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Review

Remodeling of mitochondrial plasticity: the key switch from NAFLD/NASH to HCC

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Abstract: Hepatocellular carcinoma (HCC) is the most common primary malignancy of the liver and the third-leading cause of cancer-related mortality. Currently, the global burden of nonalcoholic fatty liver disease (NAFLD) has dramatically overcome both viral and alcohol hepatitis thus becoming the main cause of HCC incidence. NAFLD pathogenesis is severely influenced by lifestyle and genetic predisposition. Mitochondria are highly dynamic organelles which may adapt in response to environment, genetics and epigenetics in the liver ("mitochondrial plasticity"). Mounting evidence highlighted that mitochondrial dysfunction due to loss of mitochondrial flexibility, may arise before overt NAFLD and since the early stages of liver injury. Mitochondrial failure not only promotes hepatocellular damage, but also release signals (mito-DAMPs) which trigger inflammation and fibrosis, generating an adverse microenvironment in which several hepatocytes select anti-apoptotic programs and mutations that may allow survival and proliferation. Furthermore, one of the key events in malignant hepatocytes is represented by remodeling of glucidic-lipidic metabolism combined to reprogramming of mitochondrial functions, optimized to deal with energy demand. In sum, this review will discuss how mitochondrial defects may be translated into causative explanations of NAFLD-driven HCC, emphasizing future directions for research purposes and for development of potential preventive or curative strategies.

Keywords: NAFLD; NASH; HCC; mitochondrial dynamics; hepatocytes; KCs, HSCs; apoptosis; metabolic reprogramming; Warburg effect

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Abbreviations: ACC, acetyl-CoA carboxylase; ACLY, ATP-citrate lyase; AFP, α-fetoprotein; AIF, apoptosis induced factor; AMLN; high trans-fat, high fructose and high-cholesterol; AMPK, 5'AMP-activated protein kinase; ATG5, autophagy related 5; ATP, Adenosine Triphosphate; BCL, B-cell lymphoma; Bax/Bcl-xL, BCL2 Associated X/Bcl-extra-large; BNIP3, BCL2/adenovirus E1B 19 kDa- protein-interacting protein 3; Ca^{2+}, calcium; CCL, C-C-motif chemokine ligand; CDE; Choline-Deficient, Ethionine-supplemented diet; CPT1/2, Carnitine Palmitoyl-Transferase 1/2; CRISPR/Cas9, Clustered Regularly Interspaced Short Palindromic Repeats/CRISPR-associated protein 9; CSC, cancer stem cells; DEN, Diethylnitrosamine; DNL, De novo lipogenesis; DRP1, Dynamin-related protein 1; EMT, epithelial-mesenchymal transition; ER, Endoplasmic Reticulum; ETC, Electron Transport Chain; FADS, fatty acid desaturase; FASN; fatty acid synthase; FFAs, Free Fatty Acids; Fis1, Fission 1;FGF21, fibroblast growth factor 21; FUNDC1, FUN14 Domain-containing 1; GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; GPX1, Glutathione Peroxidase 1;

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GLUTs, glucose transporters; GSSG/GSH, glutathione disulfide/glutathione; GST, Glutathione Transferase; H2O2, Hydrogen Peroxide; HBV, Hepatitis B viral; HCC, Hepatocellular Carcinoma; HCV, Hepatitis C viral; HFD, High Fat Diet; HEY, YRPW motif protein 1; HK, Hexokinase; HRE, hypoxia response elements; HSCs, Hepatic Stellate Cells; IL-6, Interleukin 6; IMMs, Inner Mitochondrial Membranes; IR, Insulin Resistance; IRF1, interferon regulatory factor-dependent type 1; JNK, c-Jun NH2-terminal kinase; KCs, Kupffer cells; LC3, Light Chain 3; LDH, lactate dehydrogenase; LDL, Low Density Lipoproteins LDs, Lipid Droplets; LIR, LC3-interactive region; LPA, lysophosphatidic acid mediator; LT, Liver Transplantation; LXR, Liver X Receptor; MAMs, Mitochondrial-Associated ER Membranes; MAPKs, mitogen-activated protein kinases; MDVs, Mitochondrial-Derived Vesicles; MetS, Metabolic Syndrome; Mff; Mitochondrial Fission Factor; MFNs,Mitofusins; MiD49/50, Mitochondrial Dynamics 49/50; MnSOD, Manganese Superoxide Dismutase; MOMs, Mitochondrial Outer Membranes; MPC, mitochondrial pyruvate carriers; mtDNA, Mitochondrial DNA; mTOR, Mammalian Target of Rapamycin; MTP, mitochondrial trifunctional protein; MUFAs, mono-unsatured fatty acids; NAFLD, Nonalcoholic fatty liver disease; NASH, Nonalcoholic steatohepatitis; Nf-κB, nuclear factor-kappa-light-chain-enhancer of activated B cells; NLR, NOD-like receptors; NOD, nucleotide binding oligomerization domain; NRF1/2, Nuclear Respiratory Factor 1/2; O_2, oxygen; OA, oleic acid; Opa1, Optic Atrophy 1; OXPHOS, Oxidative Phosphorylation; PA, palmitic acid; PDH, pyruvate dehydrogenase; PDM, Peri-droplets Mitochondria; PEP, phosphoenolpyruvate; PGC1s, Peroxisome Proliferator-activated Receptor-γ coactivator 1; PI3K, phosphoinositide 3-kinase; PINK1, PTEN-induced kinase 1; PK, pyruvate kinase PLIN, Perilipin; PPAR, Peroxisome Proliferator-activated Receptor; PRRs, pattern recognition receptors; PTEN, Phosphatase and Tensin homologue; PUFAs; polyunsaturated fatty acids; ROS, Reactive Oxygen Species; SCD, stearoyl-CoA desaturase; SIRT, Sirtuin; SREBP1/2, Sterol Regulatory Element-Binding Protein 1/2; STAT3, Signal transducer and activator of transcription 3; T2DM, Type 2 Diabetes Mellitus; TAMs, tumor-associated macrophages; TCA, Tricarboxylic Acid; TGs, triglycerides; CPT TLR, Toll-like receptor; TNF-α, Tumor Necrosis Factor α; TNM, Tumor Node Metastasis; TRAIL, TNF-related apoptosis inducing ligand; UCP2, Uncoupling Protein 2; UPRmt, Mitochondrial Unfolded Protein Response; VLDL, Very-Low Density Lipoproteins; YAP, Yes-associated protein

1. Introduction

Hepatocellular carcinoma (HCC) is the main subtype of liver tumor and the third-leading cause of cancer death worldwide, whose incidence reflects the etiologies of liver diseases and their geographical distribution [1, 2]. The Asian population has the highest HCC prevalence, and it is amenable to viral hepatitis for more than 50% of cases. Viral hepatitis C (HCV) and alcohol abuse prevail in Western countries, where HCC is much less frequent [2]. However, the global spreading of obesity and metabolic syndrome (MetS) have rapidly increased the incidence of nonalcoholic fatty liver disease (NAFLD) over the past two decades and, in parallel, those of NAFLD-related HCC in the industrialized society due also to the development of anti-viral therapies and effective HBV immunization programs. Nowadays, NAFLD is the most common chronic liver disorder, affecting 25-30% of general population, and it is closely intertwined with insulin resistance (IR), overweight, type 2 diabetes mellitus (T2DM), dyslipidemia, hypertension, and hyperglycemia. NAFLD is defined as hepatic fat content >5% of liver weight (steatosis), a potential reversible condition which could evolve into nonalcoholic steatohepatitis (NASH) in the 10-25% of subjects, fibrosis, and cirrhosis. It has been reported that NASH patients with advanced fibrosis (F3-F4) had 7-fold higher rate to develop HCC and, in a small proportion, HCC arises in NAFLD individuals without fibrosis [3, 4]. Additionally, the 30-40% of HCC cases occurred in subjects with cryptogenic cirrhosis, some of which were further affected by dyslipidemia, obesity, T2DM and possibly

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by NASH [5]. A study analyzing the Surveillance, Epidemiology and End Results (SEER) registries recorded a 9% annual increase of NAFLD-HCC cases from 2004 to 2009. The survey also examined the prevalence and mortality of 4.929 HCC individuals. Among them, the 14.1% of HCC was due to fatty liver, the 5% received NAFLD-related liver transplantation (LT) and the presence of NAFLD increased the risk of 1-year mortality, especially in older subjects with previous heart disease [6, 7]. Therefore, NAFLD is currently representing not only a clinical and socio-economic burden for health, but it is predicted to overcome HCV, HBV and alcoholic hepatitis thus becoming the leading cause of HCC and LT [1-4, 6].

Pathogenesis of NAFLD involves the interplay among environmental, epigenetic, and genetic factors. The outbreak of HCC results from continuous cycle of parenchymal disruption and tissue regeneration sustained by inflammation, oxidative stress, fibrogenesis and hypoxia. In this scenario, mitochondria, which are extremely adaptable in response to external cues, exert a key role as bioenergetic factories and for the regulation of liver metabolism. Compelling evidence has suggested that mitochondrial dysfunction may precede IR or arise before NASH development thereby reinforcing the concept that NAFLD may be considered a mitochondrial disorder. Loss of mitochondrial plasticity in terms of functions, morphology and dynamics may support hepatocellular injury and the onset of the *Warburg* effect, the mechanism by which hepatocytes exploit anaerobic glycolysis even in the presence of oxygen in order to sustain energy demand and cell proliferation [8-11].

Therefore, this review will focus on how environmental factors, genetics/epigenetics and metabolic alterations may impact on mitochondrial dysfunction and prime the hepatocytes to epithelial-mesenchymal transition (EMT). The main goal of the present work will be to summarize the main aspects related to NAFLD-HCC pathophysiology driven by mitochondrial failure and the current knowledge about mitochondrial dynamics, which could lay the groundwork for the development of new therapeutic approaches to prevent and/or manage NAFLD progression towards HCC.

2. Mitochondria: the workforce of the liver

The liver is enriched in mitochondria, highly dynamic organelles endowed of their own mitochondrial DNA (mtDNA) in multiple copies encoding 13 subunits of the electron transport chain (ETC), 22 transfer RNAs, and 2 ribosomal RNAs [12]. Mitochondria are vital for cell homeostasis and its metabolic activities as they provide the bulk of energy requirements through oxidative phosphorylation (OXPHOS) and adenosine triphosphate (ATP) synthesis as well as they regulate redox status, β-oxidation, tricarboxylic acid cycle (TCA), ketogenesis and glucidic/lipidic metabolism. Physiologically, mitochondria are renewed from pre-existing ones in a cycle known as mitobiogenesis, encompassing fusion and fission events, since they cannot be generated *de novo* [12, 13]. Recently, our group have proposed an extensive review about mitochondrial dynamics and its involvement in NAFLD pathogenesis [14].

