Oxidative stress in nonalcoholic fatty liver disease: a reappraisal of the role in supporting inflammatory mechanisms

Salvatore Sutti and Emanuele Albano

Department of Health Sciences and Interdisciplinary Research Centre for Autoimmune Diseases, University of East Piedmont, Novara, Italy

Correspondence should be addressed to E Albano Email emanuele.albano@med.uniupo.it

Abstract

In the last decade, non-alcoholic fatty liver disease (NAFLD) and particularly its evolution to nonalcoholic steatohepatitis (NASH) have become a leading cause of chronic liver disease and cirrhosis as well as an important risk factor for hepatocellular carcinoma. Oxidative stress is a common feature of NAFLD/NASH and plays a key role in the complex of metabolic and cellular derangements that are involved in the development of liver steatosis, as well as in the transition to steatohepatitis. This review deals with the contribution of oxidative stress in promoting hepatic inflammation which represents a key factor in NAFLD evolution to liver fibrosis/cirrhosis. We discuss in detail recent data involving oxidative stress products as triggers for hepatic innate immunity and as a source of antigens implicated in sustaining lymphocyte-mediated adaptive immune responses. Attention is also paid to emerging evidence linking oxidative stress and extra-hepatic complications of NAFLD/NASH.

Introduction

In the last two decades, the worldwide spreading of overweight and obesity has resulted in an increase in the prevalence of nonalcoholic fatty liver disease (NAFLD) which is now becoming the most common liver disease. By now, NAFLD prevalence in the general population ranges from 31 to 32% in the Middle East and South America, 24-27% in Europe, North America, and Asia, and about 14% in Africa (Younossi et al. 2018) However, in the next decade, the burden of NAFLD is expected to further increase and current projections estimate that by 2030 more than 400 million individuals all over the world will develop NAFLD (Estes et al. 2018).

NAFLD is a heterogeneous disease encompassing a spectrum of lesions. In the majority of NAFLD patients, the main feature is hepatocyte triglyceride accumulation (steatosis) which has low risk of further liver complications (Powell et al. 2021). Conversely, about 20–30% of NAFLD subjects, develop nonalcoholic steatohepatitis (NASH), in which liver steatosis combines with parenchymal damage (hepatocyte apoptosis and ballooning, focal necrosis), lobular/portal inflammation, and variable degree of fibrosis (Powell et al. 2021). Liver fibrosis in NASH often progresses to cirrhosis and is the strongest predictor for disease-specific mortality (Powell et al. 2021). Although NAFLD progression to cirrhosis is more frequent among middle-aged and elderly people (Powell et al. 2021), the diffusion of NAFLD among obese children leads to pediatric NASH and an increased risk of cirrhosis in adulthood (Mann et al. 2018). At present, NAFLD is also an increasingly frequent cause of hepatocellular carcinomas (HCCs) in Western countries (Huang et al. 2021). The actual incidence of NAFLD-related HCCs ranges from 10...
to 34%, but it is expected to increase by 122% within 2030 making NAFLD the prevalent cause of HCC in the next decade (Huang et al. 2021).

Along with the hepatic effects, the presence of NAFLD/NASH also associated with an increased risk of extra-hepatic diseases such as type 2 diabetes mellitus, cardiovascular diseases, chronic kidney diseases, and osteoporosis which represent the major cause of death and disability among NAFLD/NASH patients (Mantovani et al. 2020). Interestingly, the contribution of NAFLD/NASH to these diseases is independent of the risk factors in common with metabolic syndrome and systemic complications are more prevalent in patients with steatohepatitis (Mantovani et al. 2020). This indicates that the presence of liver inflammation not only is the driving force for the disease evolution to cirrhosis and HCC but might also specifically contribute to extrahepatic injury.

Oxidative stress in NAFLD/NASH

The involvement of oxidative stress mechanisms in the pathogenesis of NAFLD/NASH stems from the observation that an increase in oxidative stress markers is a common feature in either rodent models of NAFLD/NASH or the human disease. Experimental studies using nutritional protocol leading to simple steatosis (high-fat diets), or overt NASH (high fat/carbohydrate or choline/methionine deficient diets), have shown elevations in the liver content of lipid peroxidation products, such as oxidized lipids, malonaldehyde (MDA), and 4-hydroxynonenal (4-HNE), along with the accumulation of protein carbonyls, isoprostanes, nitrotyrosine, and oxidized DNA products (Baumgardner et al. 2008, Sutti et al. 2014, Chen et al. 2020). These observations are confirmed by several clinical studies evidencing an increase in oxidative stress markers in patients with NAFLD and even more in those with NASH (Chen et al. 2020, Sun et al. 2020). In some studies, these changes are accompanied by the lowering of the hepatic content of reduced glutathione (GSH) and vitamin E along with the impaired activity of antioxidant enzymes including superoxide dismutase, catalase, and GSH peroxidase (Chen et al. 2020, Zelber-Sagi et al. 2020). These changes correlate with the clinical severity of the disease suggesting that oxidative damages can contribute to NAFLD progression (Chen et al. 2020, Zelber-Sagi et al. 2020). Supporting this view, in an elegant study Sun and co-workers have shown that targeting oxidized phospholipids (OxPLs) by over-expressing a functional single-chain antibody that neutralizes OxPLs effectively improves steatohepatitis and fibrosis in hyperlipidemic LDL receptor-deficient (Ldlr−/−) mice (Sun et al. 2020).

