Review

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Alterations in the redox state and liver damage: Hints from the EASL Basic School of Hepatology☆

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Summary

The importance of a correct balance between oxidative and reductive events has been shown to have a paramount effect on cell function for quite a long time. However, in spite of this body of rapidly growing evidence, the implication of the alteration of the redox state in human disease has been so far much less appreciated. Liver diseases make no exception. Although not fully comprehensive, this article reports what discussed during an EASL Basic School held in 2012 in Trieste, Italy, where the effect of the alteration of the redox state was addressed in different experimental and human models. This translational approach resulted in further stressing the concept that this topic should be expanded in the future not only to better understand how oxidative stress may be linked to a liver damage but also, perhaps more important, how this may be the target for better, more focused treatments. In parallel, understanding how alteration of the redox balance may be associated with liver damage may help define sensitive and ideally early biomarkers of the disorder.

Keywords: Liver disease; Oxidative stress; Redox proteomics; Inflammation.
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Abbreviations: ROS, reactive oxygen species; RNS, reactive nitrogen species; SOD, superoxide dismutase; ALI, acute liver injuries; CLD, chronic liver diseases; HCC, hepatocarcinoma; HBV, hepatitis B virus; HCV, hepatitis C virus; NAFLD, non-alcoholic fatty liver disease; MDA, malondialdehyde; 4-HNE, 4-hydroxynonenal; APE1/Ref-1, apurinic apyrimidinic endonuclease/redox effector factor 1; TNF, tumor necrosis factor; NOS2, nitric oxide synthase 2; ETC, electron transport chain; I/R, ischemia/reperfusion; FA, fatty acids; NASH, non-alcoholic steatohepatitis; ASH, alcoholic steatohepatitis; 8OHdG, 8-hydrossyguanosine; HIF-1 α, hypoxia-inducible factor 1α; PHD, prolyl-hydroxylases; NF-κB, nuclear factor-κB; GSH, reduced glutathione; GSSG, oxidized glutathione; CVD, cardiovascular disease; ER, endoplasmic reticulum; ERS, endoplasmic reticulum stress; HH, hereditary hemochromatosis; MPO, myeloperoxidase; HLPP, Human Liver Proteome Project; 2-DE, two-dimensional electrophoresis; MS, mass spectrometry; LC, liquid chromatography.

Introduction

All living organisms have to cope with harmful by-products of oxygen such as $O_2^{\cdot-}$, H_2O_2, and $^{\cdot}OH$ (collectively called free radical or reactive oxygen species, here referred to as ROS), which appeared on Earth approximately 2 billion years ago and provoked the development of cell defense mechanisms [1]. However, the history of free radicals is much younger since their formation in cells was first documented about 60 years ago by a study demonstrating that harmful oxygen species may be produced in animals and plants as mediators of the damaging effects of radiation [2]. It was only after the discovery of the existence of superoxide dismutase enzyme (erythrocuprein or SOD), which catalyzes $O_2^{\cdot-}$ dismutation to H_2O_2 [3], that the biological interest in ROS became apparent. It soon became clear that living organisms are capable of inducing their antioxidant defense systems by relatively rapid mechanisms to cope with the oxidative stress due to an imbalance between the generation of ROS and the antioxidant defense capacity of the cell. Both animals and certain plants can induce SOD upon increased exposure to oxygen, paraquat (a known producer of $O_2^{\cdot-}$), and X irradiation [4]. Within the body, tissues with a higher oxygen consumption rate, such as liver, heart, and brain, constitutively express greater antioxidant enzymes than those with lower oxygen consumption [5].

Oxidative stress is a major pathogenetic event occurring in several liver diseases, ranging from metabolic to proliferative disorders. Main sources of ROS are represented by mitochondria and cytochrome P450 enzymes in the hepatocyte, Kupffer cells, and neutrophils [6,7]. Oxidative stress affects major cellular components including lipids, DNA, and proteins [8]. Through modulation of protein structure/function, ROS can influence gene expression profile by affecting intracellular signal transduction pathways [9]. While several enzymatic and non-enzymatic markers of chronic oxidative stress are well known in the liver, early protein targets of oxidative injury are yet poorly defined [10]. Identification of these biomarkers will enable early detection of liver diseases and allow monitoring the degree of liver damage, the response to pharmacological therapies and the development of new therapeutic approaches. In the era of molecular medicine, new proteomic methodologies promise to establish a relationship between pathological hallmarks of the disease and protein structural/functional modifications, allowing a better understanding and a more rational therapy of several liver disorders.