Energy shortage and low ATP availability stimulate hepatic mitobiogenesis by activating peroxisome proliferator-activated receptor (PPAR)-γ coactivators 1 alpha (PGC1α), which is induced by fibroblast growth factor 21 (FGF21) and promotes Krebs cycle, lipid catabolism and gluconeogenesis [15, 16]. Notably, Bhalla et al demonstrated that PGC1α over-expression may promote HCC development by coordinately sustaining mitochondrial biogenesis and β-oxidation [17]. Mitochondrial fusion supports OXPHOS and mitochondrial coupling efficiency during cell proliferation. Elongation of mitochondria is orchestrated by Mitofusin 1/2 (MFN1, MFN2) and optical atrophy 1 (OPA1) which mediate merging of mitochondrial outer membranes (MOMs) and inner membranes (IMMs), respectively [18-20].

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Conversely, during fission mitochondria are separated in two or more daughter organelles by dynamin-related protein 1 (DRP1), which is recruited around MOMs by specific adaptors like fission 1 (FIS1), mitochondrial fission factor (Mff) and mitochondrial dynamics (MiD) Proteins 49/50 [21-23]. Aberrancies in mitochondrial dynamics are drivers of HCC development and progression. In particular, alterations of MFNs and OPA1 functions may lead to metabolic reprogramming and EMT, while DRP1 de-regulation may prompt cell growth, tumor microenvironment and invasiveness [24].

Mitochondrial dynamics further includes a mechanism which allows to either repair damaged mitochondria through mitochondrial unfolded protein response (UPR^{mt}) or definitively disrupt them by autophagy (mitophagy) in order to prevent mitochondrial failure [12]. Mitophagy is fine-tuned regulated within hepatocytes to the extent that three types of mitophagy participate to preserve cell homeostasis. When mitochondria are preparing to separate, one daughter mitochondrion is transiently hyperpolarized while the other one is hypopolarized. The latter may run into complete depolarization by losing protonmotive force, uncoupling OXPHOS and dissipating mitochondrial membrane potential [25, 26]. Suboptimal mitochondria that not overcome the quality check are selected for type 1 mitophagy, which is mediated by phosphatase and tensin homologue (PTEN)-induced kinase 1 (PINK1)/Parkin signaling [25-27]. Both fructose and Western diet exploited as dietary models of hepatic steatosis may hasten mitochondrial depolarization and mitophagy thus arising mitochondrial dysfunction at early stages of NASH, possibly contributing to liver disease progression towards HCC [28]. Type 1 mitophagy is tightly linked to nutrients availability and insulin signaling, while type 2 mitophagy may either occur in parallel with PINK1/Parkin-dependent mitophagy or be induced by photodamage in a phosphoinositide 3-kinase (PI3K)-independent manner [25, 26, 29]. In both type 1 and 2 mitophagy, E3 ubiquitin ligases are enrolled on MOMs and provides polyubiquitin tails to mitochondrial receptors (*i.e* BNIP3, FUNDC1) containing light chain 3 (LC3)-interactive region (LIR) motifs. Ubiquitination of LIR domains is required for binding the autophagic compartments and deficiency in autophagic processes was associated with mitochondrial dysfunction and genomic instability in murine hepatocytes [30]. Finally, type 3 micromitophagy embedded mitochondrial-derived vesicles (MDVs) containing selected oxidized cargoes and mitochondrial fragments into multivesicular bodies which, then, will merge to lysosomes for degradation [25, 26, 31].

3. Mitochondrial alterations at early stages of hepatic steatosis: cause or consequence?

The mechanisms underlying NAFLD pathogenesis are highly complex and multifactorial and parallel hits participate to the disease onset and progression. Sedentary lifestyle and hypercaloric diet are the major risk factors that influence visceral adiposity and development of peripheral IR. It has been reported that defects in mitochondrial biogenesis may rapidly accelerate beige-to-white adipocytes transition thus contributing to adipose tissue expansion [32]. Benador et al revealed that mitochondria surrounding lipid droplets (LDs) in the adipose tissue, termed peri-droplet mitochondria (PDM), showed a different bioenergetic metabolism compared to those non-adjacent to the LDs surface thus contributing to their enlargement and adiposity [33]. Furthermore, signs of hepatic mitochondrial dysfunction have been observed in insulin resistant rats without overt NAFLD, thereby suggesting that mitochondrial failure may appear as an early event before steatosis onset [34].

Fatty liver results from the unbalance between lipid synthesis and catabolism within the hepatocytes. IR promotes adipose tissue lipolysis that consequently causes an efflux of free fatty acids (FFAs) to the liver, where they are stored

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as triglycerides (TGs) to counteract the harmful effect of FFA surplus. In addition, the compensatory hyperinsulinemia activates the hepatic *de novo* lipogenesis (DNL) through sterol regulatory element-binding proteins (SREBP1, SREBP2), ATP-citrate lyase (ACLY), acetyl-CoA carboxylase (ACC) and fatty acid synthase (FASN), which provide precursors for TG synthesis thus exacerbating LDs accumulation [35]. In response to high caloric intake, IR and obesity, hepatic mitochondria adapt in number, biomass and activity through a mechanism known as "mitochondrial flexibility". Firstly, the liver increases FFA transport into mitochondria through carnitine palmitoyltransferase-1/2 (CPT1/2) and enhanced both β-oxidation and OXPHOS. Indeed, megamitochondria were observed in liver biopsies of both NAFLD and NASH patients, suggestive of an enhanced mitochondrial density [36]. Shami and colleagues have recently proposed a detailed description of three-dimensional ultrastructure of giant mitochondria in NAFLD subjects. The authors provided a classification of these organelles distinguishing in elongated, irregular, and spheroidal shape based on the characterization of their internal ultrastructure [37], although further investigations are required to elucidate whether the raise in mitochondrial content observed in NAFLD/NASH hepatic tissues reflect high level of mitobiogenesis or reduced mitophagy [38]. Then, FFA overload overwhelms both TCA and FA catabolism, from which NADH/$FADH_2$ are generated. Both NADH and $FADH_2$ transfer electrons to the respiratory chain coupled to the synthesis of ATP and a small fraction of protons leaks from ETC, reacts with oxygen (O_2) and generates reactive oxygen species (ROS) [39]. Elevated concentrations of lipid species, especially those incorporating saturated/monounsaturated FA chains, critically promote both ROS-induced lipotoxicity and hepatocellular damage thus favoring liver disease progression and loss of mitochondrial dynamics [39, 40]. Therefore, this paragraph will discuss how lipid metabolism, in terms of composition, anabolism and catabolism, may impact on mitochondrial dysfunction and favor *per se* a pro-tumorigenic microenvironment since the early stages of NAFLD.

3.1 Fatty acids metabolism, de novo lipogenesis and β-oxidation: from steatosis to HCC

NAFLD development is directly involved in hepatocarcinogenesis and tumor adaptation to local micro-environment independently of NASH. The most studied mechanisms linked to carcinogenesis affect mitochondria and include the *Warburg* effect, from which lactate and pyruvate are produced to support energy demand [8, 9, 14], and glutaminolysis, which exploits glutamine to sustain Krebs cycle by generating high citrate concentration [41]. Notwithstanding, aberrant activation of DNL is one of the major metabolic events occurring in NAFLD-HCC onset. In this field, the role of lipid species is increasingly grabbing attention as they can modify mitochondrial functionality and participate to metabolic switching in hepatocytes to the extent that alterations of lipid metabolism are currently recognized as a hallmark of hepatic cancer.

Many malignant tumors showed LDs accumulation and activation of lipogenic pathways, which were correlated with pro-survival phenotype in human HCC cell lines and poor prognosis in HCC patients [42]. Among lipid classes, TGs, diglycerides and ceramides are enriched in steatotic livers and contribute to the onset of endoplasmic reticulum (ER) stress whose alterations in terms of functions and architecture participate to both DNL and IR [43]. It has been demonstrated that lipid overload may disrupt ER-mitochondrial communications in steatotic hepatocytes and *ob*/*ob* mice feeding high fat diet (HFD). Lipids altered the abundance of calcium (Ca^{2+}) transporters and channels leading to Ca^{2+} efflux into cytoplasm. The increased intracellular Ca^{2+} concentration enhanced ROS content and mutagenesis of both nuclear DNA and mtDNA, thereby inducing activation of oncogenes or inhibition of onco-suppressors and affecting mtDNA replication [44, 45].

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Several studies revealed that aberrantly activated DNL is critical for HCC development and progression. Inhibition of stearoyl-CoA desaturase (SCD), FASN and ACC, which provide FFAs within hepatocytes, abrogated Akt-driven HCC and reduced hepatic cancer stem cells (CSC) pool [46, 47]. Consistently, FFAs *per se* derived from adipose tissue lipolysis combined to those newly synthetized from DNL may hasten hepatocytes degeneration and foster mechanisms of tumor escape by activating anti-apoptotic programs. FFAs may be converted in mono-unsatured fatty acids (MUFAs) by desaturase enzymes as fatty acid desaturase (FADS) and SCD. Increased MUFAs, Fads1/2 and Scd2 levels have been observed in mice affected by NAFLD-HCC and human HCC specimens [42, 48, 49]. Moreover, rat hepatocytes treated with palmitic acid (PA) affected insulin signaling, enhanced β-oxidation rate and exacerbated ROS content. PA also activated c-Jun NH2-terminal kinase (JNK), which is the most constitutively activated factor in HCC involved in mitochondrial cytochrome c release, cell death and compensatory proliferation [50, 51]. Kudo et al found that hepatocyte-specific *Pik3ca* transgenic mice, a genetic model of hepatosteatosis, developed hepatocellular adenomas with abundant LDs and HCC but both without inflammation and fibrosis, supporting a direct role of lipids as pro-tumorigenic factors. Notably, they demonstrated that accumulation of oleic acid (OA) and PA promoted liver cancer development by suppressing Pten, inhibitor of the Pi3k/Akt/mammalian target of rapamycin (mTOR) signaling [52].

In response to excessive FFA concentration, mitochondria enhance CPT1/2-mediated mitochondrial lipid flux and β-oxidation to protect against lipotoxicity, a mechanism which is lost during NAFLD course. Moreover, uncoupling protein 2 (UCP2) activity is upregulated due to FA surplus thus boosting mitochondrial proton leak and increasing NASH susceptibility [39]. Defects in mitochondrial trifunctional protein (MTP), which catalyzes long-chain FA β-oxidation, induced hepatic IR and steatosis development alongside an increase in antioxidant defenses and cytochrome P-450 to counteract ROS production [53]. Reduced FA catabolism, ATP and mitochondrial membrane potential have been reported in progressive NAFLD and in many NAFLD-HCC cases. Diethylnitrosamine (DEN)-injected mice fed with HFD downregulated both CPT2 and β-oxidation during HCC onset. Suppression of FA oxidation enables HCC cells to adapt to a lipid-rich microenvironment and to escape lipotoxicity by blocking JNK [54]. CPT2 reduction results in the accumulation of acylcarnitine species which further hamper β-oxidation and provide the acquisition of stem cell properties through the signal transducer and activator of transcription 3 (STAT3)[54, 55].