Several mechanisms have been implicated in causing an excess production of reactive oxygen species (ROS) in NAFLD/NASH (Bellanti et al. 2017, Chen et al. 2020, Arroyave-Ospina et al. 2021). Among these, are the alteration of the mitochondrial respiratory chain, the induction of cytochrome P450s in the endoplasmic reticulum (ER), and the activation of NADPH oxidases in both hepatocytes and inflammatory cells play the major role (Bellanti et al. 2017, Chen et al. 2020, Arroyave-Ospina et al. 2021). In this respect, metabolic alterations leading to an excess of free fatty acids (FFAs) and lipotoxicity along with a high intake of fructose are important factors in triggering hepatocyte sources of ROS (Bellanti et al. 2017, Chen et al. 2020, Arroyave-Ospina et al. 2021). Nonetheless, equally important is the cellular capacity to efficiently modulate antioxidant responses through the action of redox-sensitive transcription factors, such as the nuclear factor-kB and the nuclear erythroid 2-related factor 2 (Nrf2) (Arroyave-Ospina et al. 2021). High levels of ROS lead to the release of Nrf2 from its cytoskeletal-anchoring protein Kelch-like ECH-associated protein 1 and by translocating to the nucleus Nrf2 promotes the transcription of genes regulated by antioxidant responsive elements (Arroyave-Ospina et al. 2021). In a recent study, Mohs and co-workers (Mohs et al. 2021) have shown that high Nfr2 expression is associated with oxidative stress in liver samples from NAFLD/NASH patients and that Nrf2 activation correlates with the grade of inflammation, but not with the grade of steatosis. Consistently, positive or negative Nrf2 modulation appreciably influences the severity of steatohepatitis and fibrosis in mice with NASH (Arroyave-Ospina et al. 2021).

Although clinical and experimental data strongly point to the association between oxidative stress and NAFLD/NASH, it is less clear the actual role played by oxidative mechanisms in the complex of metabolic and cellular derangements that are responsible for the onset of NAFLD and the transition from liver steatosis to steatohepatitis. Recent reviews have focalized the possible contribution of oxidative stress in the development of insulin resistance, in the impairment of liver lipid metabolism as well as in the mechanisms responsible for mitochondrial alterations, ER stress, and hepatocyte death (Bellanti et al. 2017, Chen et al. 2020). Here, we would like to discuss in detail the involvement of oxidative stress in supporting hepatic inflammation because inflammatory reactions are a key factor in the risk of NAFLD evolution and extrahepatic complications.
Mechanisms supporting hepatic inflammation during NAFLD evolution

The key role played by hepatic inflammation in the progression of NAFLD has led to a great number of studies investigating the contribution of both innate and adaptive immunity. It is outside the scope of this review to discuss in detail the pathogenesis of liver inflammation in NASH. Nonetheless, few aspects deserve a short mention in relation to the interplay between oxidative stress and inflammatory mechanisms.

The activation of resident Kupffer cells is currently seen as a key element in the onset of hepatic inflammation in NASH. In these settings, metabolic derangements consequent to insulin resistance are responsible for causing an excess of circulating FFAs and cholesterol which directly stimulate Kupffer cells (Kazankov et al. 2019). Within the hepatocytes, FFAs also cause ER stress and lipotoxicity (Lebeaupin et al. 2018) which favor the hepatocyte production of pro-inflammatory cytokines and of microvesicles capable of stimulating Kupffer cell responses (Srinivas et al. 2020). Additional triggers of macrophage response involve the stimulation of inflammasome (Szabo & Petrašek 2015) and gut dysbiosis associated with obesity (Aron-Wisnewsky et al. 2020). This latter can contribute to hepatic inflammation through changes in the enteral adsorption of bacterial products and metabolites (Aron-Wisnewsky et al. 2020). As a result of these stimuli, activated Kupffer cells release cytokines, chemokines, eicosanoids, nitric oxide (NO), and ROS (Kazankov et al. 2019). Kupffer cell-mediated signals also promote the liver recruitment of leukocytes such as neutrophils, monocytes, natural killer, and natural killer T (NKT) cells which further contribute to support inflammation (Cai et al. 2019). In particular, the release of chemokines, such as CCL1, CCL2, and CCL5, is responsible for parenchymal infiltration by monocytes, that by differentiating to macrophages play a major role in NASH evolution by either directly supporting inflammation and by providing the stimuli responsible for the transformation of quiescent hepatic stellate cells (HSCs) into matrix-producing myofibroblast (Loomba et al. 2021). Furthermore, by promoting the release of ROS and NO liver macrophages also represent an important stimulus for oxidative stress in NASH (Kazankov et al. 2019).