Review

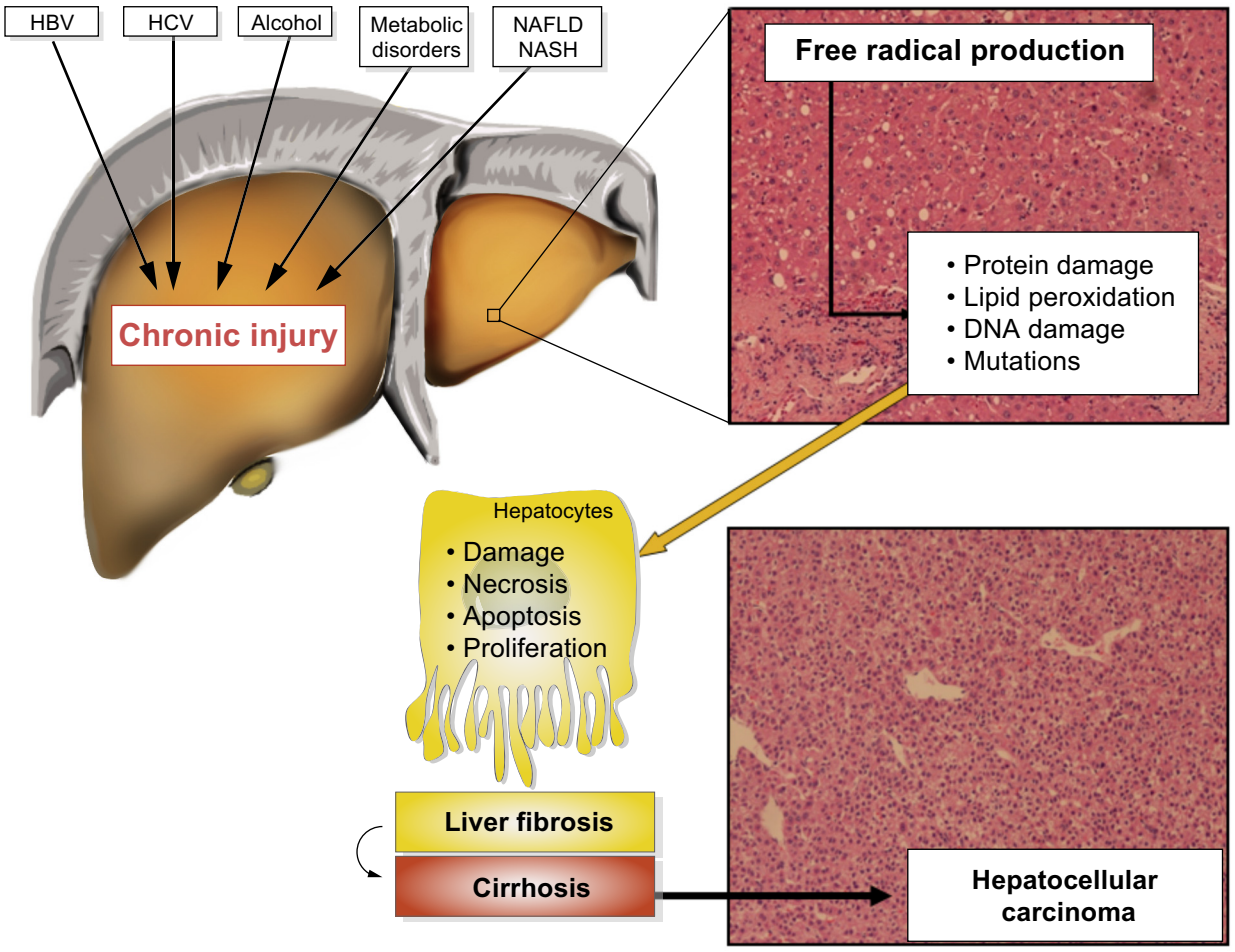

Fig. 1. Etiologic factors and central role of oxidative stress in the pathogenetic development of hepatic diseases. Oxidative stress is the major pathogenic event occurring in several liver disorders. Chronic liver injury due to HBV and HCV infection, inadequate alcohol consumption and metabolic disorders determine a pro-oxidative state causing protein and DNA damage and lipid peroxidation. Instauration of hepatocyte oxidative stress condition results in liver fibrosis and cirrhosis, which may lead to hepatocellular carcinoma.

Liver diseases are frequent pathologies worldwide [11] and may be divided into acute and chronic on the basis of the persistence of liver injury. Acute liver injuries (ALI) are characterized by a rapid resolution and a complete restitution of normal organ architecture/function after the elimination of the cause, while chronic liver diseases (CLD) are characterized by persistent liver damage with progressive alteration of organ function caused by increased cellular damage [12]. The most common causes at the basis of CLD are viral infections sustained by hepatitis C and B viruses (HCV and HBV), alcohol abuse and alterations of lipid/carbohydrate metabolism, also known as non-alcoholic fatty liver disease (NAFLD). All these clinical features are major risk factors for HCC development [13]. However, regardless of the different etiology and natural course, a common landmark of all types of liver injury is an increased production of ROS (Fig. 1) [10].