However, T2DM, obesity or NAFLD etiologies may contribute to the development of "oxidative" HCCs, a metabolic HCC variant observed in a subset of patients, as these conditions provide FFAs in bulk that, in turn, may be re-routed towards β-oxidation rather than TG re-esterification [11, 55-58]. In this context, PPARα, master regulator of FFA catabolism, is activated and stimulated the WNT/β-catenin oncogenic cascade which, in turn, sustains FFA dismissal. Additionally, β-oxidation is further supported by either hydrolysis of intrahepatic TGs, which provide FFA substrates, and acetyl-CoA, which derives from β-oxidation and promotes ketogenesis. In "oxidative" HCCs, FFA degradation efficiently feeds the ETC and supplies ATP thus rendering its synthesis addicted to FFAs oxidation at expense of ATP produced by the Warburg effect [58].

3.2 The role of LDs and lipophagy in NAFLD-related HCC

LDs, whose biogenesis starts from ER-Golgi compartments, are highly dynamic organelles stocking energy sources and working as a buffering system incorporating lipotoxic species. LDs may either re-arrange in size according to nutritional status or support cell survival by providing FFAs *via* autophagic processes during stressful conditions. Tumor cells

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largely exploit LDs breakdown in absence of energy availability and hypoxia to support cell growth and tumor expansion [59-62]. It has been shown by Tian et al. that unbalanced lipophagy is unexpectedly involved in carcinogenesis in hepatoma cell lines, NAFLD murine models and NAFLD-HCC human samples [63]. Specifically, Nogo-B oncogene, which localizes at ER-LDs interface, interacts with autophagy related 5 (ATG5) to promote LDs self-catabolism. LDs breakdown releases lysophosphatidic acid (LPA) mediator, which enhances the pro-proliferative Hippo/Yes-associated protein (YAP) cascade [63]. Moreover, oxidized low-density lipoprotein (oxLDL) and free cholesterol accumulate in the hepatic microenvironment of NASH mice [63] and NAFLD subjects [64]. Increased uptake of oxLDL in the liver *via* CD36 receptor may represent a triggering *stimulus* for metabolic rewiring of the hepatocytes as it can promote Nogo-B transcriptional activation [63].

Furthermore, in both human hepatocytes laden with large LDs and cirrhotic scars flanked by macrovescicular steatosis, nuclear localization of YAP protein was increased compared to hepatocytes accumulating small LDs and cirrhotic sections with micro-steatosis [65], suggesting a link between macro-steatosis and cancer. Conversely, characterization of LDs proteome highlighted that perilipin (PLIN1), ADRP (PLIN2) and TIP47 (PLIN3) proteins mediate LDs-mitochondria crosstalk thus regulating LDs expansion and disposal. Notably, PLIN1, PLIN2 and PLIN3 are differentially expressed during tumorigenesis and usually dwell on LDs surface according to the LDs dimension. PLIN2 and PLIN3 mainly coat small LDs and are commonly overexpressed at early stages of HCC, as the LD dimension allows rapid dynamics between synthesis and consumption to sustain phases of cell proliferation or metabolism. PLIN1 expression is lost during hepatocarcinogenesis and may reflect differentiation grade of hepatocytes [66]. Activation of SREBP1 *via* PI3K/Akt/mTOR further takes part to neoplastic steatogenesis and PLINs expression [42]. These studies pointed out a differential role of micro/macro-LDs in HCC onset and progression, although details on the mechanisms and respective roles need to be addressed in the future.

4. Mitochondria play a crucial role in the switch from NASH towards HCC

A continuous flux of FFAs into mitochondria results in increased oxidative damage associated with mitochondrial dysfunction, ER stress and tissue inflammation which may contribute to the progression from NAFLD towards NASH up to HCC [62]. Over NAFLD course, mitochondrial failure in terms of functions, morphology and dynamics occurs attempting to deal with energy surplus and to protect against FA-induced lipotoxicity, with the consequent loss of mitochondrial plasticity. In both mice and humans affected by NASH, blunted ketogenesis and mitochondrial respiration were observed, whereas the citric acid cycle was increased in the attempt to discard lipid overload [63].

Compromised OXPHOS, ketogenesis, low ATP synthesis and incomplete β-oxidation coupled to an overreactive Krebs cycle incremented ROS production causing mitochondrial abnormalities, lipid peroxidation and mtDNA damage. Increased serum levels of malondialdehyde (MDA), a byproduct derived from oxidative degradation of lipids, were detected in NAFLD patients, while antioxidants coenzyme Q10 and CuZn-superoxide dismutase (SOD) were reduced [64]. The mitochondrial ROS production triggers mitogen-activated protein kinases (MAPKs) and induces JNK phosphorylation (p-JNK), affecting the mitochondrial ETC and worsening ROS production [65]. Interestingly, if on one hand p-JNK may antagonize oxidative damage by enhancing apoptosis, on the other hand signals arising from necrotic hepatocytes may stimulate JNK cascade in Kupffer cells (KCs) in order to express the pro-tumorigenic cytokines interleukin 6 (IL-6) and tumor necrosis factor-alpha (TNF-α) which promote EMT, migration and invasiveness [67].

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ROS induce the activation of 5' AMP-activated protein kinase (AMPK), which prompts PGC1α. The latter is a powerful sensor of nutritional status which is activated in response to fasting, glucocorticoids, dietary FFAs and it plays a crucial in role in the regulation of OXPHOS, mitobiogenesis and glucidic/lipidic metabolism [15]. The PI3K/Akt/mTOR cascade mediated PGC1α activation, in turn, induces transcriptional factors (*i.e* PPARs, nuclear respiratory factors (NRF1/2,), estrogen related receptors) that increase the levels of ROS scavengers, including SOD2 and glutathione peroxidase 1 (GPX1). However, antioxidant defenses are not able to manage the long-term ROS exposure during NASH. Indeed, lower levels of SOD2 were observed in both HFD-fed rodents and humans [68], while higher circulating levels of the glutathione disulfide/glutathione (GSSG/GSH) ratio and glutathione transferase (GST) were found in a small cohort of 21 pediatric NASH patients, as a possible compensatory mechanism against oxidative stress [69].

In response to mtDNA, proteins and lipids damage induced by oxygen radicals, the nicotinamide adenine dinucleotide (NAD^+)-dependent histone deacetylase sirtuins (SIRTs), localizing on mitochondria, promote SOD2 activation through its deacetylation. In NAFLD subjects, SIRT1, SIRT3, SIRT5 and SIRT6 are down-regulated, while SIRT4 was overexpressed in response to the exacerbated DNL [70]. Moreover, several members of SIRT family may alleviate ROS-induced hepatocellular injury and promote apoptosis thus preventing compensatory proliferation and tumorigenesis [71, 72].

NASH is characterized by loss of mitochondrial flexibility, activation of Kupffer cells (KCs), hepatic macrophages, and even by the presence of fibrosis. Cytokines 'release from both hepatocytes and KCs may activate hepatic stellate cells (HSCs), which produce collagen and fibrotic scars, and further stimulate apoptotic receptors, such as Fas, TNF receptor 1 (TNFR1) and TNF-related apoptosis inducing ligand (TRAIL) on hepatocytes surface [72-74]. Consequently, the pro-apoptotic B-cell lymphoma 2 (Bcl-2) and JNK pathways stimulate the mitochondrial membrane permeabilization through their translocation on MOMs where they can create tunnels, from which apoptosis inducing factor (AIF) and cytochrome c are released, promoting mitochondrial derangement and apoptosis [74]

Furthermore, low rate of mitophagy in NAFLD hampers the disruption of degenerative mitochondria, causing the release of mitochondrial damage-associated molecular patterns (mito-DAMPs), that even trigger inflammation. Mito-DAMPs may prompt pattern recognition receptors (PRRs) by binding and activating the Toll-like receptor 4/9 (TLR4/9) plus the downstream nuclear factor kappa-light-chain-enhancer of activated B cells (Nf-κB) signaling, the nucleotide binding oligomerization domain (NOD)-like receptors (NLRs), the pyrin domain containing 3 (NLRP3) inflammasome and the interferon regulatory factor-dependent type 1 (IRF1) [70]. Ping et al demonstrated that hepatocytes-derived mtDNA secreted from damaged liver tissue in the circulation triggers the fibrotic response, by interacting with TLR9 on KCs and inducing collagen deposition by activated HSCs [71].

Although mitochondrial dysfunction has a prominent role in NAFLD progression, also ER stress contribute to the severity of liver damage. Indeed, these two organelles physically communicate through mitochondrial-associated membranes (MAMs) in response to stress conditions [72]. MAMs are required for the transport of lipids, Ca^{2+}, insulin signaling and glucose homeostasis and even for the interplay with the UPR, that is considered a sensor of ER stress [73]. As previous mentioned, fat accumulation may disturb ER-mitochondria network causing either Ca^{2+}-induced apoptosis or DNA injury, Additionally, cytosolic Ca^{2+} may contribute to hepatocarcinogenesis by activating compensatory proliferation of hepatic CSCs [75, 76]. Therefore, this paragraph aims to delineate the contribution of mitochondrial

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dysfunction and loss of mitochondrial flexibility in the progression from simple steatosis towards NASH and how these abnormalities may generate an advantageous microenvironment for tumorigenesis.

4.1 The loss of "mitochondrial flexibility" during NASH may exert a role in HCC development

As previously underlined, derangement of mitochondrial adaptability, through which mitochondria modify their function and number in response to nutrients availability, may drive and/or accelerate NASH development and its progressive forms despite further studies are required to elucidate which mechanisms among fusion, fission or mitophagy mostly influence the natural history of NAFLD [36, 77].

The disequilibrium of mitobiogenesis and accumulation of damaged mitochondria, mainly due to the failure of mitophagy, have been observed in liver tissue of several NASH subjects and it was even correlated with alterations of mitochondrial architecture [78]. In murine models, genetic deletion of *Mfn2*, which is involved in mitochondrial elongation during fusion, led to mitochondrial failure, ER stress and higher levels of H_2O_2 [79]. Contrariwise, the administration of omega-3 polyunsaturated FAs (PUFAs) in HepG2 cells, previously incubated with PA and OA to mimic steatosis, increased the expression of *Mfn2* with the consequent elongation of mitochondrial tubules [80]. It has been observed that *Mfn1*-deficent mice displayed an increase of *Mfn2* and *Opa1*, as a possible compensatory mechanism. Higher levels of *Opa1* promote the remodeling of mitochondrial cristae and avoids apoptosis by inhibiting cytochrome c release [77]. Fibrotic mice fed with high *trans*-fat, high fructose and high cholesterol (AMLN) diet showed an increased number of disrupted mitochondria paralleled by reduced OXPHOS capacity, loss of both mitochondrial integrity and cristae structure. In these rodents, the expression of *Mnf1* and *Opa1* was significantly reduced and reflected a higher number of separated mitochondria [81]. Recently, Zahng et al demonstrated that *Mnf1* expression was decreased in 34 HCC patients. To explore the role of the mitochondrial fusion, they exploited an *in vitro* model by using MHCC97-H cell line and observed that *Mnf1* inhibits cell proliferation, migration and invasion. These results suggested that the loss of mitochondrial dynamics, mainly due to the deletion of *Mnf1*, has a crucial role to impede HCC development [82].

