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Review

Role of Oxidative Stress in Liver Disorders

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Abstract: Oxygen is vital for life as it is required for many different enzymatic reactions involved in intermediate metabolism and xenobiotic biotransformation. Moreover, oxygen consumption in the electron transport chain of mitochondria is used to drive the synthesis of ATP to meet the energetic demands of cells. However, toxic free radicals are generated as byproducts of molecular oxygen consumption. Oxidative stress ensues not only when the production of reactive oxygen species (ROS) exceeds the endogenous antioxidant defense mechanism of cells, but it can also occur as a consequence of an unbalance between antioxidant strategies. Given the important role of hepatocytes in the biotransformation and metabolism of xenobiotics, ROS production represents a critical event in liver physiology, and increasing evidence suggests that oxidative stress contributes to the development of many liver diseases. The present review, which is part of the special issue "*Oxidant stress in Liver Diseases*", aims to provide an overview of the sources and targets of ROS in different liver diseases and highlights the pivotal role of oxidative stress in cell death. In addition, current antioxidant therapies as treatment options for such disorders and their limitations for future trial design are discussed.

Keywords: oxidative stress; ROS; mitochondria; antioxidant; cell death; liver disease

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1. Introduction

Oxidative stress reflects the imbalance between the excessive formation of reactive oxygen/nitrogen species (ROS/RNS) and limited antioxidant defenses. A direct consequence of excessive ROS production derives from its interaction with cellular biomolecules, such as DNA, lipids, and proteins, which are modified and may cause cell death. Besides exogenous sources, such as ionizing radiation, diet, metals, pesticides or other toxic compounds, ROS are also generated endogenously as byproducts of a variety of enzymatic reactions and metabolic pathways that require molecular oxygen and are likely involved in the pathogenesis of different human diseases including liver diseases (Figure 1). Liver is one of the most critical organs in the body due to its numerous functions. Importantly, the metabolism and detoxification of alcohol and drugs are its main tasks which produce ROS as byproducts [1]. In addition, it is responsible for the storage of vitamins (A, B, D, E, and K), glycogen, and minerals like iron and copper which are involved in ROS-generating reactions. Remarkably, all chronic hepatic disorders, independently from their etiology, share as a common feature a highly oxidative milieu that perpetuates cellular damage and contributes to the progression of fibrosis, cirrhosis, and ultimately hepatocellular carcinoma, meaning that all could benefit from the same therapeutic strategies aiming to boost the antioxidant defense system.

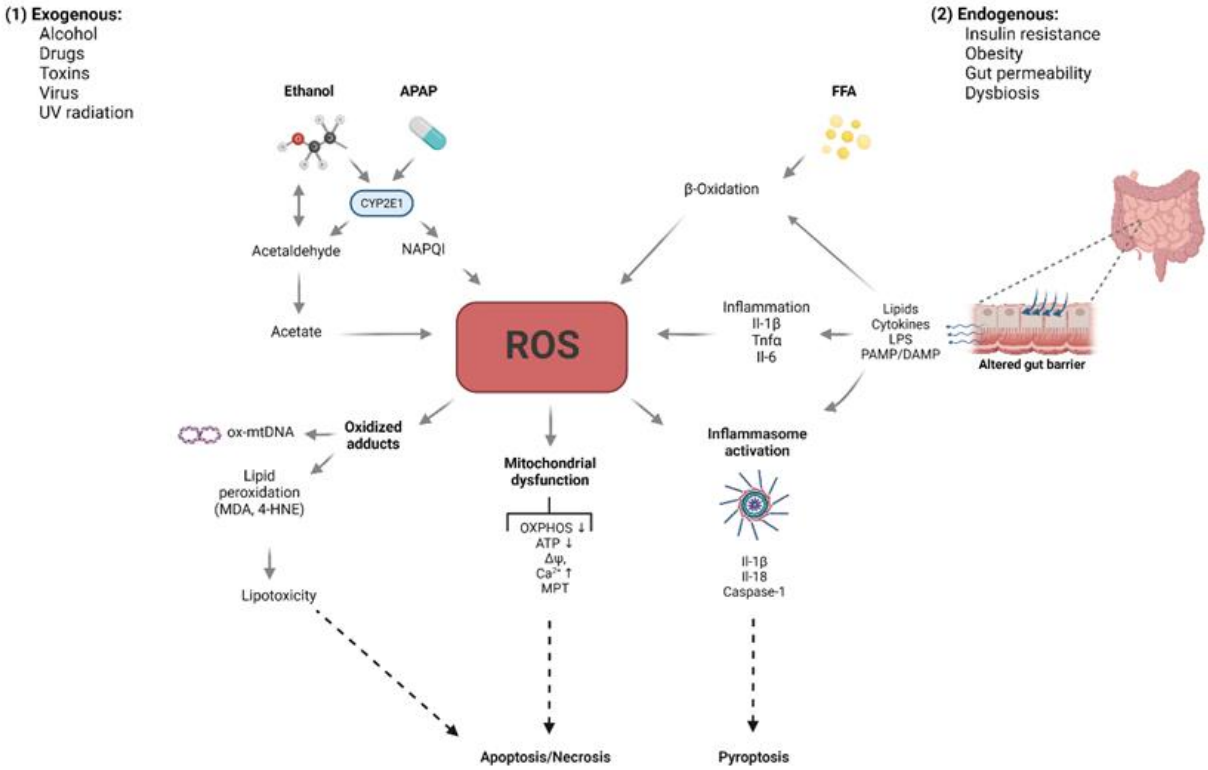

Figure 1. Reactive oxygen species can be produced by (1) exogenous sources such as alcohol, drugs, virus infection, UV light, radiation, stress, and smoking, or (2) endogenous sources during metabolic pathways in which oxygen is involved. The metabolism of molecules like ethanol, drugs (such as APAP), and FFA in hepatocytes induce an increment in ROS formation in the mitochondria that in turn provokes mitochondrial dysfunction. The accumulated ROS can react with cellular biomolecules (lipids, proteins, and DNA) and alter or impede their functioning. The generation of these oxidized adducts extends oxidative damage and favors cellular death. Moreover, ethanol consumption and lipid-enriched diets can lead to the loss of the intestinal barrier integrity permitting the entry of microbial products or saturated-FA to the circulation that release pathogen-associated molecular patterns (PAMPs)/damage-associated molecular patterns (DAMPs) and trigger the activation of cytokines (Il-1β, Il-6, Tnfα) leading to inflammation. APAP: acetaminophen; ATP: adenosine triphosphate; Ca^{2+}: calcium ion; CYP2E1: cytochrome P450 2E1 oxydase; FFA: free fatty acids; HNE-4: 4-Hydroxynonenal; LPS: lipopolysaccharide; MDA: malonaldehyde; MPT: mitochondrial permeability transition; NQO1: NAD(P)H: Quinone Oxidoreductase 1; OXPHOS: oxidative phosphorylation system; PAMPs: pathogen-associated molecular patterns; ROS: reactive oxygen species; UV: ultra violet; ΔΨ: mitochondrial membrane potential. Created with BioRender.com.

2. Free Radicals: Sources and Defense

ROS include a number of molecular species derived from oxygen, such as superoxide ($O_2^{\bullet -}$) anion, the prototype of ROS which is produced in enzymatic reactions and in the electron transport chain (ETC) of mitochondria, as well as other oxidants like hydrogen peroxide (H_2O_2) or hydroxyl radicals (•OH). The majority of ROS is produced in mitochondria [2,3], where superoxide anion can be generated as a by-product of transfer of electrons to oxygen during the respiratory chain to produce ATP [4]. Extramitochondrial sources of ROS include membrane nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, lipoxygenases, cyclooxygenases, peroxidases, other heme proteins, xanthine oxidase, peroxisomal fatty acid β-oxidation, and hepatic P-450 microsomal detoxification [5–9]. Although oxidative stress ensues through the alteration of the balance between oxidants (i.e., ROS) and antioxidants, a strict equilibrium between antioxidants (i.e., superoxide dismutase (SOD) and GSH redox cycle) is also necessary to circumvent the generation of oxidants

such as hydrogen peroxide [10]. In this regard, the cell displays antioxidant mechanisms to combat ROS of different origins which comprise enzymatic and non-enzymatic components. The enzymatic system includes different enzymes that catalyze reactions involved in ROS detoxification. Some of the most relevant are SOD, catalase (CAT), glutathione peroxidase, and reductase (GSH-Prx or Gpx), peroxiredoxins, glutaredoxins, thioredoxins, and sulfiredoxins. To increase efficiency in ROS scavenging, some of these enzymes are located in specific sites where free radicals and ROS are generated within the cells to counteract them in a more direct fashion. As an example, two isoforms of the SOD enzyme exist, the cytosolic one (Cu/Zn–SOD) and the mitochondrial one (Mn-SOD), also known as SOD-1 and SOD-2. Non-enzymatic antioxidant components include small molecules, such as glutathione (GSH), ascorbic acid (vitamin C), retinol (vitamin A), and tocopherol (vitamin E) which provide protection against radical species by accepting electrons in their structure [11]. Another key factor is the oxidative stress-induced transcriptional machinery governed by the nuclear factor E2-related factor 2 (Nrf2) which regulates the expression of various antioxidant genes [12]. Indeed, pharmacologic activation of the Nrf2-dependent antioxidant signaling pathway has been shown to protect the liver in different oxidative stress models [13]. Figure 2 illustrates the contribution of each cellular compartment to oxidative stress and the antioxidant defense mechanism to overcome ROS generation.