Oxidative stress and liver damage

ROS and reactive nitrogen species (RNS), such as $NO^{\cdot}$, NO^{+}, NO^{-}, and $ONOO^{-}$, are critical intermediates in the normal physiology and pathophysiology of the hepatocyte. When the equilibrium between ROS generation and the antioxidant defense of the cell is disrupted, a "net" oxidative stress results. In the liver, free radicals triggered by ROS and RNS are created by neutrophils, Kupffer cells, mitochondria, and cytochromes P450 (for more details about ROS and NOS generation see [14]). The damage created by oxidative stress affects all major cellular components, including lipids, proteins and DNA [15]. The relevance of cellular redox imbalance in liver pathologies is outlined by a number of studies in patients with viral, alcoholic or non-alcoholic fatty liver diseases [16–19], pointing to a correlation between organ damage and increase in pro-oxidant cellular markers, such as malondialdehyde (MDA), 4-hydroxynonenal (4-HNE) and their protein adducts, associated with a concomitant decrease of antioxidants [16,19,20]. These markers may contribute to monitor the extent of liver damage; although their alteration may represent an epiphenomenon due to the particular cellular stress [10]. Chemical modification of essential biomolecules by ROS may cause their functional inactivation and lead to either cell death or an adaptive cellular response. In particular, a complex functional modulation of the cellular protein repertoire occurs upon oxidative

insult [21,22] through the ROS-dependent modification of specific amino acids, such as Cys, Trp, Tyr, His, Arg, and Lys [23].

ROS also behave as molecular second messengers within the cell, as they can be generated during triggering of particular cellular responses by cytokines, hormones, growth factors, and other soluble mediators, such as extracellular ATP [24]. Through the activation of protein kinases and phosphatases, intracellular ROS may directly or indirectly control the function of transcription factors, such as Nrf1 and NF-κB, thus leading to profound changes in cellular gene expression profile [25,26]. Moreover, ROS perpetuate and potentiate their own effects by influencing transcription and activation of cytokines and growth factors, responsible for further ROS production leading to the onset of the so called 'vicious cycle' [20]. Depending on the cell type and intensity/duration of the oxidative stress affecting cells, ROS may therefore act either as pro-apoptotic molecules or stimulators of cell proliferation.

Cellular defenses coping with ROS generation are multiple and include enzymatic (superoxide anion dismutase, catalase, GSH peroxidases, peroriredoxins, glutaredoxins, thuoredoxins, sulfiredoxins) and non-enzymatic antioxidants (vitamins A, C, and E, GSH, urate, bilirubin) (for more details about antioxidant mechanisms see [6]). The coordinated action of antioxidant enzymes ensures efficient ROS removal. Therefore, oxidative stress may be defined as an imbalance between the generation of ROS and the antioxidant defense capacity of the cell. In spite of the fact that the innovative concept that oxidative stress requires a fine balance within antioxidant systems is gaining relevance, large-scale interventional studies in humans with antioxidants have been inconsistent in demonstrating medical benefits, particularly in cancer patients [27,28]. The idea that redox signaling may specifically involve discrete pathways within cells suggests the possibility that oxidative stress can actually occur without an overall imbalance of pro-oxidants and antioxidants, and that the disruption of redox-sensitive signaling pathways can lead to metabolic and organ specificity in oxidative stress [6].

Some enzymes have a fundamental importance in maintaining cell functions during oxidative stress conditions. The nuclear protein Apurinic Apyrimidinic Endonuclease/Redox Effector Factor 1 (APE1/Ref-1) is a paradigmatic example [29]. This protein is involved in both transcriptional regulation of gene expression during adaptive cellular response to oxidative stress and the base excision repair pathway of DNA lesions generated as a consequence of ROS-induced base damages [29]. A significant upregulation and relocalization of this protein has been described during HCC progression, accounting for a causative role of oxidative stress in the pathogenesis of HCC and suggesting APE1/Ref-1 as a new biomarker of the transformation process [30].

In most of the liver diseases, of both metabolic and viral origin and associated with transformation processes, a chronic oxidative stress condition represents the main common determinant. During acute and chronic damage, hepatocytes are exposed to increased levels of oxidants, cytokines and bile acids. In spite of their powerful antioxidant resources, hepatocytes suffer from the cytotoxic effect of oxidative stress, leading to cell death. It is still a matter of debate whether cell death induced by ROS occurs either by necrosis or apoptosis, and which are the pathways involved in dead processes [31,32].

In the liver, inflammatory cells, cholangiocytes, and Kupffer cells are the main sources of tumor necrosis factor α (TNFα). TNFα, together with other inflammatory cytokines, contribute