Regarding alterations in mitochondrial fission, it has been remarked that the deletion of *Drp1* in mice inhibited Bcl-2 and the release of cytochrome c [25]. In mice challenged with HFD, lacking *Drp1* gene ameliorated hepatic fat content and ER stress through the expression of Fgf21, that has a beneficial role on mitochondrial dynamics and prevented the release of pro-fibrotic mediators supporting that inhibition of mitochondrial separation may improve NAFLD severity [83]. Nevertheless, Pollard et al generated a liver-specific murine model in which AMPK and fission were constitutively activated (iAMPK^{CA}). Chronic mitochondrial scission protected against obesity, steatosis, necroinflammation and fibrosis as it swiftly drove damaged mitochondria to autophagic compartments [84].

4.2 Megamitochondria and mitophagy: the impact of morphological alterations in NASH

Fusion-fission unbalancing affects mitochondrial architecture and promotes the formation of megamitochondria, which were detected in both adult and pediatric NAFLD/NASH patients [36, 37]. In a cohort of 31 biopsied NASH patients, it has been observed megamitochondria localized in the central lobule adjacent to the central vein and the portal triads, that were featured by crystalline insertions [85]. In another study, liver biopsies of NASH patients showed an alteration of mitochondrial morphology characterized by megamitochondria, cristae paucity and opacity of granules [86]. Ahishali and colleagues performed a quantitative and semi-quantitative ultrastructural evaluations of liver biopsies from 23 patients, 10 with NAFLD and 13 with NASH [87]. Both NAFLD and NASH hepatic tissues were characterized by the

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presence of megamitochondria with no significant differences among the two groups. However, NASH patients, much more than NAFLD ones, showed higher mitochondrial diameters, intra-mitochondria crystalline inclusions and granules in the matrix which correlated with both mitochondrial swelling and OXPHOS failure [85]. Recently, Verhaegh et al. have observed ultrastructural changes by using transmission electron microscopy in 37 NAFLD patients, of whom 12 had NASH [88]. In this study, the presence of giant mitochondria showed no differences between patients with or without NASH, according to the results of Ahishali *et al* [87], supporting that the appearance of megamitochondria could represent a transitory phase between simple steatosis and NASH. However, their exact role in NAFLD pathophysiology needs to be clarified.

The presence of giant and/or globular mitochondria in fatty liver has been associated with suppression of mitophagy to the extent that restoring mitophagy may enable to rescue mitochondrial dysfunction in NAFLD [89]. Mice carrying *Parkin* deficiency, which is involved in type 1 mitophagy, displayed swollen mitochondria with loss of cristae [90]. Moreover, the high caloric intake in mice reduced the expression of LC3 receptors, with the consequent activation of inflammatory response, that leads to inhibition of mitophagy and NASH progression [91]. Glick and colleagues exploited two murine models deleted for *Parkin* or *BCL2/adenovirus E1B 19 kDa-protein-interacting protein 3* (*Bnip3)* genes, in which lipid synthesis was exacerbated possibly developing a NASH phenotype. In these rodents, the authors observed the presence of globular mitochondria and unidentifiable cristae [92]. Moreover, changes in mitochondrial ultrastructure were evaluated in a mouse model of NASH, in which CXCR3 factor was strongly upregulated. The presence of CXCR3 promotes inflammation in chronic liver disease and may impact on mitochondrial morphology, appearing round-shaped with disrupted cristae. On the contrary, the deletion of *CXCR3* gene ameliorated mitochondrial architecture in terms of less swollen mitochondria and more organized cristae, suggesting that its ablation could prevent morphological alterations [93].

Finally, lipidomic analysis of NASH patients revealed higher hepatic levels of dihydroceramide and dihexosylceramide species that were correlated to defects of mitophagy, oxidative stress and inflammation [94]. The accumulation of dihexosylceramide has been even found in human HCC tissue, suggesting that the reduced mitophagy may be involved in the progression from NASH up to HCC [95].

4.3 The contribution of hepatocellular mitochondrial dysfunction and inflammatory response in NASH

In the large spectrum of NAFLD pathogenesis, inflammation and apoptosis are benchmark of NASH progression. De-regulated mitochondrial activity within hepatic cells and hepatocytes-derived danger signals may directly or indirectly induce inflammation and apoptosis precipitating fibrosis, cirrhosis and, eventually, HCC development. Clinical and experimental evidence showed that ROS-induced apoptosis triggers TRAIL receptors that, subsequently, promote the release of cytokines and chemokines [73]. Nevertheless, the role of TRAIL is controversial. TRAIL$^{-/-}$ mice worsened the inflammation and fibrosis but improved the adipose tissue injury, suggesting that it is indispensable for adipose tissue homeostasis but promoted the hepatic inflammatory and fibrotic response [74].

Apoptotic signals impaired mitochondrial biogenesis as they dampened mitophagy and induced the release of mito-DAMPs. Many components of mitochondria, as formyl peptides, share structural similarities with bacteria and could allow cell-to-cell communications by binding the PRRs expressed on the surface of KCs and HSCs thus exacerbating the systemic secretion of pro-inflammatory TNF-α, IL-6, IL-1β and IL-8 [96-98]. Among mito-DAMPS, mtDNA represents

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the major active components released by damaged hepatocytes and may interact with TLR9 thereby activating inflammation and fibrosis [45]. Both mtDNA and formyl peptides could recruit and induce migration of polymorphonuclear neutrophils through MAPKs thus eliciting neutrophil-mediated hepatic injury [99]. Consistently, elevated concentrations of mito-DAMPs were detected in HFD-fed mice and serum samples of NAFLD/NASH patients, especially in those with advanced fibrosis. Moreover, circulating levels of mtDNA reflected hepatocellular injury and associated with histological parameters of disease progression in both rodents and humans, respectively [45, 100]

Inflammasome activation is closely linked to progressive NAFLD and its expression was higher in both NASH mice and patients [75]. TLRs, TNFR1 and harmful *stimuli*, including mito-DAMPs, increased intracellular levels of ATP and Ca^{2+} ions as well mitochondrial-derived ROS, activate NLRP3 inflammasome and co-adjuvate its assembly. Interestingly, excessive mitochondrial fission at expense of fusion has been associated with NLRP3 activity in hyperglycemic and diabetic conditions [101]. Wei and collaborators firstly demonstrated that the expression of NLP3 inflammasome was completely lost or significantly downregulated in 128 HCC patients. NLRP3 promoted not only the activation of inflammatory pathways but also the reestablishment of hepatic homeostasis by activating apoptosis. Therefore, NLRP3 wholly deficiency favored the compensatory proliferation of hepatocytes and HCC onset [102]. Conversely, NLRP2, belonging to inflammasome cascade as inhibitor of NF-kB signaling, may participate to NASH improvement. Suppression of *Nlrp2* gene accelerated steatosis development in mice. The hepatic expression of NLRP2 was also found lower in NASH subjects with the consequent increase of IL-1β and IL-18 by KCs [76].

The mito-DAMPs-induced inflammation combined to mitochondrial oxidative damage within hepatocytes further modulate the efficacy of nuclear receptors as liver X receptors (LXRα, LXRβ) and members of PPAR family (PPARα, PPARβ, PPARγ), which are key regulators of mitochondrial activity (*i.e.*, β-oxidation) and may be crucial in NASH progression. In particular, LXRs down-regulation may sustain fat accumulation by increasing SREBP1-c and dropping the VLDL catabolism [103]. The administration of SR9238, that is an agonist of LXR, is able to significantly reduce hepatic inflammation in NASH mice [104, 105]. Moreover, Pparγ-deficient mice displayed higher levels of TNF-α, suggesting that PPARγ may regulate KCs-mediated inflammatory response [106]. Likewise, PPARα blocks NF-κB and oxidative burst as well as it eludes the progression towards fibrosis [107, 108]. Mice fed choline-deficient, ethionine-supplemented (CDE) diet increased hepatic TG content and developed NASH alongside reduced PPARs and PGC1α, showing lower levels of mitochondrial mitobiogenesis [109]. Additionally, in a PGC1$\alpha^{-/-}$ mice exposed to an obesogenic diet, Besse-Patin et al. correlated the inefficient β-oxidation and ROS production to a fibrotic response, indicative that mitochondrial damage in hepatocytes may directly aggravate NASH condition [110]. In a cohort of 85 biopsied patients, it has been found that lower expression of PPARα correlated with the severity of liver disease, suggesting that it may represent a possible pharmacological target in NASH treatment [111].

Finally, it has been postulated an interplay between PPARα and hepatocytes proliferation. The oncogene Cyclin D1 inhibits its expression and consequently reduces levels of β-oxidation in HCC cell lines, confirming that PPARα is even involved in carcinogenesis [112]. *Ergo*, such findings provided an overview of the cross-talk among mitochondrial dysfunction occurring in hepatocytes and activated inflammatory response, highlighting the relevance of mitochondria dysregulation in active NASH.

4.4 The crosstalk among parenchymal mito-DAMPs, HSCs and inflammation in NASH-driven HCC

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As discussed above, mitochondrial dysfunctions activate both KCs and HSCs, that, in turn, exacerbate the inflammation and promote fibrotic response, respectively. The ROS production, lipid peroxidation, mito-DAMPs and cell death signals activating caspases augment the hepatic infiltration of pro-inflammatory cells and together trigger fibrogenesis [113, 114]. Particularly, 4-hydroxynonenal (4-HNE), an end-product of lipid peroxidation, acts as a potent pro-fibrogenic *stimulus* for the expression of genes involved in extracellular matrix deposition, the primary mechanism of fibrosis and cirrhosis [115, 116]. HSCs can be activated by the cytokines and chemokines, like TNFα, IL-1β, IL-6, TGF-β, C-C Motif Chemokine Ligand (CCL) 2 and 5, released from hepatocytes and KCs. In turn, KCs may enhance the production of chemoattractant factors and ROS by NADPH oxidase and further worsen the inflammatory response and HSCs-activated phenotypes. Recently, Ping et al demonstrated that the chronic administration of hepatotoxic thioacetamide (TAA) in mice for 6 weeks is able to cause hepatocytic cell death along with hepatic collagen accumulation. According to these findings, upregulation of pro-fibrotic genes such as TGFβ1, pro-collagen α1 and Tissue inhibitor of metalloproteinases-1 (TIMP-1) were detected. TAA-induced liver fibrosis further correlated with high levels of circulating mtDNA arising from death hepatocytes. Notably, freshly isolated HSCs exposed to mito-DAMPs assumed morphological features of their activation, with loss of LDs, myofibroblast-like phenotype and a robust positivity to alpha-smooth muscle actin (α-SMA), marker of HSCs activation [100]. In two independent cohort of NAFLD subjects, it has been observed that mtDNA levels were higher in NASH patients and, much more, in those with fibrosis, compared to NAFLD subjects and matched controls according to the results obtained in mice [100].