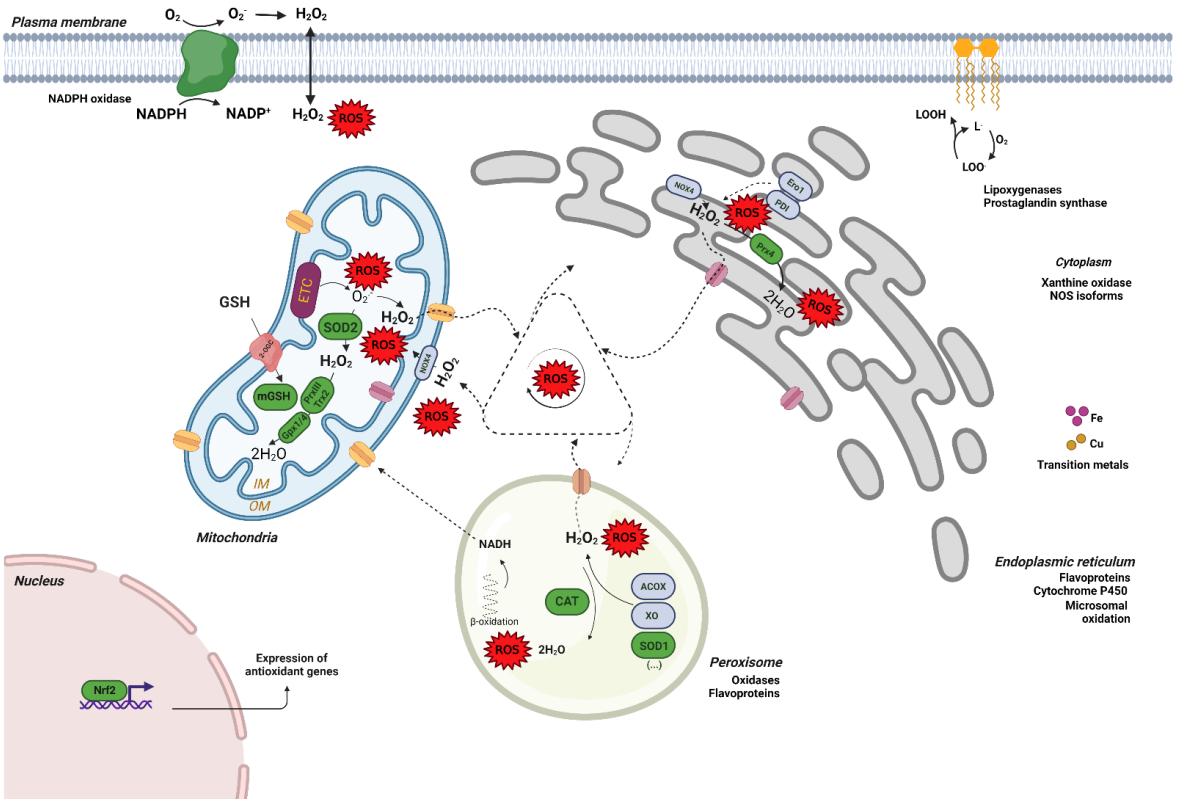

Figure 2. Major sources of ROS inside the cells. ROS is produced at different sites inside the cell such as mitochondria, endoplasmic reticulum, peroxisomes, plasma membrane or cytoplasm. Yet, ROS can be liberated through specialized channels or aquaporins and enter in neighboring organelles. To combat these highly reactive species the cell poses antioxidant mechanisms comprising enzymatic (SODs, CAT, Gpx, Trx, Prx) and non-enzymatic components (GSH, Nrf2 transcriptional activity). Interorganellar interactions are represented by dotted arrows. ACOX: Peroxisomal acyl-coenzyme A oxidase; CAT: catalase; Ero1: endoplasmic reticulum oxidoreductase 1; ETC: electron transport chain; Gpx1/4,8: glutathione peroxidase; GSH: glutathione; IM: inner membrane; NOX4: NADPH-oxidase isomerase; Nrf2: nuclear factor E2-related factor 2; OM: outer membrane; PDI: protein disulfide-isomerase; Prx: peroxiredoxin; ROS: reactive oxygen species; SOD: superoxide dismutase; Trx: thioredoxins XO: xanthine oxidase; 2-OGC: 2-oxoglutarate carrier. Created with BioRender.com.

3. Redox Control in the Liver

Due to the predominance of mitochondrial-origin ROS and the involvement of this organelle in liver diseases, attention will be focused in the strategies to attenuate mitochondrial ROS formation. Hepatocytes are well equipped with non-enzymatic and enzymatic antioxidant defense systems that neutralize free radicals. SOD enzyme is the "first line" defense in reducing superoxide radicals formed in the ETC to H_2O_2 and O_2. CAT is a major H_2O_2 detoxifying enzyme. However, mitochondrial expression of CAT is null as the localization of this enzyme is mainly peroxisomal. Therefore, the degradation of mitochondrial hydrogen peroxide is carried out by other alternative antioxidant enzymes. Mitochondrial glutathione peroxidases (Gpx1,4) and various hydroperoxides (PrxIII, Trx2) catalyze the reduction of H_2O_2 in a chain reaction with GSH as the electron donor, and the subsequent conversion of GSH disulfide (GSSG) back to GSH by the NADPH-dependent glutathione reductase (GR). Hence, mitochondrial GSH (mGSH) protects mitochondria from the lack of catalase, implying that its availability is crucial for the appropriate redox maintenance in this organelle.

GSH is exclusively synthesized in the cytosol, thus specific carriers are required to distribute GSH to different compartments including mitochondria. In the liver, GSH is transported into mitochondria via the 2-oxoglutarate carrier (OGC; Slc25a11) which imports GSH in exchange for 2-oxoglutarate (2-OG). A relevant aspect of GSH transport in hepatic disorders is the dependence of the 2-OG carrier on membrane fluidity [14] which is determined by fatty acid composition and cholesterol content. Increment of the membrane rigidity impairs this transport system, limiting mGSH availability and antioxidant protection. In this sense, previous studies in various models of alcoholic (ASH) and non-alcoholic steatohepatitis (NASH) have reported increased mitochondrial cholesterol which resulted in mitochondrial dysfunction, reduced levels of mGSH and increased susceptibility to cell demise [15–20]. In addition, by controlling cardiolipin redox status, mGSH plays a key role in regulating cell death pathways [21], which is further described later in this review.

When free radical species elude cellular antioxidant defense mechanisms, they react with macromolecules generating toxic adducts that lead to the alteration of function of all cellular components preceding apoptosis [22]. In this aspect, elevated markers of oxidative stress such as DNA damage marker 8-hydroxy-2′-deoxyguanosine (8-OHdG) and lipid peroxidation products (MDA and 4-HNE) have been observed in hepatic tissue and plasma of patients diagnosed with different liver disorders (e.g., NASH, hepatitis C, liver fibrosis or HCC), serving as potential biomarkers for disease [23–25]. Likewise, excessive oxidation of lipids modifies the physical properties of cellular membranes and can contribute to the impairment of the mitochondrial membrane potential and Ca^{2+} buffering capacity. If prolonged in time, Ca^{2+} overload elicits mitochondrial permeability transition (MPT) opening which results in a secondary burst of ROS and ignites a cascade of detrimental effects that lead to hepatocellular death as ultimate consequence [26,27].

4. Cell Aging and Cell Death Regulation in the Liver

4.1. Cell Aging in the Liver

The aging process is characterized by the gradual decay of physiologic functions that occurs with time. Several hallmark features have been identified in aging such as genetic alterations, mitochondrial impairment, loss of proteostasis, dysregulation of nutrient sensing, altered intracellular communication, and telomere shortening. Major interest has been focused in the last years in this progressive condition due to its correlation with the onset of diseases including liver disorders. Indeed, aging is associated with the severity and poor prognosis of various liver diseases including nonalcoholic fatty liver disease, alcoholic liver disease, hepatitis C, and liver transplantation [28,29]. In addition, as this organ has the capacity for regeneration, process that declines with age, fibrotic response is also altered during aging [30,31].

In the young liver solutes such as lipoproteins, insulin, and carbohydrates are able to diffuse between the blood and hepatocytes via the liver sinusoid endothelial cell (LSECs) fenestrations which are critical for maintaining hepatic homeostasis. With age, multiple changes take place in each liver cell type (hepatocytes, liver sinusoidal endothelial cells, hepatic stellate cells, and Kupffer cells (KCs)), impairing many physiological functions. Aging-related changes in liver cells include volume changes, increased polyploidy and DNA damage, accumulation of lipofuscin, impaired mitochondrial oxidative capacity, elevated oxidative stress and ROS, and presence of senescent cells with senescence associated secretory phenotype (SASP) which promote the recruitment of inflammatory cells.

Concerning liver sinusoidal endothelial cells (LSECs), these cells show reduced fenestrations, cellular autophagy, and augmented levels of cell adhesion markers during aging. These traits have been described in several species including mice, rats, non-human primates and humans, and in mouse models of premature aging [32–34]. Loss of fenestrations has particular importance as it can lead to hyperlipidemia and hepatic insulin resistance by limiting the uptake of lipoproteins and insulin [35]. In addition, LSECs also play a key role in the removal of circulating macromolecules like collagen catabolism products, hyaluronic acid or antibodies. This endocytotic activity is mislaid with aging, contributing to the disproportionate accumulation of harmless molecules in the liver [36]. Moreover, aging LSECs display a reasonable pro-inflammatory profile as evidenced by the presence of leukocytes recruited by adhesion molecules and the high expression of interleukin 6 (Il-6) cytokine and $CD68^{+}$-cells. Moreover, upregulation of p16 and downregulation of SIRT1 has been reported which might influence cellular senescence [37].