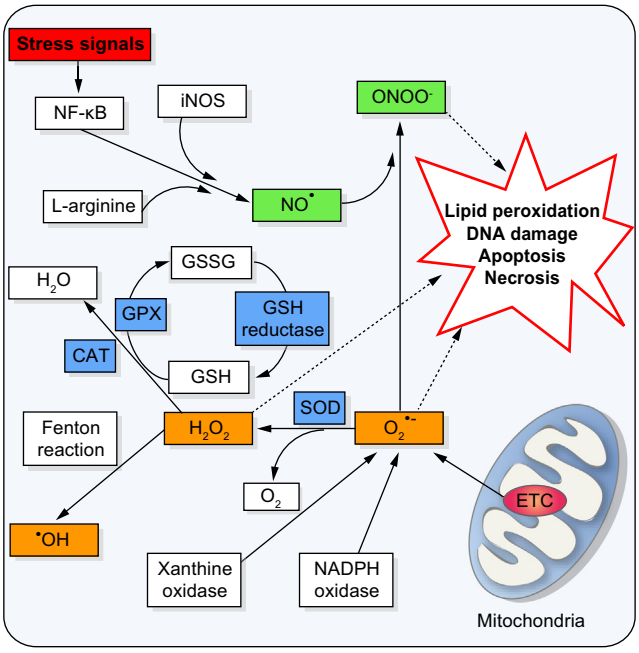

Fig. 2. Main pathways for the formation of ROS and NOS. Mitochondria are the major source of cellular ROS during respiratory processes. Electron flow leads to the formation of superoxide anion ($O_2^{\cdot -}$) that is generated by the univalent reduction of molecular oxygen (O_2). This process may also be mediated by enzymes such as NADPH oxidase and xanthine oxidase. Superoxide dismutase (SOD) catalyzes the dismutation of two superoxide anions into hydrogen peroxide (H_2O_2) and oxygen. H_2O_2 can react with reduced transition metals, *via* Fenton's reaction, to produce the highly reactive hydroxyl radical ($^{\cdot}OH$). Alternatively, H_2O_2 could be converted into water by enzymes catalase (CAT) and glutathione peroxidase (GPX). Due to its relative long half-life (10^{-5} s), H_2O_2 could damage lipids, DNA and proteins leading to cell death. Inflammatory cells are the main source of TNFα. This cytokine contribute to mitochondrial dysfunction indirectly leading to the formation of RNS as a consequence of the induction of nitric oxide synthase 2 (NOS2) and formation of nitric oxide ($NO^{\cdot}$), which reacting with $O_2^{\cdot -}$ generates peroxynitrate ($ONOO^-$).

to mitochondrial dysfunction by interfering with the mitochondrial respiratory chain and by forming $O_2^{\cdot -}$ [33]. An indirect effect of TNFα in promoting mitochondrial dysfunction is the increased production of RNS as a consequence of the induction of nitric oxide synthase 2 (NOS2) [34]. RNS, such as $NO^{\cdot}$, NO^+, NO^-, and $ONOO^-$, play an important role in controlling the cellular redox state (Fig. 2) [35]. Interestingly, some of the physiological effects of RNS are mediated through the formation of S-nitroso-Cys or S-nitroso-GSH intermediates [36]. RNS may functionally inactivate proteins of the mitochondrial respiratory chain through nitration of their Tyr residues or intermediate formation of S-nitrosated protein adducts at Cys residues [36].

Mitochondria play a crucial role in controlling apoptotic cell death, particularly in the hepatocyte. Activation of death receptors induces amplified apoptotic pathways involving caspase 8 and mitochondrial membrane proteins, which abolish the flow of electrons in the electron transport chain (ETC), increase mitochondrial ROS production and finally trigger the apoptosome (Fig. 2) [37]. ROS can also damage the mitochondria directly, by oxidizing various mitochondrial biomolecules, or by further increasing lipid peroxidation. Mitochondria are involved in both fatty acids (FA) β-oxidation and ROS generation and increasing

evidence indicates that respiratory-chain defects are a key determinant of mitochondrial dysfunction, which in turn occurs as a result of ischemia/reperfusion (I/R) and excess of FA (lipotoxicity) [38]. Mitochondrial impairment causes enhanced ROS production, which initiates a self-sustaining loop through the generation of MDA and 4-HNE, resulting from cellular lipid peroxidation, which are able to inhibit cytochrome c oxidase of mitochondrial complex IV. On the other hand, ROS *per se* damage both mtDNA and Fe–S cluster enzymes of the respiratory chain, leading to chronic organelle damage [39].

The increased availability of FA in hepatic pathologies characterized by fat accumulation (NASH or ASH) determines the activation of microsomal cytochrome P-450 isoforms CYP2E1 and CYP4A10/4A14, involved in FA ϖ-oxidation, leading to an increased ROS production and uncoupling mitochondrial respiration [38,40]. Of notice is the finding that a non-mitochondrial source of ROS, such as the NADPH-oxidase system of Kupffer cells, is also activated in NASH models, probably as a consequence of lipoperoxide or endotoxin phagocytosis [41]. This pathway is also present in activated hepatic stellate cells, possibly contributing to the general oxidative stress condition. Moreover, some viral proteins, such as the HCV protein NS5A, may affect intracellular Ca^{2+} concentration and signaling, thus triggering the elevation of ROS concentration in mitochondria and the translocation of NF-κB and STAT-3 into the nucleus with the consequent activation of target genes [42–44].