Pro-inflammatory pathways activated by mitochondrial dysfunction and oxidized mtDNA not only contribute to fibrosis but also to HCC onset. For instance, it has been demonstrated that NF-κB cascade is involved in cell survival [117]. Park et al demonstrated that TNF-α and IL-6 produced by KCs may activate pro-oncogenic pathways *via* JNK signal transducer and activator of STAT3, Janus kinase 2 (JAK2), MAPK, and PI3K. In dietary and genetic models of hepatosteatosis, it has been shown that IL-6 induced STAT3 activation and trigger the hepatocyte proliferation leading to HCC [118]. Uysal and coworkers proposed a panel of circulating markers of liver damage, including proinflammatory cytokines (TNF-α, IL-6, IL-8), ferritin, nitric oxide and mediators of mitochondrial damage (i.e., MDA) that combined to clinics may predict NASH progression up to HCC [119]. Finally, upregulation of hypoxia inducible factor 1-alpha (HIF-1α) occurs n response to intrahepatic ROS production. HIF-1α is stable under hypoxia, while it is degraded under normal oxygen condition (normoxia). In NASH, oxygen is unevenly distributed in the hepatic lobules due to both inflammation and fibrotic scars thus promoting hypoxia. Interestingly, non-tumoral cells such as mesenchymal stem/stromal cells (MSCs) are typically recruited to the injured or hypoxic area. In mice treated with DEN and exposed to high-fat high-cholesterol high-sugar diet (HF-HC-HSD), which triggers liver fibrosis, it has been observed that the increase of HIF-1α coupled to the release of inflammatory cytokines mediated metabolic reprogramming, angiogenesis and proliferation thereby prompting switching from NASH to HCC [120, 121].

4.5 "Evasion" from mitochondrial-induced apoptosis drives HCC

The development of HCC is almost never a sporadic event, but rather a Darwinian selection of those clonal cells able to trigger mechanisms to escape to intrinsic cues and to adapt to exogenous restraints imposed by the environment. During NASH, lipid peroxidation, mito-DAMPs, oxidative/ER stress and inflammation activate apoptotic receptors, like FAS, TNFR1 and TRAIL, resulting in parenchymal disruption and regeneration through fibrotic scars [122]. The infiltration

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of pro-inflammatory cells and the presence of apoptotic bodies have been observed in the liver of several NASH patients [123]. Although NASH progression is characterized by an extensive apoptosis, non-hepatocytes and non-cellular components (as extracellular matrix) allow generation of tumor microenvironment, in which hepatic stem cell progenitors may carried out strategies to elude cell death and induce compensatory proliferation [124]. Recently, Anstee and colleagues have proposed a review to provide a detailed description of the key NAFLD/NASH mechanisms that promote HCC evading programs [122].

Inami and collaborators revealed that liver-specific loss of autophagy is crucial for hepatocellular survival. Since the early stages of NAFLD, FFAs and TG overload inhibit autophagy through the activation of mTOR. The abundance of lipid species induced oxidative stress that may also promote the activation of p62, involved in the p62-KEAP1-NRF2 pathway. Phosphorylated p62 triggers NFR2 expression which, in turn stimulated antioxidant defences [125-127]. Improvement of mitochondrial ROS production through p62-KEAP1-NRF2 signaling may represent one of the pro-survival events allowing tumor initiation. The activation of KEAP1-NRF2 pathway has been reported in more 25% HCC subjects and the inhibition of autophagic proteins as ATG7 and beclin1 may provide an escaping strategy from ROS-induced apoptosis [128-130].

Moreover, mitochondrial oxidative stress altered MAMs and incremented the ER-induced efflux of Ca^{2+}, thus contributing to hepatocytic cell death. HFD-fed mice treated with cyclosporin A, that inhibited mitochondrial permeabilization by regulating Ca^{2+} turnover, displayed lower levels of apoptosis [131]. Guerra et al examined the contribute of Ca^{2+} signaling in HCC pathogenesis. Authors exploited HCC cell lines, murine models and human liver samples to investigate the role of inositol 1,4,5-triphosphate (InsP3) receptor which acts as Ca^{2+} channel and observed that its release was linked to the proliferation of both hepatocytes and tumor cells. Such findings may suggest that inhibition of Ca^{2+} entry into mitochondria may sustain tumorigenesis as an escaping mechanism to apoptosis [132].

Interestingly, Huang et al demonstrated that even the defects in mitochondrial biogenesis during NASH may enhance anti-apoptotic signals and favour cell growth [133]. In human HCC cell lines, such as Bel7402 and SMMC7721, *MFN1* genetic ablation increased mitochondrial fission, leading to ROS generation and ROS-induced mutagenesis, as constitutive activation of the PI3K/Akt/mTOR network. Akt drives downstream degradation of *TP53* onco-suppressor gene via the E3 ubiquitin-protein ligase MDM2 and, on the other side, promotes the transcriptional activity of Nf-κB and inhibits autophagy. Indeed, increased levels of PI3K/Akt/mTOR axis has been observed in about 40-50% of HCC patients [134, 135].

Long-lasting exposure to inflammatory response and oxidative burst represents the major workforce that encourage genomic instability, sensitizing hepatocytes to JAK/STAT, ERK and MAPK growing signals. Cytokines, like TNF-α and IL-6, was correlated with carcinogenesis and cell division. In particular, TNF-α and IL-6 stimulate the compensatory growth of hepatocytes through Nf-κB, mTOR and STAT3 in response to mitochondrial-induced apoptosis and, simultaneously, sustain a pro-survival tumor microenviroment by the paracrine/autocrine release of chemokines (CCL2, CCL7 and CXCL13) and cytokines (IL-1β, IL-6, TNF–α) [118, 135-138].

The progression from NASH towards HCC in obese people is even mediated by the unbalance between leptin and adiponectin. Leptin is involved in the inflammatory and fibrotic response by triggering the JAK/STAT, PI3KAkt and ERK signalling pathways [139]. Increased serum leptin was found in HCC patients with or without cirrhosis [140]. On the contrary, adiponectin can suppress the tumor growth by either activating JNK-mediated mitochondrial apoptosis

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and caspases or inhibiting Akt and STAT3 [139]. Nevertheless, low levels of adiponectin are unable to repress KCs-mediated inflammatory response and promote HCC development. [141].

5. Metabolic reprogramming and mitochondrial dysfunction in HCC

Metabolic reprogramming is one of the key events determining the shifting from NASH up to HCC and a growing body of evidence has recognized it as a hallmark of hepatic cancer and not as an epiphenomenon of malignant transformation. Inflammation, fibrosis, ROS, fat accumulation, hypoxia and apoptotic signals challenge the hepatocytes to rewire liver metabolism in terms of cell survival, proliferation and, eventually, invasiveness. To support the high energy demand and macromolecule biosynthesis (lipids, nuclei acids, proteins), and to escape from disadvantageous conditions that may lead to programmed cell death, transformed hepatocytes metabolize glucose through glycolysis to produce ATP in presence of oxygen rather than proceeding with the mitochondrial respiratory chain. This rapid but low-efficiency phenomenon is paradoxically known as aerobic glycolysis or *Warburg* effect discovered by Otto Warburg in 1950 [9, 11] and allows to quickly introduce and process glucose by accelerating glucose transport into the hepatocytes, increasing the kinetics of glycolytic enzymes and secreting glycolytic by-product as lactic acid.

Many studies revealed the prognostic value of glucose transporters (GLUTs) upregulation in human HCCs. Among them, it has been recently suggested by Gao et al that GLUT3 overexpression correlated with elevated α-fetoprotein (AFP) levels, tumor size, poor histological differentiation, tumor node metastasis (TNM) stages and it may predict survival of HCC patients [142]. Hexokinase (HK), which traps glucose inside hepatocytes by its phosphorylation at six-carbon (glucose-6 phosphate), further corroborates to increase glycolytic rate and, as GLUTs, high HK activity sensitizes HCC cells to invasiveness and metastasis [143, 144]. A microarray analysis of 153 HCC subjects revealed that HK2 was overexpressed in both dysplastic and carcinogenic tissues. Consistently, liver-specific HK2 knockout (KO) mice reduced the incidence of DEN-induced HCC, ameliorated oxygen consumption rate (OCR) and response to Sorafenib, a multikinase inhibitor exploited as pharmacological approach in advanced HCCs [143]. Notably, a novel HK domain containing 1 (HKDC1) isoform led to liver tumorigenesis by Wnt/β-catenin cascade and associated with HCC poor outcome and metastasis [145]. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), catalyzing the sixth step of glycolysis, exerts pleiotropic roles and it is stably expressed within cells to the extent it is duly used as reference gene in research fields. However, it has been demonstrated that GAPDH may regulate mTOR-C1 signaling and cell growth according to glucose availability [146]. In addition, GAPDH can interact with voltage-dependent anion channel (VDAC1) to activate apoptosis by inducing IMMs permeabilization, loss of the inner transmembrane potential, matrix swelling, release of cytochrome c and AIF [147]. In GAPDH transgenic mice and in HCC murine models induced by DEN, GAPDH overexpression accelerated tumor development and progression by regulating inflammatory cytokines (Il-6, Il-1β, Mcp1, Icam1, Vcam1) at transcriptional level and redirecting metabolic intermediates of glycolytic flux towards one-carbon cycle, essential for cell proliferation [148]. Higher mRNA levels of GAPDH have been also observed in HCC biopsies compared to non-HCC adjacent sides and to normal liver [149].

According to the Warburg's theory, aerobic glycolysis adopted by tumoral cells results from changes of mitochondrial metabolism and, specifically, it involves pyruvate fate. At final phases of glycolysis, pyruvate kinase (PK) converted phosphoenolpyruvate (PEP) into pyruvate which, in turn, can be used to generate acetyl-CoA by pyruvate dehydrogenase (PDH) thus linking cytosolic glycolysis to mitochondrial TCA. In HCCs, hypoxic conditions stimulate

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HIF-1α to inhibit PDH and pyruvic acid is re-routed towards lactate production through lactate dehydrogenase (LDH). Several findings revealed that HFD models alter hepatic GLUT1/3 expression and aggravate LDH levels supporting the pathogenic role of lipids on mitochondrial dysfunction and glucidic reprogramming since the early events of NAFLD [150]. Moreover, lactate overflowing may participate to H_2O_2 production in isolated mitochondria from murine hepatic tissue, directly taking part to oxidative damage [151]. A meta-analysis including 10 non-randomized controlled studies highlighted that pre-operative increase of LDH levels significantly associated with poor life-expectancy in individuals affected by HCC, thus appearing a promising factor to evaluate HCC prognosis [152].

Finally, the abundance of mitochondrial pyruvate carriers (MPC) which transfer pyruvic acid from cytosol to mitochondria for its oxidation is reduced in HCCs thereby sustaining tumorigenesis and glucose utilization. It was observed that low MPC1 protein expression may represent an attractive biomarker to monitor tumor relapse over the time period following hepatectomy in HCC patients [153]. Notwithstanding, Tompkins et al showed that MPC disruption in a murine model of HCC induced by N-nitrosodiethylamine plus carbon tetrachloride (CCL_4) administration impairs hepatocarcinogenesis by hijacking glutamine onto Krebs cycle rather than glutathione synthesis, whose downregulation impedes tumor growth [154].