According to several studies, liver volume decreases by 20–40% as one gets older [28,38]. These changes are linked to a reduction of blood flow in the liver, as an approximately 35% reduction in blood volume of the liver is observed in individuals above 65 years of age compared with those aged less than 40 years [39]. These changes are associated with increases in portal pressure and vascular resistance leading to reduced hepatic blood flow due to downregulation of the vasodilatory pathways involving NO bioavailability in LSECs [40]. In addition, aged LSECs exhibit increased expression of intercellular adhesion molecule 1 (ICAM-1) which promotes leukocyte adhesion and contributes to further reduction of sinusoidal blood flow [40]. Regarding other hepatic cells, excessive lipid loading with marked increase in the number and size of lipid droplets and overproduction of collagen and laminin that are deposited in the basal lamina membrane account for the effect of aging on hepatic stellate cells (HSCs) [41]. Finally, KCs, major inflammatory cells in this organ, accumulate within the liver with age and attach to the adhesion markers expressed on LSECs, contributing to Il-6 release and extending inflammatory processes in the liver [37,42].

These molecular alterations lead to low-grade inflammation and facilitate multiple phenotypic changes in hepatocytes and other liver cells by the release of inflammatory signals, and will provoke cellular senescence and death as ultimate consequence. Notably, all these cellular events are observed in most types of liver disease including non-alcoholic

fatty acid liver disease, alcoholic liver disease, primary biliary cholangitis, liver fibrosis, and hepatocellular carcinoma, which will be discussed in detail in the following section.

From the therapeutic point, prevention of age-associated alterations or inhibition/modulation of senescent cells might provide benefit to the health of patients with chronic liver diseases. As an example, several compounds have already shown positive effects by increasing fenestrations in LSECs isolated from old mice, including 7-ketocholesterol, sildenafil, amlodipine, cytochalasin D, bosentan, 2,5-dimethoxy-4-iodoamphetamine, and TNF-related apoptosis-inducing ligand. In addition, two drugs that delay aging by regulating the nutrient sensing pathways, nicotinamide mononucleotide and metformin, were also found to increase fenestrations in LSECs from aged mice [43,44]. Definitively, the identification of specific markers and senescence-related pathways will provide important knowledge to improve translational application of preclinical studies in human disease.

4.2. Cell Death Regulation in the Liver

Hepatocellular death participates in most types of acute and chronic liver disorders, being a crucial pathologic factor in disease progression. Despite the fact that it essentially occurs to accomplish the specific removal of injured cells as an adaptive reaction to diverse disturbances, it also takes place as a result of inability to cope with excessive stress, involvement in the promotion of inflammation, liver injury, fibrosis, cirrhosis, and hepatocellular carcinoma.

Depending on the nature of insult, trigger mechanisms and morphologic phenotype, cell death can be divided into different types. In this review, we will focus on apoptosis, necrosis, and pyroptosis types of cell death occurring in liver cells under various liver disorders.

4.2.1. Apoptosis

Apoptosis, or active programmed cell death, is deeply organized, caused by concrete signaling cascades and characterized by nuclear fragmentation, chromatin condensation, and cellular shrinkage. It commonly occurs to control cell populations in tissues during development and aging. Apoptosis is mainly dependent on caspase activation, although caspase-independent apoptosis also exits. Apoptosis can either be initiated via intrinsic mitochondrial or extrinsic death receptor-mediated pathways, both leading to the activation of the executioner caspases -3 and -7.

Intrinsic apoptosis is induced by different aberrant events such as DNA damage, oxidative stress, ER stress, starvation, and mitotic defects [45] which trigger BAX/BAK-induced mitochondrial outer membrane pore formation (MOMP) and the leakage of mitochondrial apoptotic components such as cytochrome c and SMAC/DIABLO which bind to APAF1 and pro-casp9 forming the apoptosome which activates CASP9. In turn, CASP9 induces the activation of executioner caspase-3 and caspase-7 [45–47]. MOMP is regulated by the balance between the pro-apoptotic and anti-apoptotic members of the B cell lymphoma-2 (Bcl-2) family [46]. In contrast, the binding of ligands to death receptors such as FAS and TNFR1 triggers the extrinsic death receptor apoptotic pathway, activating CASP8 via FADD and TRADD, and subsequently CASP3 and CASP7, and apoptosis [46].

ROS are powerful inducers of apoptosis and can trigger the intrinsic mitochondrial pathway, the extrinsic death receptor pathway and ER stress pathway [48], being mitochondria crucial to activating apoptosis in all those pathways [49]. Proteins are considered the principal targets of ROS and they are very frequently oxidized [50]. Indeed, increased ROS levels can induce reversible post-translational alteration of cysteine [51], selenocysteine [52], histidine [53], and methionine residues [54]. ROS drive to the oxidation of thiol groups of critical cysteine residues in many proteins including kinases, phosphatases, and transcription factors [55,56], achieving the regulation of cellular functions via the redox-balance of cysteine residues of redox-sensitive proteins. Thus, ROS regulate apoptosis via diverse mechanisms, including death receptor activation, caspase activation, Bcl-2 family proteins, and mitochondrial dysfunction. The activities of assorted protein kinases, including

MAPK (mitogen-activated protein kinases), protein kinases-B/C, inhibitor of-I-kappaB kinases, and their equivalent phosphatases influence the apoptotic program depending on cellular context. In this line, it has been shown that ROS induce apoptosis via JNK activation [57,58] by intrinsic in the mitochondria or extrinsic apoptotic signaling by death receptor pathways [59–62]. Of note, a dual role of JNK has been demonstrated by early-transient activation of JNK by TNF promoting cell survival, whereas the long-sustained JNK activation following TNF administration in cells with blocked NF-kB-driven gene expression is associated with elevated ROS production which sustains JNK activity and triggers cell death [63–65]. In addition, JNK affects the activity of cytoplasmic proteins involved in the control of programmed cell death, such as Bim, Bid, and cFlip [66–69].

Additionally, cellular and organelle membranes are strongly susceptible to ROS damage. Oxidized phospholipids act as cell death signals triggering programmed cell death, activating both the intrinsic and extrinsic apoptotic signaling pathways [70]. It has been demonstrated that lipid peroxidation products form adducts with ERK, JNK, and p38, and activate caspase signals promoting apoptosis [71,72]. In addition, ROS may oxidize cardiolipin, a mitochondrion-specific inner membrane phospholipid, and thus activate the intrinsic apoptotic pathway [73]. Indeed, oxidized cardiolipin possesses lower affinity for cytochrome c which drives the detachment of cytochrome c from the MIM [21,74,75]. In addition, oxidized cardiolipin controls the permeabilization of MOMP via oligomerized Bax [21,74,76], supporting the hypothesis that cardiolipin is a critical upstream target in mitochondria-dependent apoptosis. Since mitochondrial ROS are involved in cardiolipin oxidation, mGSH arises as an important modulator of apoptotic cell death by indirectly controlling the redox state of cardiolipin [21,74]. In fact, apoptosis induced by ROS is linked to GSH oxidation and decreased GSH levels, increasing ROS production and the loss of redox homeostasis [77,78].

Apoptosis (both intrinsic and extrinsic) is involved in ASH, NASH, and cholestatic liver injury [79,80]. In ALD, metabolic, toxic, and inflammatory factors drive to mitochondrial dysfunction, ROS formation, Bax translocation to mitochondria, and caspase activation [81,82]. Alcohol and acetaldehyde are highly reactive and induce ROS generation and ER stress which can activate IRF3. In turn, IRF3 interacts with Bax leading to hepatocyte apoptosis [83]. In addition to ER and mitochondrial stress, alcohol also drives to apoptosis and inflammation via lysosomal malfunction raising lysosomal pH [84]. In addition, acute and chronic alcohol consumption modifies the intestinal permeability, driving to increased bacterial lipopolysaccharide (LPS) which activates Kupffer cells (KCs) and subsequently TNF generation [85]. High levels of circulating TNF and TNFR have been found in patients with alcoholic liver disease and ASH, while in animal models anti-TNF antibodies treatment protects against alcoholic liver injury [82,86]. In addition, apoptotic TUNEL positivity staining and CASP3 positive hepatocytes have been detected in liver biopsies of patients with alcoholic hepatitis with high bilirubin (>3 gr/dL) and elevated steatohepatitis [87]. Unfortunately, treatment with infliximab, an anti-TNF antibody, has been proven harmful in patients with alcoholic steatohepatitis because of the elevated infection and mortality rates, regardless of the evident link between TNF and alcoholic liver disease [88].

There are robust data indicating that hepatocyte cell death leads to inflammation and fibrosis in NASH [89]. Activation of caspase-3 and -7, elevated expression of FAS receptors and hepatocyte apoptosis are crucial events in experimental models and human NAFLD correlated with disease severity and progression [90,91]. Indeed, fragments of CK-18 produced by caspase 3 are used as indicators of NASH in patients with potential NAFLD [92,93]. In addition, ER stress provoked by free fatty acids (FFA) is related with the activation of JNK in human steatosis [94]. P-JNK and mitochondrial Sab (Sh3bp5) interaction drives to ROS generation, maintains JNK activation and apoptosis-related lipotoxicity, leading to NASH pathogenesis [95]. Indeed, JNK plays a critical role in lipotoxicity induced by palmitic acid [96–98]. Evidence supports a role for Src-dependent activation of the MAP3K, MLK3 [99,100]. The role of JNK in palmitic acid toxicity is associated with the induction and activation of PUMA and Bim [96,101], both pro-apoptotic Bcl2 family

members involved in mitochondrial permeabilization. In addition, JNK phosphorylates Sab, an outer membrane mitochondrial protein containing C terminal JNK docking sites facing the cytoplasm, and directly drives to mitochondrial respiration impairment and the increase of ROS. This, in turn, sustains JNK activation, as ROS activate the MAPK pathways and further block mitochondrial function, inducing MOMP via regulation of Bcl2 proteins in TNF- and ER stress-induced apoptosis. Other possible players of palmitic acid toxicity might act upstream or downstream of JNK. These include NADPH oxidase [102,103] as a source of oxidative stress [104], induction or posttranslational regulation of Bcl2 family members, and lysosomal permeabilization [105]. Thus, highly elevated JNK1 signaling has been described in human NAFLD livers [106].