Besides the involvement in development of CLD, oxidative stress is also generated under physiological conditions, such as during I/R upon liver transplantation. The I/R damage of the transplanted liver is one of the main determinants of primary non-function or initial poor function of the graft, which may contribute to a poor transplant outcome [10]. Hepatic I/R injury can be categorized into warm I/R and cold-storage reperfusion injury [45]. ROS are generated very early after warm ischemia (within the first 4 h of reperfusion) and revascularization of ischemic tissue, in which the initial cell death triggers an inflammatory response with activation of tissue macrophages and recruitment of neutrophils [46]. Primary sources of ROS during hepatic I/R are impairment of mitochondrial ETC, activation of NADPH oxidase in neutrophils and Kupffer cells and conversion of xanthine dehydrogenase into the ROS-producing form xanthine oxidase [47] (Fig. 2). Although the latter mechanism was often considered as a critical source of ROS during I/R injury, recent evidence tends to limit the contribution of this pathway in ROS formation [47].

Role of oxidative stress during HCV infection

A close relationship exists between HCV infection and oxidative stress, and hepatitis C virus infection is associated with severe alteration of the host redox status [48]. Lipid peroxidation products are increased in serum, peripheral blood mononuclear cells (PBMC), and liver tissue, and 4-HNE and 8-hydroxyguanosine (8OHdG) are also elevated [49]. In addition, there is a significant reduction of hepatic, plasmatic, and lymphocytic GSH levels in patients chronically infected with HCV associated with an increased percentage of oxidized GSH (GSSG), suggesting an increased GSH turnover [49].

This increased oxidative stress in hepatitis C may be explained by chronic inflammation, and the continued generation of ROS and NOS may be due to an increased activity of NADPH oxidase (Nox 2 protein) of Kupffer and polymorphonuclear cells in the liver [50]. NS3 protein of HCV has been shown to activate Nox 2 protein of phagocytes and trigger apoptosis and dysfunction of T cells, natural killer cells, and natural killer T cells. Nox 2 protein is located on phagosomal and plasma membranes, leading to increased generation of ROS and other reactive species that can exert oxidative stress to the nearby cells.

The excess iron deposits found in the liver tissue of some HCV patients may promote the generation of free radicals in these individuals [51]. The mRNAs of *TNF-α* and cytochrome P-450 (*CYP2E1*), both of which can increase ROS production, might also be elevated in hepatitis C patients [52]. Furthermore, there is some indication that HCV can directly induce oxidative stress in the hepatocyte [53]. Gene expression in HCV core has been associated with increased ROS, decreased intracellular and/or mitochondrial GSH content, and increased levels of oxidized thioredoxin and lipid peroxidation products [54,55]. Therefore, it may be suggested that HCV produces oxidative stress through multiple mechanisms that include chronic inflammation, iron overload, and liver injury. Some of the HCV proteins may contribute to this process.

Sensitive oxidative stress biomarkers may be important in the diagnostic approach to HCV infection and help monitor disease progression and the efficacy of therapies. However, in spite of the amount of information available on the pathogenic role of oxidative stress in HCV infection and the significant advances made in the last few years, the translation to clinical practice is still far [56].

ROS and hypoxia in HCC

Human HCC is the fifth most frequent neoplasm worldwide and the third cause of cancer-estimated deaths. There are multiple etiological agents that are associated with the development of HCC, the most frequent being HBV and HCV infections and metabolic disease as NASH [57]. Globally, up to 80% of HCC is attributable to HBV or HCV [58]. The risk of HCC is increased 5- to 15-fold in chronic HBV carriers [59] and 11.5- to 17-fold in HCV-infected patients [60]. Antiviral therapy is effective in preventing HCC in only a proportion of patients [61]. Moreover, sustained clearance of HBV or HCV may be difficult to accomplish, particularly among cirrhotic patients.

It has been shown that oxidative DNA damage in cirrhotic HCV-infected patients is associated with increased risk of developing HCC [62]. In patients with chronic hepatitis C, an increased 8OHdG in DNA extracted from liver tissue was reported [49,63,64]. Although these reports suggest that oxidative stress may be involved in the progression of liver disease, they did not show a direct participation of oxidative stress in hepatocarcinogenesis.

Intracellular ROS generation may also represent a critical event linking hypoxia to angiogenesis and HCC development. Indeed, hypoxic areas are very common in HCC and hypoxia-related induction of ROS formation has been reported to stabilize the hypoxia-inducible factor 1α (HIF-1 α), through inhibition of prolyl-hydroxylases (PHD) or redox-dependent activation of protein phosphorylation cascades, and to lead to upregulation of HIF-1 target genes [65]. Mitochondria are the major source of ROS generated during hypoxia [66]. Hypoxia increases mitochondrial ROS *via* the transfer of electrons from ubisemiquinone

to molecular oxygen at the Qo site of complex III of the mitochondrial ETC [67]. Release of reactive oxygen species from the inner mitochondrial membrane to the intermembrane space leads to the activation of transcription factors, including HIF-1 α [68]. Furthermore, hypoxia-induced ROS generation has been shown to enhance the DNA binding of NF-κB through a redox dependent mechanism, leading to transcriptional activation of target genes [69].