5.1 Mitochondrial dynamics in HCC: embarking on new paths for novel therapeutic targets

Contradictory results concerning the alterations in mitobiogenesis and mitophagy have prompted to misleading information about the role of mitochondria in NAFLD-related HCC and need to be deeply elucidated. Alterations of mitochondrial morphology towards a fragmented shape are suggestive of carcinogenesis and a metastatic phenotype. Consistently, low levels of fusion proteins have been observed in human HCC micrographs compared to non-cancerous counterpart and were associated to cancer development, progression and refractoriness to medical treatments. MFN1 reduction was correlated either with EMT and reprogramming of glucose metabolism alongside vascular invasion and poor outcome of HCC patients [82], while MFN2 overexpression induced cell death by enhancing cytochrome c release, Ca^{2+} entry and lowering mitochondrial membrane potential in HepG2 cells thus inhibiting HCC cell growth [155, 156]. Up to 40% of HCC patients decreased hepatic expression of OPA1 isoforms [157, 158]. Among them, OPA1-Exon4b is mandatory for maintenance of mitochondrial respiration and IMMs potential as well as it regulates mitochondrial bioenergetics at transcriptional level by binding D-loop region of mtDNA [158]. In HCC, inhibition OPA1-Exon4b causes low ATP synthesis and compromises membrane potential along with alterations of TFAM distribution, an essential factor in determining the abundance of the mitochondrial genome by regulating its packaging, stability, and replication [158]. Low mtDNA copy number in HCC has been associated with reduced proliferation and migration although it improved chemotherapy sensitivity [159].

In contrast to aforementioned evidence, Li et al revealed that *Mfn1* and *Opa1* knockdown hampered cell proliferation paralleled by apoptosis, low O_2 consumption and ATP production in HCC organoids and *in vivo* models [160]. Furthermore, mitochondrial dimension was found larger in 10 HCC specimens compared to non-tumoral tissues suggesting that mitochondrial elongation may sustain cancer metabolism and growth [160]. These findings were supported by analyzing 5 HCC cohorts from the Oncomine microarray database (https://www.oncomine.org), including more than 400 HCC patients of whom RNA-seq data were available [160]. It has been reported that OPA1 expression is strictly involved in sensitizing HCC cells to cytotoxicity induced by Sorafenib [161]. Zhao and

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collaborators demonstrated that Sorafenib exposure led to mitochondrial fragmentation by downregulating OPA1 and enhancing apoptosis. Additionally, Sorafenib mixed to FH535, a β-catenin antagonist, synergically targeted complexes of respiratory chain and reduced aerobic glycolysis supporting that Sorafenib-FH535 treatment may overcome the low efficacy of current single pharmacotherapies used in HCC [162].

Emerging evidence has pointed out that mitochondrial fission promoted proliferation, tumor microenvironment and invasiveness. In 15 HCC tissues, Huang et al observed a prevalence of globular mitochondria with a lower length compared to those of matched non-HCC tissues accompanied by high DRP1 protein expression and reduced mitofusins. Fragmented mitochondria staunching in the hepatocytes exacerbate ROS production, which stimulates Akt-mediated NF-kB activation and cell cycle activity alongside the inhibition of *TP53* gene and autophagy [133]. DRP1 also enhances HCC growth by promoting G1/S phase transition through coordinately modulating NF-kB and p53 pathways. Consistently, *Drp1* silencing led to cell cycle arrest in both *in vitro* and in preclinical models [24]. In HCC mice, Drp1 overexpression caused mtDNA stress promoting Ccl2 secretion and infiltration of tumor-associated macrophages (TAMs). Moreover, DRP1 levels correlated with the percentage of TAMs in 69 HCC biopsies [163] and modulates the efficacy of chemotherapy response. Cisplatin coupled to DRP1 inhibitor (Mdivi-1) synergically activated apoptosis by augmenting Bcl2-associated X/Bcl extra-large (Bax/Bcl-xL) ratio and increasing both mitochondrial membrane permeability and cytochrome c release [164].

5.2 Recovery of mitophagy in HCC: friend or foe?

Mounting evidence highlighted that mitochondrial quality control and mitophagy may attenuate chronic liver injury in the NAFLD/NASH background and, consequently HCC occurrence, by rescuing mitochondrial integrity and functions [20, 77, 89, 165-167]. As previously described, deranged mitochondria increase oxidative damage and release of hepatocyte-derived mito-DAMPs (*i.e.* mtDNA) which may recruit Kupffer cells, exacerbate innate immune response and activate HSCs thus promoting carcinogenesis [100]. Empowering mitophagic processes by overexpressing E3 ubiquitin ligases (*Fundc1*, *Bnip3*) prevent DEN-induced HCC in mice and alleviate inflammasome cascade, JNK signaling, cell proliferation, migration and invasiveness [168, 169]. Excess of mitophagy may cause ATP shortage and Ca^{2+} mobilization from ER affecting filamentous actin polymerization and lamellipodium-based migration of malignant hepatocytes [169]. Consistently, *Parkin* null mice reduced body weight but showed liver enlargement as they spontaneously developed advanced HCC paralleled by high AFP and β-catenin expression thus recapitulating human HCC [170]. Parkin downregulation was detected in up to 80% of HCC cases and PINK1 inhibition was associated with poor clinical outcome [171-173]. Notably, hypoxia may reduce PINK1/Parkin-dependent mitophagy resulting in loss of mitochondrial cristae, mass and augmented CSC progenitors [171].

Notwithstanding, unbalanced mitophagy may initiate and/or accelerate hepatocarcinogenesis to the extent that blocking mitophagy may restore Sorafenib sensitivity [174]. Huang et al showed that number of mitochondria in fission state was highly frequent in HCC tissues compared to adjacent non-tumors ones and these morphological alterations were accompanied by enhanced autophagic processes [133]. Furthermore, ERK/HIF-1α-mediated BNIP3 upregulation promoted the acquisition of anoikis resistance, a type of programmed cell death occurring when cells lose their attachment to the ECM and to neighboring cells [175]. Liu and coworkers demonstrated that activation of PINK1-

dependent mitophagy both provides a selective removal of the onco-suppressor p53 and maintains hepatic stem cell population [176].

5.3 Metabolic and epigenetic dysregulation of mitochondrial metabolism in HCC: a huge variability

Regulation of mitochondrial turnover mostly occurs in response to low-energy conditions and through AMPK/SIRT/PGC1α nutrient sensors. Controversial pre-clinical and human studies reported that both up and down-regulation of AMPK/SIRTs/PGC1α network can led to glycolytic reprogramming and HCC [177] thereby rendering mandatory to focus research efforts to elucidate discrepancies.

Several investigations reported that PGC1α deficiency was associated with degeneration of mitochondrial morphology [178], de-differentiation of cultured hepatocytes [178, 179] and high glycolytic rate in human HCCs [180]. Downregulation of PGC1α expression has been reported in publicly available repositories as The Cancer Genome Atlas (TCGA, https://www.cbioportal.org/) and GSE14520 (https://www.ncbi.nlm.nih.gov/geo/info/overview.html) containing transcriptomic analysis of HCC patients, and in clinical findings [178, 181]. Recently, loss of PGC1α correlated with poor prognosis and favored the *Warburg* effect through the WNT/β-catenin/pyruvate dehydrogenase kinase (PDK) axis, and metastasizing in HCC patients [181, 182]. Likewise, low SIRT3 expression was associated with serum AFP levels, which has both diagnostic and prognostic value in the context of HCC, tumor multiplicity and high relapse rate thus representing a prognostic marker of overall survival in HCC patients [71]. Importantly, it has been reported that SIRT3 can link to four hallmarks of cancer such as genomic instability, sustained proliferation, dysregulated energetic status, and tumor-promoting inflammation. However, in HCCs, SIRT3 acts as tumor suppressor as it mitigates ROS-induced hepatocellular injury and interacts with glycogen synthase kinase 3 beta (GSK-3β) to induce Bax translocation to the mitochondria causing apoptosis [72].

On the other hand, it has been reported by Bhalla et al. that *Pgc1α*$^{-/-}$ mice were protected against DEN-induced liver cancer and Pgc1α overexpression in mice induced gene expression reprogramming supporting DNL, glycolysis and oxidative metabolism [183]. In keeping with these findings, several studies showed that sirtuins, NAD^+-dependent epigenetic modifiers of liver metabolism, mitochondrial functionality, telomere length and genomic stability, were repeatedly found overexpressed in human HCC cell lines such as HKC1-4, SNU-423, HKC1-2, PLC5 SNU-449, SK-Hep-1, Huh-7, HepG2, Hep3B and in multiple malignancies [184-188]. SIRT1 upregulation triggers mitobiogenesis *via* PGC1α and correlates with tumor microvascular invasion, advanced TNM score and predicted HCC recurrence [188]. *Sirt1* genetic ablation (*Sirt1*$^{-/-}$) mitigated tumor growth and invasiveness in both *in vitro* and *in vivo* models. Interestingly, when mitochondrial biogenesis was re-activated by overexpressing Pgc1α in *Sirt1*$^{-/-}$ mice, they re-developed an aggressive HCC phenotype, suggesting that mitobiogenesis may sustain tumor progression [188]. It has been shown that SIRT6 overexpression favors hepatoma cell proliferation through extracellular signal-regulated kinases 1/2 (ERK1/2) pathway [187, 189], whereas SIRT2 plays a critical role in promoting HCC metastasis and invasion rather than cell growth by re-routing liver cancer metabolism [186]. SIRT2 mediated mitochondrial deacetylation and stabilization of phosphoenolpyruvate carboxykinase (PEPCK), involved in gluconeogenesis by catalyzing the conversion of oxaloacetate into PEP, and glutaminase (GLS), which converts glutamine into glutamate, thus replenishing Krebs cycle of glucidic and aminoacidic substrates [186]. Either PGC1α-mediated mitochondrial biogenesis or the fueled citric acid cycle enhanced NADH/ATP production and O_2 consumption rate, and both participate to the occurrence of HCC

metastasis [186, 190]. In addition, both SIRT2 and SIRT6 inhibits the adhesion properties of E-cadherin proteins further supporting its role in cell migration and invasiveness [186, 187].

Just recently, Zhao and collaborators provided the first evidence that long non-coding RNA (lncRNA) MALAT1 controls metabolic reprogramming in hepatocytes and its downregulation increased the number of swollen mitochondria along with dampened OXPHOS, ATP production and mtDNA copy number. Additionally, *MALAT1*-deficient cells affected mitophagy by reducing PINK1, SQSTM1/p62, NDP52, BNIP3 and LC3 expression thereby supporting its oncogenic role as lncRNA in HCC onset [191].

5.4 The impact of hypoxia on hepatic metabolic reprogramming and mitodynamisms

The liver is the major organ that stocks nutritional surplus into glycogen, LDs, cholesterols, and supplies energy-producing substrates to the peripheral tissues even under fasting conditions. A remarkable amount of O_2 is required to regulate anabolic and catabolic processes occurring in the hepatocytes, thus causing a steep O_2 gradient throughout the hepatic lobules that may get back to normoxia through cell adapting systems [121]. HIF-1 and HIF-2, oxygen-sensitive transcription factors recognizing hypoxia response elements (HRE) on promoter regions, mediate cellular adaptation to low oxygen and regulate both glucose and lipid metabolism, respectively [192]. Excessive dietary intake together with fatty liver, necroinflammation, fibrotic scars and tissue regeneration may bother hepatic oxygen distribution thus precipitating pathological hypoxia. It has been reported that HFD provoked hepatic hypoxia and impaired mitochondrial dynamics and functions (*i.e* defective β-oxidation) through HIF-2/PPARα pathway thereby exacerbating NAFLD progression [192-194]. Fat-laden hepatocytes receiving cobalt chloride (CoCl2) to mimic hypoxia increased mitochondrial superoxide production and released extracellular vesicles enriched in chemoattractant cytokines (i.e., IL-1β, IL-6, TNF-α, iNOS, NLRP3) for KCs and inflammasome activation [171, 195]. DEN-treated mice fed with Western diet increased the expression of both HIF-1α and pro-inflammatory cytokines, allowing the recruitment of TAM with a M2 phenotype and the switching from NASH to HCC [120].