Moreover, in patients with NAFLD and NASH, hepatocytes undergoing apoptosis activate immune cells and hepatic stellate cells (HSCs), a crucial event during initiation and progression of fibrosis [107,108]. Accordingly, CASP3 and CASP8-deficient mice fed a methionine-choline deficient (MCD) diet were protected from apoptosis, and showed decreased inflammation and fibrosis [109,110]. In line with this, elevated lipid peroxidation, TUNEL positivity, augmented CASP3 and CASP8 activities, and fibrosis were found in mice fed a high fat diet (HFD) [111] which were significantly reduced using a pan-caspase inhibitor. Remarkably, presence of CASP6 was reported in NASH human livers. In NASH, inflammation-induced CASP3 and CASP7 activation leads to casp6 cleavage and activation which in turn cleaves Bid. Truncated Bid promotes the leakage of mitochondrial cytochrome c which activates CASP3 and 7 driving to a continuous apoptosis loop in hepatocytes [112]. Therefore, controlling hepatocyte cell death pathways might have therapeutic implications [113] and hence, assorted apoptosis inhibitors have been suggested as potential treatments for NASH [114–118].

Additionally, elevated FAS expression, cytoplasmic shrinkage, nuclear condensation, and TUNEL positivity of cholangiocytes have been described in liver biopsies of primary biliary cholangitis (PBC) patients [119]. Indeed, apoptosis blockage and enhanced survival was demonstrated in FAS KO mice [120]. Ursodeoxycholic acid (UDCA), a non-toxic bile acid used to treat cholestatic diseases, protects against toxic bile acid-induced apoptosis [121]. In addition, cleaved CASP3 and CASP8 have been found in PBC patients' liver biopsies, indicating an activation of apoptosis [122]. Moreover, ameliorated AST/ALTs and reduced cleaved CASP3 and p-JNK were reported in liver-specific CASP8 KO mice after bile duct ligation (BDL) surgery, pointing at apoptosis as the presumable type of cell death in BDL mice. Nevertheless, cholestatic liver disease models promote areas of necrotic resembling cell death, as bile infarcts after BDL or in *Mdr2* KO mice [79].

Despite apoptosis possessing a minor pro-inflammatory potential compared with necrosis, KCs phagocytosis of apoptotic bodies augments death ligands and cytokines in cholestatic liver damage in mice [123]. Thus, Fas-involved apoptosis of hepatocytes is linked with activation HSCs and liver fibrosis, connecting apoptosis to liver injury [124].

4.2.2. Necrosis

Traditionally, necrosis has been considered a passive and accidental form of cell death characterized by cell swelling, membrane disruption, and the leakage of inflammatory cellular components (DAMPs), leading to an inflammatory response. However, recent evidence indicates that necrosis is mostly mediated by MPT, characterized by the development of permeability transition pore at inner and outer mitochondria membranes, driving to a rapid disappearance of the membrane potential gradient, disruption of ATP synthesis, osmotic failure of both membranes, and cell death [46]. Thus, various regulated necrotic modalities exist, including necroptosis, pyroptosis and ferroptosis, that possess important morphologic characteristics as passive necrosis combined with specific and regulated causal mechanisms [46,125].

Necroptosis is a lytic form of regulated necrosis occurring usually after death receptor (RD) (TNFR1, CD95, TRAIL-R) and Toll-like receptor activation (TLRs, TLR3, and TLR4), but only when apoptotic signaling is inhibited. It is caspase-independent and initiated

by activation of receptor interacting protein kinase (RIPK1/RIPK3) and the mixed lineage kinase domain like pseudokinase (MLKL) forming the necrosome [126], and increased the plasma membrane permeability which causes an important inflammatory response associated with most chronic liver diseases including viral hepatitis, autoimmune hepatitis, NASH, and ALD [80,127,128].

Several studies have demonstrated the relation between ROS production and necrosome signaling [129–131]. In some types of cells, mitochondrial ROS is critical for necroptosis by triggering RIPK1 autophosphorylation and RIPK3 recruitment which in turn mediate mitochondrial disruption and elevated ROS production in a feedforward mode, facilitating necrosome formation [129,130,132]. In addition, necroptosis is prevented by JNK inhibition, suggesting that JNK-induced mitochondrial dysfunction is a crucial trigger of necroptosis [133]. ROS can also induce ferroptosis, an iron-dependent form of cell death, different from apoptosis and necrosis [134] which requires redox cycling of Fe^{2+}/Fe^{3+} that promotes membrane phospholipids peroxidation [135]. Free intracellular redox-active iron elevates ROS production via Fenton chemistry and/or raised lipoxygenase activity [136,137] which consecutively modify pores, integrity or curvature of membranes [138], demonstrating that redox signaling firmly regulates apoptosis and ferroptosis [139].

An important crosstalk between apoptosis and necroptosis pathways has been described [46,80]. The equilibrium between these two modalities of cell death not only regulates the degree of inflammation, but also influences the type of liver cancer caused. Recently it has been described that a necroptotic environment with an inflammatory cytokine component induced intrahepatic cholangiocarcinoma caused by genetic oncogenic activation, while an apoptotic background promoted HCC development [140]. Furthermore, the role of necroptosis is determined by the background, and a switch from necroptosis to apoptosis may be more inflammatory and fibrogenic in NAFLD than in an ALD model [113,128].

4.2.3. Pyroptosis

Pyroptosis is a modality of lytic regulated cell death which develops in response to intracellular pathogens or PAMPs, mainly LPS. Pyroptosis is associated with caspase-dependent pore formation in the plasma membrane, swelling, rupture of the cell, and pro-inflammatory IL-1β and IL-18 leakage [141]. In addition, the pore-making gasdermin D (GSDMD) has been recognized a necessary executioner of pyroptosis [142,143]. Indeed, pyroptosis is mediated by the inflammasome via the pro-inflammatory activity of GSDMD. Pyroptotic caspases include caspase-1, caspase-11 and its human homologs caspase-4 and 5 [144,145].

Cleave of GSDMD and subsequent pyroptosis occur through three different mechanisms: (1) caspase-1-dependent canonical inflammasome pathway activated by PAMPs, DAMPs, and cholesterol crystals [146], (2) caspases -4, -5 or -11-mediated non-canonical inflammasome mechanism [147], and via (3) caspase-8-mediated inflammasome-independent pathway [148].

The NLRP3 inflammasome activation driving pyroptosis has been linked with inflammation, fibrosis, and cell death in the liver [149]. Thus, the NLRP3 inflammasome is an important pathway for the release of pro-inflammatory cytokines in the liver and is strongly associated with the pathogenesis of liver fibrogenesis [150]. The activation of NF-κB is the first step in the canonical activation of NLRP3 inflammasome, and ROS strongly control the activity of the transcription factor NF-κB. Most canonical NLRP3 inducers elevate ROS generation via NADPH. The inflammasome activation is dependent on ROS accumulation upon inhibition of autophagy and mitophagy, where ROS production is controlled [151]. Moreover, GSDMD has a thiol group located in the cysteine residues that can be affected by oxidative stress to form disulphide bonds and stabilize pore formation in the plasma membrane [152]. In addition, antioxidants increase Nrf2 activity, reducing ROS generation and decreasing gasdermin D, TXNIP and NF-kB oxidation which is essential for inflammasome activation and pyroptosis. Indeed, treatment with antioxidants reduces ROS and consequently prevents inflammasome activation in a carbon tetrachloride-driven acute

liver injury model [153,154], demonstrating the association between ROS generation and NLRP3 activation in liver disease.

Inflammatory caspases, including murine caspase-1 and -11, and human caspase-4/5, play critical roles as inflammation intermediaries [143], locating pyroptosis in NAFLD development to NASH progression [155,156]. Indeed, activation of IL-1β signaling, downstream of inflammasomes, drives liver inflammation, steatosis, and fibrosis in experimental NASH [157,158] and amplifies the response of other cytokines [159]. Thus, preventing inflammasome activation could be a promising therapy to hinder disease progression [160].

Since gut dysbiosis is frequently associated with ASH, NAFLD and NASH, it is reasonable that NASH might be connected to the pyroptotic GSDMD pathway activation [161]. Indeed, CASP11-GSDMD activation has been described in livers from AH patients [162], exacerbating neutrophilic infiltration via the leakage of DAMP, bacteria, PAMPs, IL-1β, and IL-18 from pyroptotic cells in AH. The involvement of NOD-like receptor family pyrin domain containing 3 (NLRP3) inflammasome in ALD has been demonstrated in several studies [163]. Indeed, an interaction has been recently described between activated NLRP3 inflammasome and mitochondrial dysfunction in the context of ASH and NASH [164].

The role of NLRP3 activation in ALD is supported by the protection of caspase-1, apoptosis-associated speck-like protein containing a caspase recruit domain (ASC), and IL-1β receptor global knockout mice which exhibit diminished steatosis and inflammation [165]. In addition, upregulation of caspase-1 and GSDMD processing were observed after using a Western diet combined with intragastric ethanol administration to induce ASH, indicating a critical role for pyroptosis in alcoholic hepatitis [162].