Hypoxia represents a strong selective pressure reported to favor cancer progression through the activation of adaptive transcriptional programs that promote angiogenesis, cell survival, motility, and invasiveness of malignant cells, including the ability to induce epithelial mesenchymal transition (EMT) and increased invasiveness in human epithelial cancer cells [70–72]. This novel, ROS- and hypoxia-related perspective is particularly relevant to HCC that usually develops on the background of a chronically damaged liver. Indeed, hypoxia, through redox signaling, has been described to induce EMT and increased invasiveness in human liver cancer cells [71], which is relevant for a tumor like HCC that is more invasive than metastatic [70,72]. Moreover, aberrant angiogenesis and vascular remodeling are critical events for HCC growth [70,72]. The angiogenic switch is sustained by hypoxia, cytokines and GFs as well as by mutation in suppressor genes and oncogenes, with hypoxia being the most relevant one.

The metabolic syndrome: role of oxidative stress in the progression of NAFLD

The definition of metabolic syndrome involves a group of metabolic and cardiovascular risk factors that help identify subjects at high risk of type 2 diabetes and cardiovascular disease (CVD). Even if the term "syndrome" implies a specific causative aetiology, there is not a clear, unifying pathophysiological cause for the metabolic syndrome. Nevertheless, abdominal adiposity, physical inactivity, and insulin resistance appear to be at the core of this condition [73]. The latest definition of the metabolic syndrome, supplied by the International Diabetes Federation, shows as typical marks: abdominal obesity, defined by increased waist circumference ($\geqslant$94 cm in men and $\geqslant$80 cm in women), and two or more of the following factors including elevated blood pressure, raised fasting plasma glucose, increase serum triglyceride levels and/or low HDL cholesterol [74]. An increase in triglyceride levels is one of the most prevalent metabolic syndrome components. Recent epidemiological data indicate that NAFLD is the most frequent hepatic lesion in Western countries. This condition is often benign, but in about 20–30% of NAFLD patients the disease can progress to NASH and cirrhosis.

Increased levels of oxidative stress have been proposed to play a relevant pathogenic role in NAFLD progression according to the original 'two-hit' theory of Day and James [75] and to the more recent insights coming from reliable animal models [76]. Along these lines, several mechanisms favoring increased generation of oxidative stress mediators in NAFLD have been proposed, including mitochondrial, increased fatty acid oxidation in either endoplasmic reticulum (ω-oxidation), by CYP-2E1 and CYP4A isoforms, or in peroxisomes (β-oxidation) by acyl-CoA oxidase [77]. Moreover, ROS and other oxidative stress-mediators, such as aldehydic end-products of lipid peroxidation are believed to sustain fibrotic progression of chronic liver diseases of different aetiology towards the end point of cirrhosis, being able to elicit cell injury and death as well as fibrosis and inflammatory response [77,78].

Molecular events in NASH

The accumulation of triglycerides in hepatocytes is the hallmark of NAFLD, a spectrum of hepatic abnormalities usually associated with altered metabolism [74]. In approximately 30% of the cases, steatosis is associated with hepatocellular damage, evident as ballooning, inflammation and fibrosis. This more aggressive form of the disease, known as NASH, may be associated with fibrosis and has the ability to progress to cirrhosis and its complications, including hepatocellular carcinoma [79]. The mechanisms leading to the appearance of NASH and its progression to fibrosis are still uncertain and focus of active investigation. Genetic factors certainly play a role, as shown for the polymorphisms of adiponutrin (*PNPLA3*), which are associated with steatosis and predisposition to injury and fibrosis [80]. Studies in animal models have increased our understanding of the molecular mechanisms that lead to the appearance and progression of NASH. A central point in the emergence of NASH is the toxic effects of lipids accumulating in the liver, a process known as lipotoxicity [81]. This event is mediated by fatty acids and some of their metabolites, whereas triglycerides represent a relatively safe form of fat storage. Of interest was the observation that cholesterol *per se* may play a role in the progression of fatty liver to more steatohepatitis. Both nutritional and genetic models of hepatic steatosis showed that the accumulation of cholesterol in mitochondria was associated with a selective glutathione depletion which in turn was associated with a higher sensitivity to TNF and Fas [82]. This observation pointed to the conclusion that not only the amount but also the type of lipids accumulated in the liver may be important, a concept confirmed in different experimental models [83]. One of the consequences of lipotoxicity is the appearance of endoplasmic reticulum stress (ERS), which results from improperly folded proteins accumulating in the ER [84]. ERS is linked to the activation of NF-κB, c-Jun N-terminal kinase, and oxidative stress pathways. More recently, autophagy has also been implicated in the pathogenesis of insulin resistance and fat-mediated damage [85].

Another pathway strictly linked to lipotoxicity is the generation of oxidative stress-related products, which contribute to hepatocellular damage, inflammation and fibrosis [86]. Inflammation is part of the wound healing response and is regulated by a complex network of soluble mediators, including cytokines such as TNF-α or IL-6. In this context, chemokines such as CCL2 or CCL5 also play a relevant role [87]. The chemokine system is not only linked to inflammation but also contributes to the development of fibrosis *via* direct actions on hepatic stellate cells. In this respect, activation of the receptors CCR5 and CCR2 play a major role [88].