Under hypoxic environment, HIF-2 overexpression may drive NAFLD-HCC development by triggering the PI3K/Akt/mTOR cascade and inducing lipid reprogramming [196], while HIF-1α stabilization forces the switching from OXPHOS to aerobic glycolysis by upregulating glycolytic enzymes and LDH [197]. Moreover, low oxygen availability induced high mobility group box 1 (HMGB1)-TLR9 binding which mediates PGC1α phosphorylation and activation, thereby sustaining mitochondrial biogenesis and cell proliferation in *in vitro*, *in vivo* and in human HCC samples [198]. It has been shown that hypoxia causes inefficient electron transfer to mitochondrial respiratory chain and affects mitophagy. Chiu and collaborators found HRE consensus sequence on promoter of the Hairy/enhancer-of-split related with YRPW motif protein 1 (HEY1) gene, belonging to NOTCH signaling. HEY1 was overexpressed in HCC patients who underwent surgical resection and in 49 HCC cases from TCGA dataset. HEY1 transcriptionally represses PINK1 in Huh7, reduced mitochondrial mass and altered inner cristae morphology [171].

6. The link among NAFLD, mitochondrial dysfunction and HCC: the relevance of genetics

Environmental factors, above all IR and obesity, influence NAFLD pathogenesis. However, it has emerged that there is a substantial variability in hepatic lipid deposition among individuals with the same grade of adiposity, raising the possibility that several other risk factors may participate to steatosis development. Familial, twin, and epidemiological studies indicated that NAFLD has a strong heritable component which contributes to the huge inter-indivitual

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phenotypic variability. Dongiovanni et al demonstrated that hepatic fat accumulation represents the main driver of the progression to the end-stage of liver damage in genetically predisposed individuals and recently proposed a detailed review including all the candidate genes related to NAFLD susceptibility [199, 200].

Currently, the rs738409 C>G single nucleotide polymorphism (SNP) in the *Patatin-like phospholipase domain containing 3* gene (*PNPLA3* or adiponutrin) is the major genetic variant associated to NAFLD onset and its progressive forms, including HCC. PNPLA3 mainly localized on ER and LDs surface in hepatocytes, adipocytes and HSCs and it may be transcriptionally induced or post-translationally modified to provide TG hydrolysis during post-prandial or hyper-insulinemic state. Patients carrying the G allele lost PNPLA3 enzymatic activity, which impedes TG disposal and interferes with the activity of other lipases as PNPLA2 [200, 201]. Beyond the triacylglycerol remodelling, PNPLA3 exerts widespread effects on human liver metabolome [202], influencing mitochondrial functions, glucose reprogramming and tumorigenesis. Huh-7 hepatoma cells overexpressing the PNPLA3 I148M variant showed high levels of lactate and γ-glutamyl-amino acids, thus mirroring the metabolic switching to aerobic glycolysis and mitochondrial failure, respectively [202]. Moreover, the rs738409 SNP impacts on retinol secretion in HSC cells leading to the myofibroblast-like phenotype, collagen deposition and boosting fibrogenesis in NASH subjects [203]. In a small cohort of 54 NAFLD subjects, it has been demonstrated that carriers of G risk allele had a severe profile of liver disease, characterized by enhanced steatosis, activation of pro-inflammatory pathways and an increased proliferative activity of hepatocytes [204]. Interestingly, Bruschi et al demonstrated that the presence of I148M substitution in the *PNPLA3* gene further affected metabolic reprogramming in TGFβ-activated HSCs, shifting towards aerobic glycolysis, lactate release and activation of YAP/Hedgehog signaling [205].

The rs641738 C>T variant in the *Membrane bound o-acyltransferase domain-containing 7/Transmembrane channel-like 4* (*MBOAT7/TMC4*) locus, encoding the MBOAT7 enzyme, was associated with the entire spectrum of NAFLD, including HCC [206]. Recently, the role of *MBOAT7* variant in the NAFLD progression has been evaluated in a large meta-analysis. Data were collected from 1047.265 subjects, of whom 8303 had liver biopsies, and displayed a correlation between the T minor allele and hepatic fat deposition, ALT levels and advanced stages of NAFLD, like fibrosis and HCC. In particular, carriers of the rs641738 variant show the 30% risk to develop HCC compared to non-carriers [207]. Physiologically, MBOAT7 localizes on MAMs and mediates phosphatidylinositol (PI) acyl-chain remodeling in the Land's cycle. Our group demonstrated that hepatic MBOAT7 expression is reduced during hyperinsulinemia and by the presence of the rs641738 C>T variant [208, 209]. MBOAT7 downregulation induces an enrichment of saturated PIs which are shunted towards the synthesis of TGs thus participating to fat accumulation. Though no evidence linking MBOAT7, mitochondrial lifecycle and metabolic reprogramming has been currently reported, it could be postulated that the wealth of saturated lipids induced by the MBOAT7 downregulation may affect membrane composition and dynamics, possibly breaking ER-mitochondria communications.

The rs58542926 C>T variant in the *Transmembrane 6 Superfamily member 2* (TM6SF2) gene induces TM6SF2 loss-of-function and hastens its hepatic protein turnover [210]. TM6SF2 dwells on ER-Golgi compartments where occur fat biosynthesis, LDs and lipoprotein formation. TM6SF2 inactivation induced by the presence of the polymorphisms impair assembly and trafficking of very low-density lipoprotein (VLDL) which remains trapped into hepatocytes [210]. In Huh-7 cells, *TM6SF2* deficiency reduces the amount of PUFAs along with alterations of mitochondrial β-oxidation and higher number of lysosomal compartments [211]. In small intestine of zebrafish, the *TM6SF2* loss-of-function

induced changes in ER architecture appearing with enlarged cisternae, supporting the notion that TM6SF2 may impact on organelles' morphology [212]. However, the rs58542926 polymorphism has been associated with NAFLD/NASH spectrum, its role in HCC development remains to be still explored. A meta-analysis including 24,147 subjects affected by chronic liver disorders, revealed that the presence of the T risk allele was correlated to higher risk to develop NAFLD and its advanced stages as HCC [213]. Raksayot et al performed a cross-sectional study in a cohort of 502 NAFLD patients and observed that carriers of T allele are led to a higher risk of HCC progression [214].

To deepen inside the mechanisms underlying NAFLD pathogenesis and to investigate possible synergisms among *PNPLA3*, *MBOAT7* and *TM6SF2* leading to hepatocytic metabolic rewiring, our group has generated *in vitro* models of genetic NAFLD. We stably silenced *MBOAT7* (MBOAT7$^{-/-}$), *TM6SF2* (TM6SF2$^{-/-}$) or both genes (MBOAT7$^{-/-}$TM6SF2$^{-/-}$) in HepG2 cells, homozygous for the I48M *PNPLA3* variant, by exploiting Clustered Regularly Interspaced Short Palindromic Repeats/CRISPR-associated protein 9 (CRISPR/Cas9) technology [10, 11, 208]. MBOAT7$^{-/-}$ spontaneously accumulated giant LDs associated with a dramatic increment of ROS and peroxides levels, while TM6SF2$^{-/-}$ and MBOAT7$^{-/-}$TM6SF2$^{-/-}$ models showed mitochondria with small and globular shape, loss of cisterns 'architecture and ultrastructural electron density, suggestive of mitochondria degeneration. Number of mitochondria were progressively increased in all mutated cell lines supporting that either *MBOAT7* and/or TM6SF2 deficiency impact on mitochondrial biogenesis. Notably, the compound knockout re-routed its metabolism towards a glucose-dependent ATP production, enhancing glycolytic enzymes, LDH and lactate release thereby supporting that the depletion of both *MBOAT7*/*TM6SF2* combined to the genetic background of I148M *PNPLA3* may affect mitochondria turnover possibly accelerating metabolic reprogramming [10, 14].

Recently, it has emerged the opportunity to translate the genetics into clinics by aggregating these genetic variants into polygenic risk scores which may better discriminate NAFLD patients at-risk to develop progressive liver damage and HCC [215]. In a large cohort of biopsied NAFLD patients, it has been observed that the cumulative number of risk alleles associated with serum markers of disease severity and increased risk to develop HCC [10, 216, 217]. A cross-sectional study consisting of 2566 NAFLD partecipants has evaluated the impact of these genetic polymorphisms on hepatocytic fat accumulation in HCC progression. For this purpose, the authors generated a polygenic risk score based on the presence of variants in *PNPLA3, TM6SF2 MBOAT7* and *GCKR* genes, which was able to predict HCC occurrence much more than the presence of a single genetic variant [215].

6.1 Mitochondrial polymorphisms are correlated with NAFLD pathogenesis

Several polymorphisms in mitochondrial genes have been associated with NAFLD development and progression. In genetically modified mice, the non-synonymous nt7778 G>T genetic variation in the mitochondrial ATP synthase protein 8 (mt-ATP8) increased the susceptibility to diet-induced NASH [218]. Identification of mitochondrial haplotypes were even associated to NAFLD predisposition opening new avenues for mito-genetic screening in patients and new experimental purposes [219].

Manganese-dependent SOD, encoded by the nuclear *SOD2* gene, mitigates oxidative damage by catalysing the conversion of superoxide radicals to hydrogen peroxide. The rs4880 C47T variant in the *SOD2* gene encodes the valine to alanine amino acid substitution at the position 16 in the signal region targeting the protein to the mitochondrial matrix. The C47T mutation causes a reduction of MnSOD2 activity and the consequent failure to neutralize superoxide

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radicals. In case-control and familial studies, Al-Serri et al. demonstrated that the inherited T risk allele was an independent predictor of NASH severity and strictly associated with fibrosis in both adult and children [220]. Conversely, the -866 G>A polymorphism localized in the promoter region of *UCP2* gene, involved in heat dissipation, has been associated with a reduced risk of obesity [221]. The A allele promotes UCP2 overexpression in the liver and has a protective role in the progression from simple steatosis to NASH [222]. Likewise, the rs1800849 -55 C/T *UCP3* variant ameliorates the circulating lipid profile and correlated with loss of body weight [223]. These findings were not confirmed by Aller at al and Qian at al, who associated both the -866 G>A polymorphism and the rs1800849 variant to higher risk of IR, obesity, lower levels of adiponectin, severe steatosis and inflammation in NAFLD subjects [224, 225]. Polymorphisms in Sirtuins further take part to regulation of mitochondrial functionality and dynamics, possibly contributing to NAFLD/NASH advancement and its cardiovascular comorbidities [226]. Patients carrying the rs11246020 variant (V208I) in *SIRT3* gene displayed a higher susceptibility to NAFLD. Consistently, *Sirt3* knockout mice feeding HFD showed IR and worsened adiposity and NASH [227]. The rs107251 in *SIRT6* gene affected SIRT6 activity influencing its role in DNA repair and maintenance of telomeric chromatin [228]. It has been described that the rs7895833 G>A in *SIRT1* gene represents a risk factor for body fat content and high diastolic blood pressure [228, 229]. Interestingly, low SIRT1 levels were detected in 70 cirrhotic HCC patients carrying the rs7895833 variant and SIRT1 reduction inversely correlated with high AFP , Child-Pugh score and tumor stage [230].