In addition, NASH is ameliorated in the GSDMD-knockout mice compared with WT mice which possess enhanced hepatic NLRP3 inflammasome expression [149,162]. Moreover, the NLRP3 inhibitor MCC950 prevented liver injury in a murine NASH model induced by feeding mice with a Western diet, decreasing transaminases, liver fibrosis, and cytokine levels compared with control mice [158]. Additionally, inflammasome-induced cholesterol crystallization in KCs is a critical factor of hepatic inflammation in NASH progression [166,167]. Sphingomyelin synthase 1 (SMS1) is involved in hepatocyte pyroptosis via a diacylglycerol (DAG)–protein kinase Cδ (PKCδ)–NLR family CARD domain-containing protein 4 (NLRC4) axis in NASH [168]. In addition, sphingosine 1 phosphate receptor 4 (S1PR4) mediates NLRP3 inflammasome activation in hepatic macrophages. SLB736, an antagonist for S1PR4 receptor, prevented NASH and hepatic fibrosis development, and could be considered a new therapeutic target in NASH development [169].

Remarkably, different cell death modalities can coincide simultaneously in pathological backgrounds, leading to the concept of PANoptosis as a novel form of inflammatory cell death during which the principal cell death pathways, apoptosis, necroptosis, and pyroptosis can be activated in parallel, driving to the formation of a large multiprotein complex named the PANoptosome [170].

Information summarizing the role of ROS in the various cell death types discussed above is included in Figure 3. Knowledge about the specific signaling pathways and cell death modalities involved in each disease will enable researchers to design treatments targeting specific molecules to ameliorate injury, inflammation and disease progression, opening novel therapy opportunities.

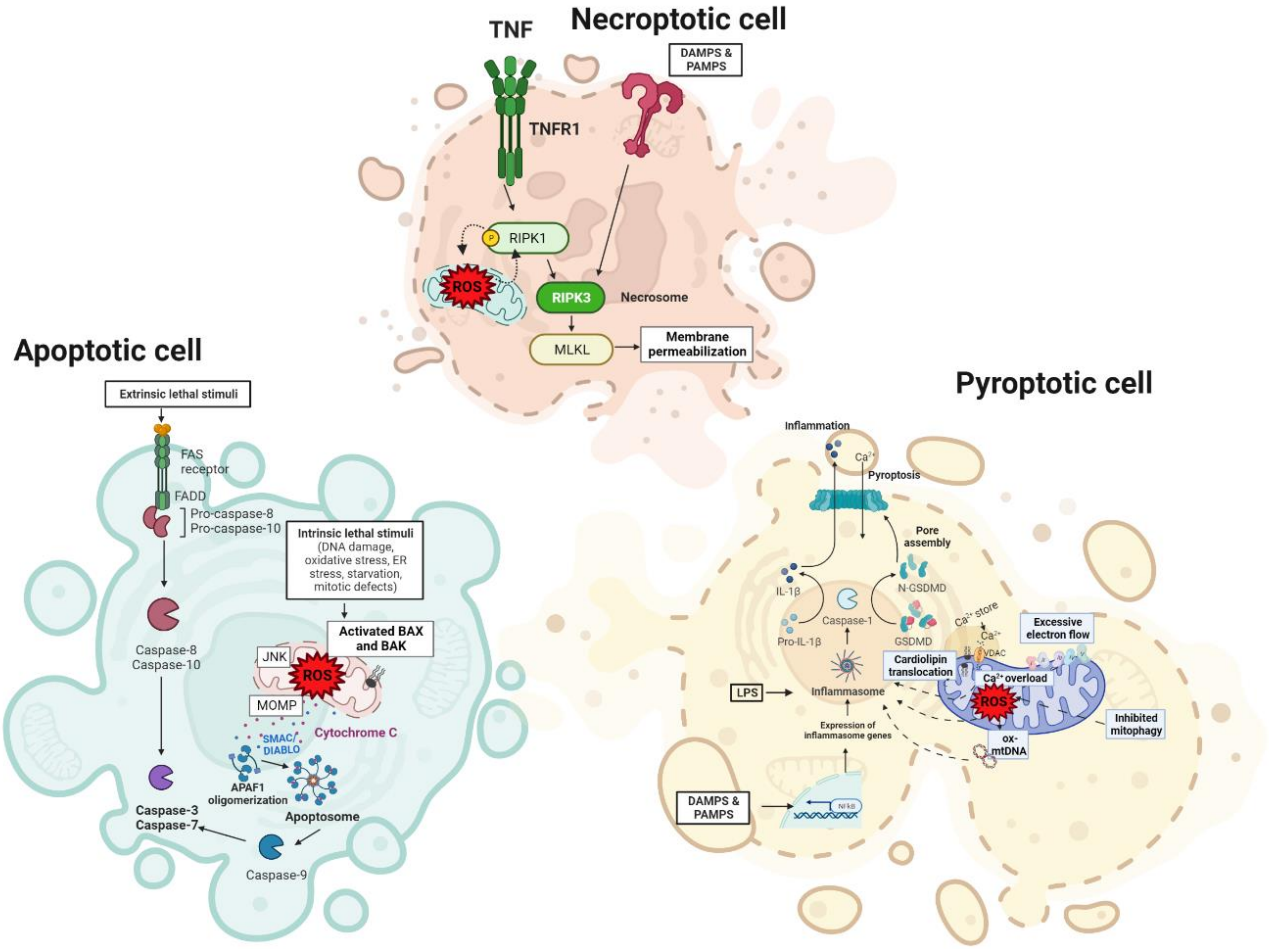

Figure 3. Scheme of the pathways associated with apoptosis, necrosis and pyroptosis cell death. Necrosis is a passive cell death caused by DAMPs and PAMPs recognized by TLR, or TNF recognized by TNFR1. The phosphorylation of RIPK1 and RIPK3 induces phosphorylation and the subsequent activation of inactive MLKL. Necrosis is triggered by ROS production that induces RIPK1 autophosphorilation and RIPK3 recruitment, following the recruitment of MLKL. They form the necrosoma that is translocated into the plasma membrane causing cell swelling and membrane ruptures. Apoptosis is a form of regulated cell death that is triggered by two different pathways. The apoptosis extrinsic pathway is activated by the FasR with the FADD adaptor protein which ends activation of the caspases cascade (caspase-8, -10, -3, and caspase-7). Intrinsic apoptosis pathway is dependent on mitochondrial damage (DNA damage, oxidative stress, ER stress, starvation, and mitotic defects) which triggers BAX/BAK-induced ROS, activation of JNK pathway and MOMP, causing the release of Cytochrome C and SMAC/DIABLO which form with the APAF-1 the apoptosome, activating caspase-9 and, subsequently, caspase-3 and -7. In pyroptosis, a regulated cell death type, DAMPS and PAMPS activate NF-kB-signaling pathway, promoting the transcription of inflammasome genes and the activation of the inflammasome complex. Subsequently, the active form of caspase-1 cleaves pro-IL-1β to mature IL-1β, and GSDMD in to an N-terminal fragment (N-GSDMD) which leads to gasdermin-mediated pore formation. The release of ROS by damaged mitochondria caused by excessive electron flow, calcium overload, inhibited mitophagy, mtDNA oxidation and cardiolipin translocation is essential for the inflammasome activation and pyroptosis. DAMPS: damage-associated molecular patterns; FADD: Fas-associated death domain; GSDMD: gasdermin D; IL-1β: interleukin-1β; LPS: lipopolysaccharide; MOMP: outer membrane pore formation; mt-DNA: mitochondrial DNA; NF-kB: nuclear factor kappa B; PAMPs: pathogen-associated molecular patterns; ROS: reactive oxygen; TLR: toll-like receptor; VDAC: voltage-dependent anion-selective channel. Created with BioRender.com.

5. Role of Oxidative Stress in Liver Diseases

5.1. Non-Alcoholic Fatty Liver Disease (NAFLD)

NAFLD is an intricate and multifactorial disease associated with multiple genetic, epigenetic, and environmental factors that comprises a spectrum of alterations beginning with simple steatosis (non-alcoholic fatty liver, NAFL) which can progress to non-alcoholic steatohepatitis (NASH) that can culminate in cirrhosis and hepatocellular carcinoma (HCC) [171]. NAFL is characterized by macrovesicular steatosis (more than 5%) without the existence of hepatocellular injury, whereas NASH encompasses hepatic steatosis, plus inflammation, ballooning of hepatocytes and hepatocellular injury in the presence or absence of fibrosis. A "two-hit" hypothesis was originally proposed to define the pathogenesis of NAFLD. Insulin resistance (IR) acts as the first hit, promoting liver steatosis with augmented hepatic lipogenesis and defective degradation of FFA. The accumulation of fat sensitizes the liver to inflammation and cell death by a second hit that causes oxidative stress, concluding in NASH and fibrosis [172,173]. In the last years, the "multiple hit" theory, which includes different factors acting in parallel, has emerged to describe more accurately the pathogenesis of NAFLD. Oxidative stress is considered to be the main contributor of the "multiple hits" to liver injury and disease progression in NAFLD [174,175]. Currently, metabolic dysfunction-associated fatty liver disease (MAFLD) has been proposed to classify the heterogeneous population of patients with this disease. In this way, MAFLD includes patients with hepatic steatosis in combination with overweight/obesity, presence of type 2 diabetes, or evidence of metabolic dysregulation [176], excluding individuals with excessive alcohol consumption. The new nomenclature encloses coexisting toxic (e.g., alcohol) or viral factors that do not exclude the link to metabolic liver disease.