The development of steatohepatitis and fibrosis is also influenced by extrahepatic factors. Differences in the microbiota have been recently suggested to participate in the pathogenesis of NASH [89]. These actions are mediated, at least in part, by activation of the toll-like receptors, which recognize microbial patterns providing danger signals. Adipokines, cytokines secreted at the level of the adipose tissue, represent another group of signals relevant to the development of steatohepatitis

and fibrosis. Leptin and adiponectin exert many opposing actions on inflammation and fibrosis. In addition, adiponectin limits the development of insulin resistance. Modulation of adipokine imbalance can explain some of the favorable effects of weight loss [90].

Iron induced liver damage

Accumulating evidence indicates that deregulation of iron metabolism is involved in the pathogenesis of ischemic/reperfusion injury, neurodegeneration, insulin resistance and diabetes mellitus, atherosclerosis, and liver diseases [91]. The most studied disease model of iron toxicity is represented by hereditary hemochromatosis (HH), where defective regulation of systemic iron metabolism due to defective release or activity of the hormone hepcidin leads to hepatic and later parenchymal systemic iron overload [92]. In HH, iron accumulation within hepatocytes has been associated with oxidative damage to DNA and activation of fibrogenesis [93], and often results in progressive liver disease. In association with obesity and metabolic syndrome, it represents the leading cause of hepatic iron overload in Western countries [94], with frequent progression to advanced fibrosis and HCC [95].

Although a long-lasting hypothesis is that the damaging effect of iron is related to its ability to catalyze the generation of ROS *via* the Fenton reaction, the molecular pathways of cellular and tissue dysfunction are less clear. Recent data have implicated an upregulation of the p53 pathway, which would lead to an initial induction of antioxidant enzymes, and later to cell senescence and death [96]. Furthermore, fatty acids would further increase ROS production when iron accumulation is associated with fatty liver, and, when present, excess alcohol.

A recent study by Dixon *et al.* describes a non-apoptotic form of cell death dependent on the oncogenic RAS-selective lethal small molecule erastin. This molecule triggers a unique iron-dependent form of non-apoptotic cell death that the authors termed "ferroptosis". Ferroptosis is dependent upon intracellular iron and is morphologically, biochemically, and genetically distinct from apoptosis, necrosis, and autophagy. Erastin, like glutamate, inhibits cystine uptake by the cystine/glutamate antiporter (system x(c)(−)), creating a void in the antioxidant defenses of the cell and ultimately leading to iron-dependent, oxidative death [97].

The role of ROS in the pathogenesis of iron-related liver damage was supported by the demonstration that a functional promoter polymorphism of myeloperoxidase (MPO), a major site of ROS production in phagocytes, is associated with cirrhosis and HCC in HH patients [98]. Similarly, in NAFLD it was recently demonstrated that the C47T polymorphism, resulting in a decreased import and activity of the mitochondrial SOD2 involved in the detoxification of ROS, was strongly associated with the susceptibility to advanced fibrotic disease even when I148M *PNPLA3* genotype was controlled [99]. The same genetic variant predisposes to cardiomyopathy and HCC in HH [100].

Recent observations suggest that as in NASH, ERS plays an important role in iron-induced liver injury and that ROS are mediators of the ERS response. ERS is an adaptive response to cellular stress induced by the accumulation of unfolded proteins, caused by unbalanced oxidative stress overcoming the protein folding capacity of ER. An excessive and protracted ERS results in cellular dysfunction by the alteration of glucose and lipid metabolism and by favoring cell death. Finally, the complex interplay between iron, ROS, and ERS response was emphasized by the evidence that accumulation of unfolded proteins in ER induces hepcidin expression *via* the ERS response [101], thus linking ERS to iron homeostasis. On the other hand, oxidative stress has also been shown to decrease hepcidin release by altering the chromatin structure of its promoter region during HCV infection and alcohol abuse [102,103], thus contributing to the increased iron absorption characteristic of these diseases.

Increased ROS production leading to unbalanced oxidative stress plays a key role in the pathogenesis of liver disease associated with hepatic iron accumulation, due to both genetic and acquired factors, and excess iron which may synergize with steatosis. The mechanisms include direct stimulation of fibrogenesis, mitochondrial damage and activation of the p53 pathway, activation of the ERS response, and DNA damage, collectively favoring HCC occurrence. Conversely, the oxidative stress and ERS response affect iron metabolism by influencing hepcidin expression. Therefore, the modulation of oxidative stress represents an attractive therapeutic target for the prevention of clinical complications of iron overload and steatosis.

Modern proteomics will help in new biomarker discovery for early diagnosis of oxidative stress-based liver pathologies

Antioxidant supplements for liver diseases did not provide convincing evidence to support or refute antioxidants in treating liver diseases [104]. This conclusion may be due to the lack of biomarkers predictive of severity and progression of the disease. Accordingly, the understanding of the sources of ROS formation during liver injury process is critical for the interpretation of experimental results and for designing an effective therapeutic intervention.

