of NASH [128]. However, it must be stressed that severe dieting and rapid weight loss can have the opposite effect, that is to promote the occurrence of NASH [169]. Indeed, severe dieting or total fasting increase adipose tissue lipolysis and the release of FFAs, that increase the pool of fat in liver and can also impair mitochondrial respiration [3, 12]. Fasting may also cause glutathione depletion [170], which enhances lipid peroxidation and cytokine-mediated cell death [171]. Rapid weight loss due to starvation, severe dieting, jejunioleal bypass or gastroplasty paradoxically increases liver inflammation and fibrosis in these patients [3].

In the past few years, data from several experimental and clinical investigations suggest that different drugs could be useful for the prevention and/or the treatment of NASH. Drugs that seem promising include metformin [172, 173] and rosiglitazone [174], which both increase sensitivity to insulin, some lipid-lowering agents such as fibrates [101, 175, 176] and probucol [177], and also betaine, which may improve VLDL secretion [178]. Another compound that also deserves deeper interest is L-carnitine [179], a cofactor mandatory for the mitochondrial oxidation of long-chain fatty acids [12], that also favors the removal of toxic endogenous metabolites, such as acyl-CoA intermediates [180]. Finally, vitamin E could also become adjunct therapy [127, 128], if its beneficial effects are confirmed. Investigations will be required in the future in order to determine whether these pharmacological approaches are able to improve mitochondrial function in NASH and if so by which mechanism(s).

### Conclusions

In wealthy countries, new life-style habits combining rich diet and lack of exercise have caused a surge in obesity. Excess of weight can trigger insulin resistance in adipose tissue, muscle and liver. Insulin resistance increases glucose/insulin levels and causes persistent adipocyte lipolysis, which can cause fatty liver (*Fig 1*). Because insulin resistance causes hepatic steatosis, and steatosis can cause NASH, there is a final, almost universal association of primary NASH with insulin resistance. However, insulin resistance is also present in patients with hepatic steatosis but without NASH [64], and, conversely, steatohepatitis can occur when hepatic steatosis is triggered by mechanisms other than insulin resistance. Indeed, long-term treatment with drugs such as amiodarone, tamoxifen or some antiretroviral drugs can induce steatosis and steatohepatitis in some individuals.

There is growing evidence that mitochondrial dysfunction plays a key role in NASH whatever its origin. Not only mitochondrial dysfunction participates to fat accretion but it also leads to the generation of ROS (*Fig 2*). Consequently, several vicious cycles involving lipid peroxidation, mitochondrial damage, ROS formation, depletion of anti-

oxidants and cytokine release may cause necroinflammation and fibrogenesis in genetically susceptible patients (Figs 2 and 3).

Further studies are required to better understand how these diverse effects interact with each other, which genetic, environmental and nutritional factors are involved in individual susceptibility, and which treatments can be best used in patients who fail to lose weight, despite medical advice. As there is an epidemic of obesity and related diseases in prosperous countries, reducing the incidence of NASH during the next decade is a major challenge for hepatologists.

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