Liver steatosis, the first stage of MAFLD, although considered reversible, sensitizes to diverse secondary insults driving the development of NASH [177]. Different lipid species accumulate in the transition of MAFLD to NASH, including, among others, triglycerides, diacylglycerol, free fatty acids, ceramides, and cholesterol, underlining that the type rather than the amount of lipids is responsible for the progression from steatosis to NASH [15,178]. Therefore, the unbalance of lipid metabolism is the most direct etiology of MAFLD.

The mechanisms by which ROS contribute to MAFLD progression are multifactorial and involve both accumulative oxidative biomolecular damage and dysregulated redox signaling [179], since ROS induce oxidative alterations to macromolecules that lead to liver injury [172,173], and possess important signaling functions [180]. In the liver, the metabolism of lipids is regulated by nuclear receptors that activate diverse ligands and modulate important enzymes involved in lipogenesis and lipoxidation [181], acting as redox triggers that in turn affect several metabolic pathways due to particular ROS [182]. AMPK activation leads to the inhibition of lipogenesis [183], downregulating several nuclear receptors and enzymes implicated in lipid metabolism, such as acetyl-CoA carboxylase, PPARα, PPARγ, and SREBPs. Oxidative stress influences AMPK signaling, since both cytoplasmic and mitochondrial ROS can upregulate AMPK activity [184]. In addition, in MAFLD, an increment in the AMP/ATP ratio stimulates AMPK [185], and H_2O_2 can precisely modulate AMPK activity [186]. H_2O_2 also increases SREBP-1c expression, leading to an increment in fatty acid synthetase and promoting lipogenesis [187]. Remarkably, various antioxidants reduce hepatic steatosis through the downregulation of SREBP-1c expression [188]. Hence, ROS-mediated signaling may partially be responsible for the alterations in lipid metabolism homeostasis. Additionally, as an effort of liver cells to control the prooxidant effects of metabolic stress in MAFLD, stress-responsive transcription factors (such as AP-1, NF-κB and Nrf2) and their regulated genes are also redox sensitive [189]. Elevated Nrf2 expression is related to ROS pathological levels in liver biopsies of patients with NASH [190]. In diverse NASH models, using MCD and HFD diets, Nrf2-knockout mice display elevated susceptibility to NASH development [191,192]. Further, pharmacological activation of Nrf2 decreases IR, liver steatosis, and fibrosis in animals fed with a high-fat and high-fructose diet [193], linking antioxidant signaling and lipid metabolic pathways.

Excessive lipid accumulation produces an important hepatic metabolic stress which causes inflammatory pathways activation. After exposure to harmful stimuli, KCs are the principal effectors involved in ROS generation and the transcription of cytokines/growth factors/enzymes modulating redox balance, metabolism, inflammation, and fibrosis [194,195], functioning as a driving force in MAFLD progression at several levels [196–198]. Hence, the innate immune and ROS signaling collaborate and synergistically influence disease progression.

MAFLD is also linked to "mitochondrial dysfunction" which is shown by modified respiratory complex activity and fatty acids oxidation [199]. ETC activity gradually reduces during MAFLD progression, and livers with simple steatosis already show a mild deterioration of ETC activity. Besides targeting lipids, ROS generated in the mitochondria can also modify mitochondrial DNA, causing deletions and mutations [200]. Of note, an important reduction in mitochondrial DNA in MAFLD patients has been shown [201]. Thus, mitochondrial DNA damage and mutations induced by ROS may affect ETC and ATP generation which in turn activates mitochondrial ROS production through the mitochondrial MPT opening, causing a vicious cycle of damage amplification termed "ROS-induced ROS release" (RIRR) [202].

Oxidative stress biomarkers normally measured in clinical samples of MAFLD comprise DNA oxidation products (8-OH-dG), lipid damage products (thiobarbituric acid reactive substances or TBARS), MDA, 4-HNE, hydroperoxides, and 8-isoprostane), and protein oxidation products (protein carbonyl, nitrotyrosine). In general, the concentrations or activities of these biomarkers are elevated in NASH patients [24,203]. In addition, antioxidants commonly evaluated in MAFLD/NASH clinical samples include enzymatic antioxidants (e.g., catalase, SOD, and GPX) and nonenzymatic antioxidants (e.g., GSH, TrxR, α-tocopherol, and ubiquinone) which are reduced in most patients with MAFLD/NASH, although they are increased in a few exceptions [204,205]. Further, several lipid peroxidation circulating biomarkers have been detected in patients with MAFLD, their concentrations positively associated with the disease severity [206,207]. Overall, these data indicate that oxidative stress and mitochondrial antioxidant defense depletion plays an important role in disease progression in MAFLD/NASH.

5.2. Alcoholic Liver Disease (ALD)

ALD is a prevalent form of chronic liver disease caused by the persistent abuse of alcohol consumption. In 1967, Comporti and colleagues proposed for the first time the involvement of lipid peroxidation as a mechanism for ethanol-induced fatty liver [208]. The oxidative metabolism of ethanol triggers a complex array of mechanisms that contribute to the progression of ALD from its initial step of steatosis to alcoholic hepatitis, cirrhosis and eventually HCC. Unfortunately, the pathogenesis of ALD is not entirely understood, which has limited the availability of therapeutic opportunities. Alcohol-induced liver injury is linked to an exacerbated ROS generation resulting in the onset of oxidative stress in hepatocytes [209] due to the metabolism of alcohol in the liver which begins with the alcohol dehydrogenase (ADH) that metabolizes ethanol to acetaldehyde. Acetaldehyde is subsequently catabolized to acetate by the acetaldehyde dehydrogenase (ALDH). Unlike acetate, which is stable, acetaldehyde is very reactive and forms adducts with DNA, promoting tissue damage. Acetaldehyde and its derivative MDA form hybrid adducts which can be recognized by KCs, endothelial, and stellate cells that produce cytokines and activate an inflammatory response that contributes to the progression of ALD [210]. In addition, ethanol is also metabolized via the microsomal system cytochrome P450, CYP2E1, which converts alcohol to acetaldehyde during alcohol chronic consumption. CYP2E1 is inducible and becomes the preferred pathway of oxidative ethanol metabolism, due in part to the saturation of ADH for alcohol, resulting in the generation of ROS and the onset of oxidative stress. ROS can also sensitize hepatocytes to LPS released from the gut and tumor necrosis factor-alpha (TNFα) which in turn generates more ROS [211], lending further support for the interconnection between oxidative stress and inflammation during ALD.

Several pathways contribute to liver injury during ALD. ADH and ALDH enzymes reduce NAD^+ to NADH. The modified NAD^+/NADH ratio induces fatty liver through the inhibition of gluconeogenesis and fatty acid oxidation [212]. CYP2E1, increased in persistent alcohol intake and stabilized by alcohol itself, produces free radicals via the trans-formation of NADPH to $NADP^+$ [213]. CYP2E1-produced ROS can peroxidase mitochondrial and peroxisomal enzymes involved in β-oxidation, resulting in the accumulation of fatty acids and hepatic steatosis development. Alcohol promotes a gradient of hypoxia from the portal vein to the central vein which contributes to hepatic injury [214]. Altered ROS metabolism increases hypoxia-inducible factor-1 alpha (HIF-1α) expression which increases TNFα secretion and impairs mitochondrial function, leading to an immune response that amplifies liver injury. Furthermore, oxidative stress induces hepatocyte apoptosis and mainly necrosis [57], a response that is accentuated due to the limited antioxidant defense that contributes to inflammation and disease severity described in patients with ALD [215]. Alcohol feeding induces mGSH depletion via cholesterol overload in the mitochondria [216,217] which is reversed after alcohol withdrawal [218]. Moreover, recent data described a mitochondrial cholesterol accumulation resulting in mGSH depletion and ASH in rats fed an ethanol-polyunsaturated fatty acid treatment, effects that are prevented by betaine cotreatment [219]. Remarkably, alcohol-induced endoplasmic reticulum (ER) stress elevates cholesterol synthesis and modifies its trafficking into mitochondria, thus indirectly affecting mGSH levels. In accordance, blocking ER stress by TUDCA restored the mGSH pool in ethanol-fed rats [216,220]. Finally, ROS contribute to the transformation of HSC into myofibroblasts and the activation of matrix metalloproteinases, leading to remodeling of the extracellular matrix in the liver, culminating in excessive liver fibrosis and cirrhosis during severe ALD. These effects are accentuated by impaired regenerative capacity of mature hepatocytes [221].

5.3. Primary Biliary Cholangitis (PBC)

PBC is a chronic and progressive cholestatic liver disease, in general affecting middle-aged women, which may lead to liver failure and transplantation [222,223]. PBC is associated with hypercholesterolemia and deficiencies of antioxidant vitamins. Patients with PBC display symptoms of endothelial dysfunction, inflammation and antioxidant deficiency [224]. Moreover, PBC is considered to possess an autoimmune etiology, since specific antimitochondrial antibodies are found in most patients. The abnormal immune response in PBC drives the induction of autoreactive T and B lymphocytes, and the generation of diverse inflammatory mediators [225,226] that subject the liver to several harmful factors including oxidative stress. Serum samples from PBC patients displayed increased oxidative stress biochemical marker levels such as lipid peroxidation and cholesterol self-oxidation products [218] which further increase the immune response. Indeed, PBC patients have increased IgGs levels against malondialdehyde adduct and human serum albumin [227]. It has been described that oxidative stress and steatosis are cofactors promoting liver injury in PBC. Besides altering lipid composition, oxidative stress in PBC also promotes the accumulation of protein oxidative products and alters GSH metabolism [228]. Hence, treating PBC with UDCA improves redox changes in serum and liver tissues [229–231].