The liver has been the subject of dedicated investigations since the first introduction of basic proteomic technologies in the early '90s [105]. After the launch of the Human Liver Proteome Project (HLPP) in 2002, a number of holistic studies have been undertaken on human and mouse/rat models in an attempt to reach a functional map of the organ, expanding the proteomic description to its "physic-ome" and "path-ome", with the aim to accelerate the development of liver-specific diagnostics and therapeutics [106]. Thus, various dozens of investigations, based on the use of 2D chromatography/electrophoresis combined with different MS approaches, have been published to describe the quantitative level of liver proteins under basal conditions [107]. These researches have been performed directly on liver tissues or, in other cases, on hepatocyte, hepatic stellate, Chang and Kupffer cells. Moreover, a large number of differential studies have been undertaken to describe the quantitative proteomic variations in hepatic cells during embryo organ development [108,109] or senescence [110], after organ transplantation/resection [111,112], following treatment with various toxic agents [113,114], or different liver diseases. In particular, a great effort has been spent in the proteomic analysis of *in vitro* models or liver tissue of HCV [115,116] and HBV infection [117,118], fibrosis and cirrhosis [118,119], HCC [120–123], and NAFLD

[124,125]. Sensitive and specific biomarkers of some liver diseases have also been searched in the serum of patients by 2-DE/MS or SELDI-MS approaches [126–128], proposing a number of disease-associated biomarkers. Developments in immunoaffinity depletion and various fractionation approaches in combination with substantial improvements in LC–MS platforms have enabled the plasma/tissue proteome to be profiled with a considerably greater dynamic range of coverage, allowing the identification of several proteins at low ng/ml levels. Despite these significant advances, major challenges associated with the dynamic range of measurements and extent of proteome coverage, confidence of peptide/protein identifications, quantitation accuracy, analysis throughput, and robustness of present instrumentation must be addressed before a proteomics profiling platform suitable for efficient clinical applications may be routinely used in the accurate diagnosis of early liver damage, as well as to monitor disease progression and assess treatment efficacy [129].

Based on previous investigations describing the main targets of ROS and RNS-dependent protein oxidation/nitrosation, redox proteomics approaches [130] were performed *in vitro* experiments by treating liver tissue or hepatocytes with specific oxidants, such hydrogen peroxide, diamide, menadione, *t*-butyl-hydroperoxide and S-nitroso-L-cysteine [130–133]. These studies identified early protein markers of thiol modification, which are subjected to glutathionylation, sulphenation or nitrosation reactions. Although still preliminary and inconclusive, this scenario emphasizes the potential role of proteomics and redox proteomics technologies in the development of liver-specific diagnostics and therapeutics. These holistic hold promise for the identification of biomarker proteins implicated in the development of CLD and of protein targets of the ROS/RNS insult during pathophysiological phenomena. It has to be mentioned, however, that while various proteomic studies have described the quantitative changes in the protein profile of hepatic cells or tissues during the pathological income, a very limited number of investigations have described their redox-associated modifications.

Conclusions and perspectives

Treatment with antioxidants has been shown to be beneficial under controlled conditions in cell-free or *in vitro* cell systems, but the expectation of this translational application to human liver disease has not been yet fulfilled. Too often the evidence for a general ROS formation is provided without the evaluation of cellular sources, the nature of ROS and identification of initiating events. This can lead to misinterpretation of the inner mechanisms and ultimately jeopardizes the translation of the findings to the human pathophysiology. This may explain the reason why despite the fact that ROS appear to be important in every experimental model of inflammatory liver disease, no drug that target ROS is currently available for human therapy. More detailed studies are warranted to elucidate: (i) the molecular mechanisms of liver tissue injury and repair during an acute inflammatory response and (ii) the role of ROS in these processes to translate into better management of the use of antioxidant and ROS scavengers in the therapy of liver diseases. We hope and expect the future will fill the gap.

Key Points

- A correct balance between pro- and anti-oxidant events (RedOx state) is crucial for a correct function of any living cell. Oxidative events lead to the production of Reactive Oxygen Species (ROS), and are rapidly counteracted by several, different mechanisms
- Intracellular ROS may control the function of transcription factors, such as Nrf1 and NF-κB, leading to profound changes in cellular gene expression profile which are at the basis of several pathological conditions in the liver
- Acute and chronic liver diseases are characterized by an imbalance of the redox state toward an oxidative status. This may be at the basis of common cellular events as inflammation and cell damage
- HCV infection, NAFLD and NASH, cirrhosis and iron accumulation are good examples of liver diseases associated with an altered redox state, though clinical data are still poorly defined
- Few, scattered data are available on molecular biomarkers of ROS production and redox alteration in clinical settings. This prevented so far investigation of the potential diagnostic and prognostic values of this status
- A better understanding of the redox state in different liver diseases will allow to better explore the potential efficacy of antioxidant treatment

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Conflict of interest

The authors declared that they do not have anything to disclose regarding funding or conflict of interest with respect to this manuscript.

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