Recently, it has been described the rs2642438 A165T polymorphisms at N-terminal domain of the *Mitochondrial amidoxime reducing component 1* (*MARC1*) gene, localizing on MOMs. The A165T variant has been associated with low fat content in the liver and reduced risk of NALD progression toward cirrhosis. Such findings were independently validated by Lukkonen et al, which showed that carriers of the rs2642438 variant alleviated NASH severity accompanied by an improvement of hepatic lipid profile, mainly consisting of polyunsaturated phosphatidylcholines, thus supporting that *MARC1* could represent a candidate therapeutic target [231, 232].

6.2 Rare NAFLD pathogenic variants are involved in the switching towards HCC

Part of the missing hereditability in NAFLD may be attributed to rare genetic variants with a large effect size.

Rare mutations in the *telomerase reverse transcriptase* (*TERT*) promoter may arise in NAFLD-cirrhosis, in 10-20% of both low-grade and high-grade dysplastic nodules and in familial HCC, supporting that *TERT* germline genetic variants may be involved in tumor initiation [233]. In a cohort of 40 NAFLD-HCC, 45 cirrhotic patients and 64 healthy controls, telomere length decreased with the progression of NAFLD towards cirrhosis and mainly with HCC [234]. Four rare mutations have emerged in the *hTERT* gene among NAFLD-HCC subjects such as the Glu113Arg_fs*79 frameshift in the second exon and 3 missense mutations (Ala67Val, Pro193Leu, Glu668Asp) which correlated with shorter telomere length. In particular, the Ala67Val and Glu668Asp SNPs led to TERT loss-of-function and decreased its hepatic expression. On the contrary, the Pro193Leu substitution did not affect TERT catalytic activity but reduced its chromatin binding capacity [235]. Furthermore, in a cross-sectional study, it has been observed that NAFLD-HCC patients showed an enrichment of rare genetic variants in *Regulator of telomere elongation helicase 1* (*RTEL1*) and *Telomeric repeat binding factor 2* (*TERF2*) genes, that are involved in telomere preservation, and in *RB1* which mediates the oxidative stress response. Mutations in *STK11*, *TSC1*, *TSC2*, *NF2* and *SMAD4* candidate genes, which regulate cell growth and proliferation, were also strongly correlated to HCC risk [236].

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HCC surveillance may be addressed to NAFLD subjects with family history of hypobetalipoproteinaemia caused by *ApoB* mutations. *ApoB* gene is involved in hepatic lipid metabolism and its genetic variants lead to an impaired synthesis of ApoB100 with the consequent alteration of hepatic VLDL export. Uncommon variants in *ApoB* gene result in the impairment of VLDL export and development of severe hepatic steatosis. Some of the genetic variants causative of ineffective ApoB100 synthesis may even alter ApoB48 isoform expressed in the enterocytes provoking malabsorption of fat and insoluble vitamins, retention of chylomicrons and alterations of intestinal barrier [237].

Finally, a novel association between variants in Sequestosome 1 (*SQSMT1*) and HCC onset have been identified in NAFLD-HCC patients. *SQSMT1* encodes the ubiquitin-binding protein p62, an autophagosome cargo protein that targets other proteins for selective autophagy. p62 takes part to Mallory-Denk bodies (MDBs), a cytoplasmatic protein aggregates found in several chronic liver diseases including NAFLD as well as in HCC, and it is involved in the hepatocytes transformation through the activation of mTOR pathway and regulation of telomere length machinery [30, 238].

Concluding remarks

Over the last two decades, NAFLD endured an upsloping trend mirroring both lifestyle changes (wealth in food supplies, lack of physical activity, etc.) and increasing incidence of MetS worldwide. Up-to-now, NAFLD represents the commonest chronic liver disorder affecting both adult and pediatric populations and it will rapidly become the foremost cause of LT and HCC even due to the high-efficacy of anti-viral therapies.

One of the key steps of NAFLD pathophysiology is the burst of mitochondrial dysfunction, which may arise from the earliest events of fatty liver in response to environmental, genetic and epigenetic factors, supporting a new viewing of NAFLD as a mitochondrial disorder. Ever-growing findings pointed out that mitochondria adapt in number, size, morphology, and organelle communications to overcome energy demand and to provide essential macromolecules for cell viability. Throughout NAFLD course, hepatocytes are exposed to endogenous hardship and extrinsic constrains rendering burdensome survival of parenchymal cells. In this context, mitochondria actively participate to evade apoptotis, to escape inflammation, to induce mutagenesis through oxidative damage and to rewire hepatocellular metabolism with the goal to favor clonal selection of malignant hepatocytes forming tumor mass (**Figure 1**).

The study of mitochondrial dynamics in NAFLD/NASH and NAFLD-related HCC is challenging and currently still at dawn due to the high phenotypic variability which features this complex disease. Liver resection and transplantation represent the ongoing curative options in NASH-related HCC. Therefore, the deep knowledge of mitochondrial lifecycle and networking in metabolic disorders, especially those severely impacting on HCC susceptibility, may offer novel insights for research purposes. On the other hand, it may pave the way for the development of new mitochondrial-based strategies which could be introduced into clinics for either preemptive use or as coajuvators of approved treatments exploited in HCC patients 'care.

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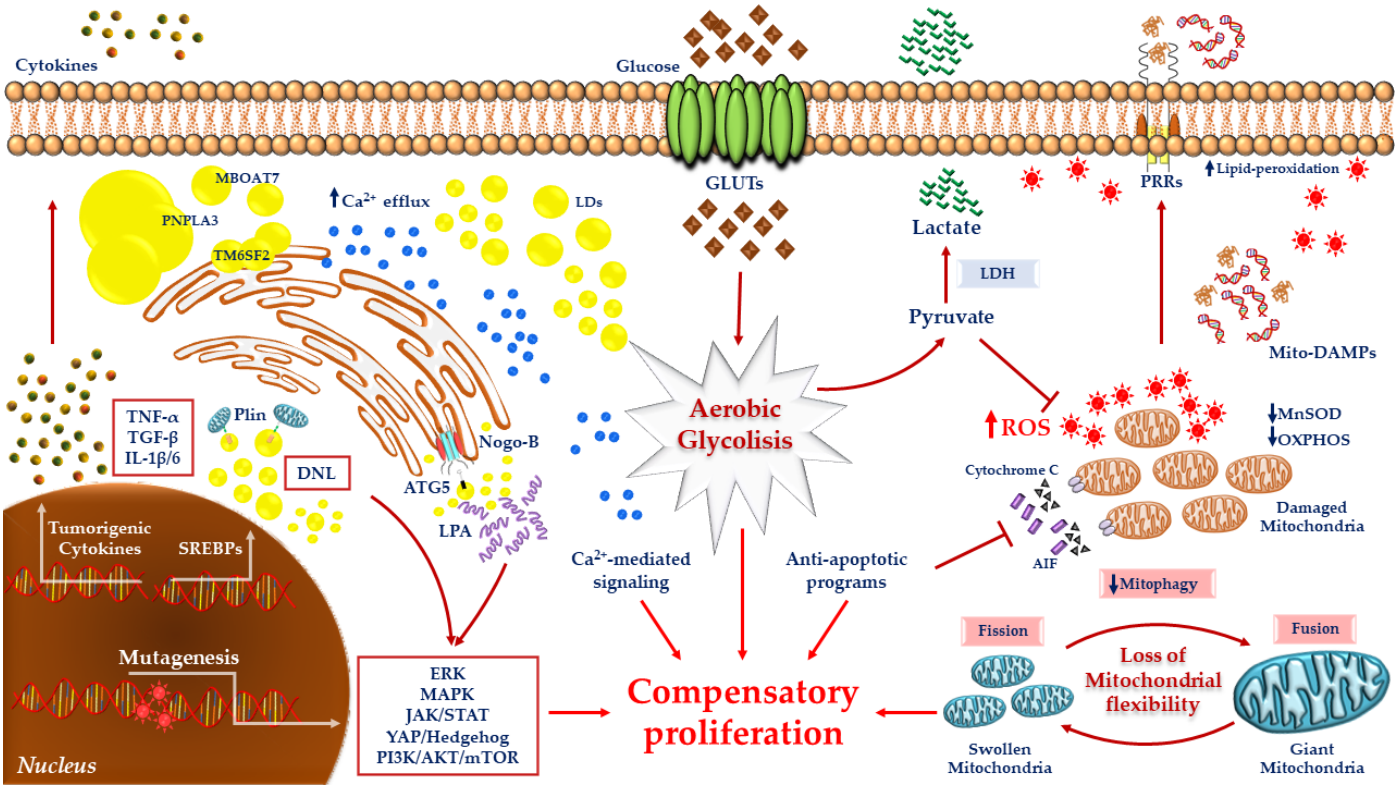

Figure 1**: **Mitochondrial pathways implicated in the pathogenesis of NASH-related HCC. Sedentary lifestyle, dietary habits and genetic background contribute to NAFLD development and its progression to NASH-driven HCC. The excess of hepatic fat accumulation promotes DNL, mitochondrial dysfunctions and distruption of ER-mitochondrial contact sites. Consequently, the increased content of LDs may favour either lipotoxicity or energetic substrates for cell viability. Loss of mitochondrial flexibility and reduced mitophagy exacerbate the number of degenerated mitochondria, which produce ROS and release cytochrome C and AIF, thus activating apoptosis. The oxidative stress overwhelmes anti-oxidant defences, affects OXPHOS capacity and triggers inflammatory signals (tumorigenic cytokines). In addition, ROS induce mutagenesis of both nuclear DNA and mtDNA causing aberrant activation of proliferative pathways and delivery of mito-DAMPs, respectively. Mito-Damps, including mitochondrial formyl-peptides and mtDNA fragments, could activate PRR receptors on hepatocellular surface, KCs and HSCs, prompting inflammation and fibrosis. ROS-induced pro-survival signaling (i.e. JAK/STAT, ERK, MAPK) are able to counteract cell death by inducing degradation of onco-suppressors and expanding the amount of hepatic progenitor cells. Moreover, the interruption of ER-mitochondria communication rises the efflux of cyotosolic Ca^{2+}, further participating to ROS production and mutagenesis. Finally, hepatocytes undergo metabolic reprogramming, characterized by enhanced glucose uptake, high rate of glycolisis, and lactate production, that is rapidly secreted to avoid the cytosolic acidification. Overall, a lipid-rich microenviroment combined to early loss of mitochondrial adaptability, both hallmarks of NAFLD onset and progression, may rearrange hepatocellular metabolism and the interplay between hepatocytes and non-parenchymal cells in order to overcome an adverse environment and trigger tumorigenesis.

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