As previously mentioned, Nrf2 induces many cytoprotective genes that influence xenobiotic metabolism, antioxidant and anti-inflammatory responses. It has been reported that Nrf2/Keap1 axis-mediated protection against oxidative stress is impaired in PBC. Thus, defects in the Nrf2/Keap1 integrity system may influence self-defense mechanisms against oxidative stress in PBC [232]. Moreover, recent observations demonstrated that the administration of S-Adenosyl-L-methionine (SAMe) may prevent autoimmune events in PBC patients via its antioxidant and S-glutathionylation properties, providing new awareness into the molecular events inducing PBC progression and pointing at the potential therapeutic use of SAMe in PBC [233]. Oxidative stress also induces apoptosis of bile duct cells in PBC, promoting biliary damage that is a consequence of reduced glutathione-S-transferase (GST) levels. Thus, the intracellular GSH reduction directly drives biliary

epithelial cells to apoptosis, the modulation of these events crucial to decreasing the immune-mediated injury [229].

5.4. Viral Hepatitis

Worldwide, over half a billion of individuals are chronic carriers of viral hepatitis. The three dominant types of viral hepatitis are hepatitis A virus (HAV), HBV and HCV. The last two are increasing in prevalence and 12% of all cancers arise from them, including the hepatitis delta virus (HDV) which can lead to accelerated disease progression [234].

Oxidative stress also plays a pathogenic role in viral hepatitis. ROS and free radicals are generated in human diseases caused by viruses, among others. Viruses enter in to the cell by endocytosis and act as an intracellular parasite using the host cell synthetic processes for their replication [235]. These processes affect the physiological statement of the ER and the mitochondria producing ROS and antioxidant system depletion (GSH, GSHPx) [236]. HBV and HCV infections are characterized by the increased levels of a wide array of oxidative stress markers in liver and blood of infected patients. Such markers include MDA, lipid peroxides, protein carbonyl content, oxysterols, and thioredoxin, which are responsible of inflammatory pathways activation [237–240]. NADPH oxidases, CYP2E1 and Ero1a are also increased in HCV [241].

Furthermore, Zn deficiency occurs in HCV, causing lipid peroxidation, loss of mitochondrial energization and oxidative DNA damage. Its supplementation therapy has been shown to improve the prognosis of patients with HCV [242]. On the contrary, the antioxidant therapy is not always effective: antioxidants can become easily pro-oxidants if they are not used at the right dose and schedule [243]. Therefore, detailed investigation of the mechanisms by which viral proteins induce oxidative stress is needed to develop effective treatments.

5.5. Liver Fibrosis

Fibrosis is characterized by progressive and excessive deposition of extracellular matrix (ECM) between hepatocytes and sinusoids, impairing the physiological architecture of the liver. It is associated with disease progression in chronic inflammatory diseases [244]. In addition, chronic portal hypertension induced by liver fibrosis is the major cause of clinical complications, bleeding events and hepatic encephalopathy [245].

As described above, steatohepatitis from all etiologies is associated with an increase in ROS by vicious cycles encompassing steatosis, lipid peroxidation, ROS formation, antioxidants reduction, modified mitophagy, and mitochondrial danger signal-induced expression of inflammatory cytokines which cause apoptosis and necrosis of hepatocytes. The pro-fibrotic mediators such as superoxide, H_2O_2, and other hydroxyl radicals are generated in hepatocytes, HSCs, and macrophages [246]. Among the ROS production enzymes, the NAPDH oxidases (NOXs) mediate fibrogenic responses by inducing angiotensin II, PDGF, and TGFβ in HSCs and macrophages that activate collagen-producing myofibroblasts that account for excessive accumulation of ECM [108,247]. Necrotic hepatocytes release DAMPs that activate danger signals to neighboring cells (HSCs and KCs). In this regard, NF-κB plays a key role in the regulation of inflammation and is considered an important modulator of liver fibrosis progression. Its activity is linked with HSC proliferation [248]. Fibrosis development principally determines the quality of life and prognosis correlated with liver function, thus being a critical risk factor for HCC development [249].

5.6. Hepatocellular Carcinoma (HCC)

HCC is the second driving cause of cancer-associated deaths worldwide due to late diagnosis and poor therapeutic outcomes [250,251]. HCC is an inflammation-related cancer, as most of HCCs emerge in the background of hepatic inflammation [252,253]. HCC incidence is significantly associated with liver inflammation from exposure to different risk factors such as hepatitis B virus, hepatitis C virus, metabolic diseases, persistent alcohol drinking, obesity and type 2 diabetes [254,255]. Chronic liver inflammation promotes oxidative stress and lipid peroxidation, producing excess ROS and aldehydes which form promutagenic DNA adducts interacting with DNA bases. Therefore, oxidative stress acts as a critical factor promoting carcinogenesis. Further, blocking antioxidant defenses strongly elevated the rate of liver cancer [256]. Moreover, mild/excess iron deposition positively correlates with HCC and p53 mutations in patients with hemochromatosis, indicating a potential carcinogenic role of oxidative stress induced by iron via Fenton reactions [257,258].

Oxidative stress is a critical factor in NASH progression and NASH-driven HCC [174,259]. NASH-related HCC incidence is expected to increase worldwide due to its association with the obesity and type 2 diabetes mellitus epidemic. Recently we have provided evidence that cholesterol rather than steatosis per se plays a role in NASH-induced HCC development [260], giving further support to strategies aimed at targeting liver cholesterol homeostasis as potential therapeutic treatments with relevance in NASH-driven HCC. Indeed, recent findings indicated that ezetimibe [260] and atorvastatin [261] improved high-fat high cholesterol diet-induced HCC development, correlating with previous results in a subcutaneous HCC model [262]. Future research may explore whether combination therapy between ezetimibe and statins may be superior for the treatment of HCC. Moreover, our laboratory also uncovered an important role of StARD1 in non-alcoholic steatohepatitis-driven HCC, where it stimulates bile acid production in the mitochondrial acidic pathway, and, in turn, activates hepatocyte pluripotency and self-renewal as well as inflammation [263].

In this regard, as HCC is closely linked to elevated oxidative stress through viral proteins or persistent inflammation and lipids, modulating oxidative stress may emerge as a promising approach against progressive liver disease. However, the administration of antioxidant therapies as potential HCC treatment remains to be fully established and should be administered with attention to the fact that a strict antioxidant balance must be guaranteed to avoid the generation of unwanted prooxidants and free radicals.

Altogether, all this data indicate that ROS is present in many liver diseases and is a determinant hallmark contributing to the progression of diseases. Molecular mechanisms underlying the pathological events described in this section need to be further investigated. Whether the formation of ROS is in fact the cause or consequence in those pathologies is also an unanswered question. As a summary, Figure 4 illustrates the molecular events leading to the development of the liver diseases discussed in this section:

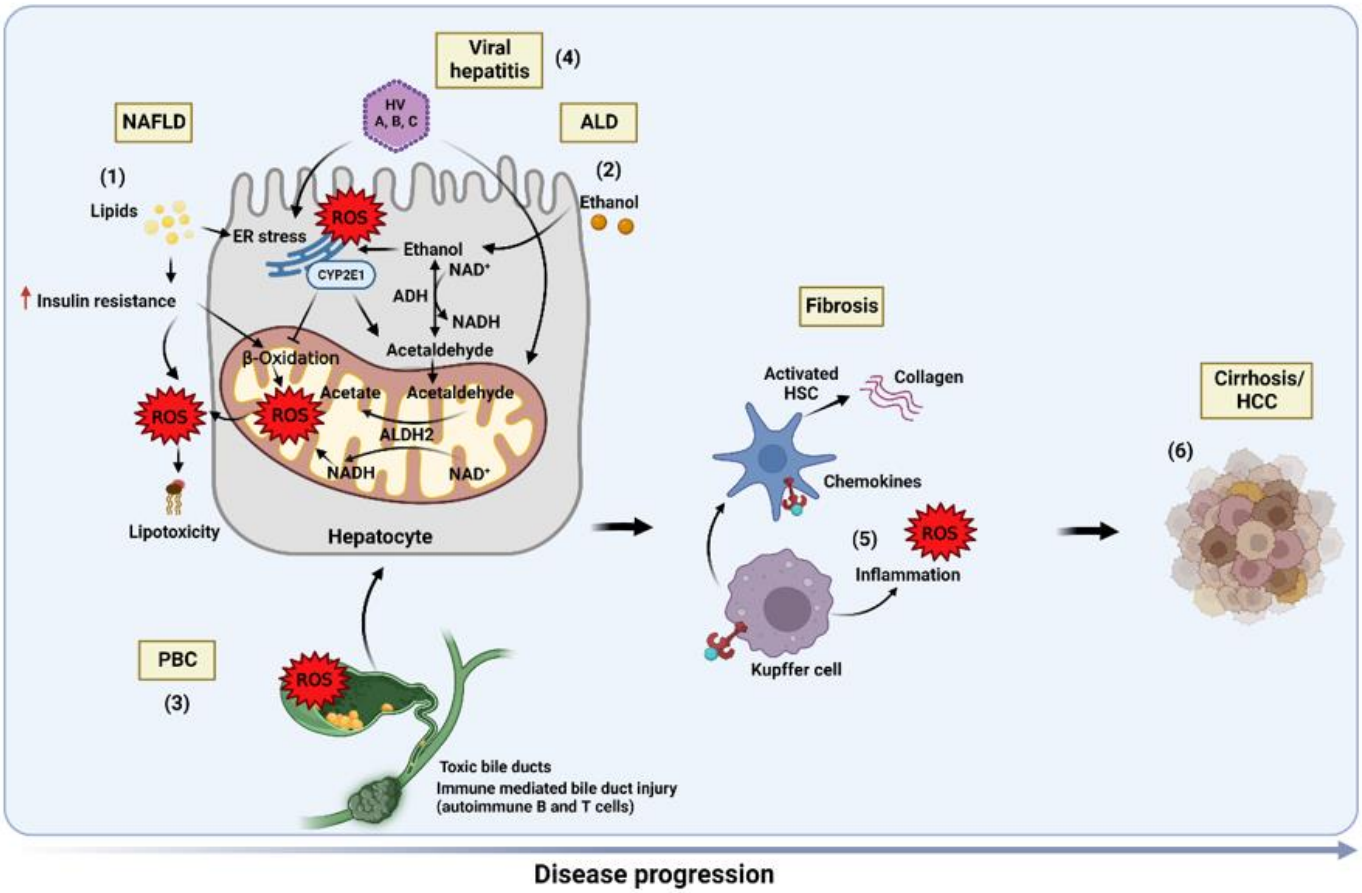

Figure 4. Outline of the pathophysiology of various liver diseases. (1) The increased hepatic lipid flux leads to an extended accumulation of triglycerides and 'toxic' levels of fatty acids, free cholesterol, and other lipid metabolites which cause mitochondrial dysfunction, ER stress, oxidative stress, and overproduction of ROS, all leading to hepatic inflammation. (2) Ethanol is metabolized in the liver by ADH or via the microsomal system cytochrome P450, CYP2E1, to acetaldehyde which is further metabolized to acetate. Acetaldehyde is very reactive and forms adducts, such as MDA, with biomolecules provoking tissue damage. (3) In PBC, autoreactive B and T cells lead to the gradual destruction of intrahepatic bile ducts, resulting in periportal inflammation and cholestasis (impeded flow of the bile to the duodenum). Prolonged hepatic cholestasis causes cirrhosis and portal hypertension. (4) Hepatitis virus A, B or C enter into the cells by endocytosis. Pathogenic mechanisms induced by viruses include persistent liver inflammation and immune-mediated oxidative stress damage to ER and mitochondria, oxidative damage induced by viral proteins, and deregulation of cell signaling pathways by viral proteins, leading to liver cell destruction. (5) Extensive ROS formation and steatohepatitis induces the release of inflammatory cytokines which cause apoptosis and necrosis of hepatocytes. Necrotic hepatocytes send danger signals to neighboring cells (HSCs and KCs) and induce the activation of matrix metalloproteinases, leading to fibrotic remodeling of the extracellular matrix. (6) Progressive fibrosis culminates in cirrhosis and is a determinant risk of factor for HCC development. Created with BioRender.com.

6. Antioxidant Therapies

Given the role of oxidative stress in liver disorders, boosting the pool of endogenous antioxidants or the intake of dietary antioxidants was proposed as an effective therapeutic strategy with many antioxidants undergoing clinical studies [264–266]. In vitro and animal model studies support the beneficial role of various antioxidant compounds in liver diseases. However, when translated to clinical trials, results were controversial or failed to be replicated in larger studies [267,268]. Potential reasons for these disappointing results include inaccurate dose schedules, inadequate choice of a certain antioxidant for a particular disease, and the redundancy of symptoms with other pathologies. Problems such as small sample size, short follow up, inappropriate endpoints, inefficacy to establish tissue delivery and antioxidant effect, and the heterogeneous character of antioxidants also complicate the analysis of results of clinical studies. Although in general the outcomes are promising, the precise cellular mechanisms of these compounds are still obscure evidencing the urge for additional research to study their potential as therapeutic option.

Non-enzymatic antioxidants currently undergoing clinical trials can be classified according to their origin: endogenous and exogenous, from diet or synthetic, antioxidants. As such, well-known endogenous antioxidants include melatonin, GSH, coenzyme Q10, lipoic acid, inosine, and carnosine. GSH is mainly produced in the liver and is one of the most important ROS scavengers in the organism. As described above, GSH replenishment has shown protective effects in liver disorders [269,270]. Antioxidant co-enzyme Q10 is localized in the inner membrane of the mitochondria, and thus represents a crucial target to prevent or minimize oxidative damage as it can directly act in the ETC. Lipoic acid is a dithiol compound produced in mitochondria that exerts a key function as it can recycle other oxidants including GSH and vitamins C and E. Importantly, both CoQ10 and lipoic acid are frequently given as adjuvant therapy in combination with other antioxidant compounds for treating degenerative diseases [271]. N-Acetyl cysteine (NAC) is a thiol-compound with antioxidant capacities as it modulates redox signaling and halts lipid peroxidation [272]. An important feature of NAC is that it is a GSH precursor by providing the rate-limiting amino acid cysteine. However, it is not able to replenish mGSH in the presence of mitochondrial cholesterol overload. Therefore, its ability to restore mGSH levels in NASH/ASH or other diseases in which cholesterol is accumulated in mitochondria is limited.

In some, if not all, pathological conditions, the endogenous pool of antioxidant defenses is not sufficient to combat the excess of ROS production. In this situation, the ad-ministration of dietary antioxidants as therapy or supplements has been proposed as it can be beneficial for improving the overall redox status and compensating the impaired endogenous antioxidant mechanisms. The list of dietary compounds with antioxidant properties includes selenium, carnitine, flavonoids, polyphenols (such as resveratrol, curcumin), carotenoids, lutein, and vitamins (A, C, E), among others. Vitamin E is considered the archetypal antioxidant vitamin and is the compound with the most encouraging results in the therapy of NASH so far [273]. Unluckily, the translation of these compounds from pre-clinical to clinical stages has several limitations largely related to pharmacokinetic and pharmacodynamic constraints, as many natural antioxidants do not fulfil the absorption, distribution, metabolism, and excretion (ADME) requirements of drugs [274].

The development of synthetic antioxidant compounds, small molecules with drug-like properties, has boomed in recent years. New benzoquinone-based antioxidants such as Mitoubiquinone (MitoQ), vatiquinone and idebenone have been developed and are included in several clinical trials for the treatment of liver disorders [275]. Another newly-designed synthetic drug with antioxidant capacity is despramipexole, a benzothiazole renowned for its effects on preserving mitochondrial function [276]. Moreover, it inhibits cytochrome P450 and confers protection against N-acetyl-p-benzoquinoneimine (NAPQI) [277], a highly reactive electrophilic intermediary produced in the liver after APAP overdose that is capable of depleting reduced GSH. To summarize the information on the latest advances in

antioxidant therapy, antioxidant molecules currently undergoing clinical trials in the field of liver disorders are briefly described in Supplementary Table S1.

7. Conclusions

As it is well known, free radical species are physiological signaling molecules that, when produced in excess or accumulated due to an unbalance with antioxidants, trigger oxidative stress, producing paramount changes in cell function and even cell death. Given the strategic function of the liver in xenobiotic detoxification and protection against toxic radicals, growing evidence has reinforced the impact of ROS and oxidant species in the progression of many liver diseases. Here, we have reviewed the disease mechanism in a disparate group of liver disorders that share oxidative stress as a common entity. In addition, we addressed the most important pathogenic processes involved in the development of liver diseases, pointing out oxidative stress as a key player in its pathophysiology.

Despite the use of antioxidants for the treatment of liver diseases gaining attention in recent years, when the benefit of antioxidant compounds has been assessed in clinical trials, mixed and controversial results have been reported and many of the promising results obtained in animal models failed to be reproduced when translated into human disease. If the design of clinical trials is improved, the data extracted from them could provide valuable information promoting enthusiasm for the possible future of antioxidants in liver diseases.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/livers2040023/s1. Table S1. Antioxidant compounds under clinical trial for the treatment of liver disorders. Source: ClinicalTrials.gov (NIH). Only trials in "Recruiting", "Enrolling by invitation", "Active, not recruiting", "Terminated" and "Completed" status are included.

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Abbreviations

ADH	alcohol dehydrogenase
ALD	alcoholic liver disease
AMPK	5′ adenosine monophosphate-activated protein kinase
ATP	adenosine triphosphate
ASC	apoptosis-associated speck-like protein containing a caspase recruit domain
ASH	alcoholic steatohepatitis
BDL	bile duct ligation
Ca^{2+}	calcium ion
DNA	deoxyribonucleic acid
ER	endoplasmic reticulum
ETC	electron transport chain
GSH	glutathione
GSSG	oxidized glutathione
$FADH_2$,	flavin adenine dinucleotide (reduced form)
FFA	free fatty acid
H_2O_2	hydrogen peroxide
HCC	hepatocellular cancer
HFD	high fat diet
HSC	hepatic stellate cells
MAFLD	metabolic-dysfunction-associated fatty liver disease
MCD	methionine-choline deficient diet
MDA	malondialdehyde
MMOs	microsomal monooxygenases
MPT	mitochondrial permeability transition
mDNA	mitochondrial deoxyribonucleic acid
NAD^+	nicotinamide adenine dinucleotide phosphate, (oxidized form)
NADPH	nicotinamide adenine dinucleotide phosphate, (reduced form)
NAFLD	non-alcoholic fatty liver disease
NASH	non-alcoholic steatohepatitis
NLRP3	NOD-like receptor family pyrin domain containing 3
NOX	NAPDH oxidases
Nrf2	nuclear factor E2-related factor 2
$O_2^{\bullet -}$	superoxide anion
$^1O_2$	singlet oxygen
•OH	hydroxy radical
-OH	hydroxy group
ONOO−	peroxynitrite
OXPHOS	oxidative phosphorylation system
PBC	primary biliary cholangitis
Prx	peroxiredoxin
ROS	reactive oxygen species
SAMe	S-(5-Adenosyl)-L-methionine
SOD1	superoxide dismutase 1
SOD2	superoxide dismutase 2
StARD1	steroidogenic acute regulatory protein
TNFα	tumor necrosis factor alpha

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