The characterization of liver macrophage expanding in either human and rodent NASH using single-cell RNA sequencing has revealed that during NASH progression embryonically derived Kupffer cells are progressively lost and replaced by monocyte-derived macrophages that have a specific phenotype characterized by the expression of the triggering receptor expressed on myeloid cells 2 (TREM-2), CD63, and the glycoproteins CD9 and NMB (GP-NMB) (Xiong et al. 2019, Seidman et al. 2020, Daemen et al. 2021). The prevalence of these cells, also called NASH-associated macrophages (NAM) correlates with the severity of NASH (Xiong et al. 2019, Seidman et al. 2020), likely in relation to their phenotypic analogies with scar-associated macrophages identified in human fibrotic livers (Ramachandran et al. 2019). In line with these findings, blocking monocyte recruitment by CCL2 and CCL5 ameliorates the severity of NASH reducing the evolution to fibrosis (Tacke & Weiskirchen 2021), whereas interference with the anti-inflammatory signals that control macrophage responses worsens NASH evolution (Locatelli et al. 2014, Hou et al. 2021).

Besides the role of innate immunity, growing evidence indicates that adaptive immune reactions involving B and T lymphocytes also play a role in directing liver inflammation in NASH. Indeed, one of the histological features of NASH is lymphocytes’ presence in either lobular or periportal infiltrates (Yeh & Brunt 2014). Furthermore, in NASH patients B- and T-lymphocytes often form focal aggregates, resembling ectopic lymphoid structures the prevalence of which positively correlates with the disease severity (Bruzzì et al. 2018). The hepatic infiltration by B- and CD4+ and CD8+ T-lymphocyte is also evident in different experimental models of NASH where it parallels with the worsening of parenchymal injury and lobular inflammation (Sutti & Albano 2020). From the functional point of view, NASH B-lymphocyte response involves the fraction of CD43+/CD23+ B2-cells, while CD4+ T-lymphocytes can acquire polarization as interferon-γ (IFN-γ)-producing T-helper 1 (Th-1) or T-helper 17 (Th-17) cells characterized by the prevalent secretion of interleukin-17 (IL-17) (Sutti & Albano 2020). Recent studies have also characterized the phenotype of NASH-associated liver cytotoxic CD8+ T-lymphocytes showing that they feature activation/exhaustion markers, the chemokine receptor CXCR6, and a high expression of the immunomodulating molecule programmed cell death protein 1 (PD1) (Ramadori et al. 2022).

The contribution of adaptive immunity to NASH is supported by the observation that increasing liver lymphocyte recruitment greatly worsens steatohepatitis and liver fibrosis, whereas steatosis, parenchymal injury, and lobular inflammation are lowered in Rag1−/− mice, lacking mature B, and T cells (Sutti & Albano 2020). In a
similar manner, selective ablation of B-lymphocytes or CD8+ T-cells also improves experimental NASH evolution (Barrow et al. 2021a, Ramadori et al. 2022). From the mechanistic point of view, available evidence indicates that the cytokine network generated by Th-1 and Th-17 CD4+ can provide a stimulus for the pro-inflammatory activity of macrophages (Sutti & Albano 2020, Huby & Gautier 2021), while metabolic stimuli induce CD8+/CXCR6+/PD1+ T-cells to exert an antigen-independent cytotoxicity toward hepatocytes (Ramadori et al. 2022).

In turn, macrophages can support lymphocyte functions through the release of a variety of mediators including interleukins 12 (IL-12), 15 (IL-15), and 23 (IL-23), as well as lymphocyte chemokines CXCL9-10-11 (Sutti & Albano 2020, Huby & Gautier 2021). The combinate action of IL-15 and of the chemokine CXCL16 is also important for modulating the activity of liver NKT which by expanding in advanced NASH support hepatic parenchymal injury, inflammation, and fibrosis. (Huby & Gautier 2021).

**Interplay between oxidative stress and inflammation in the pathogenesis of NASH**

As mentioned above oxidative damages are a common cause of cell stress and significantly contribute to causing hepatocyte death by apoptosis and necrosis in overt NASH. Thus, the release of damage-associated molecular patterns (DAMPs) such as high-mobility group box-1, heat shock proteins, mitochondrial and nuclear DNA, N-formyl peptides, and S-100 proteins represents a common mechanism through which fat-laden hepatocytes can stimulate inflammatory and immune cells (Brenner et al. 2013). It is also likely that, as a component of the lipotoxic response, enhanced ROS generation might contribute to the production of pro-inflammatory cytokines by hepatocytes through signals mediated by the stress-responsive kinases, including c-Jun N-terminal kinases 1/2 (JNK1/2) (Lebeaupin et al. 2018) or STAT1 (Grohmann et al. 2018) (Fig. 1). In fact, by inactivating hepatocyte tyrosine phosphatase non-receptor type 2 (PTPN2) oxidative stress...
promotes STAT1 signaling in NASH liver, while PTPN2 deletion in hepatocytes enhances lymphocyte recruitment to the liver through the production of the lymphocyte chemokine CXCL9 (Grohmann et al. 2018). Interestingly, obese patients with NAFLD show a higher expression of CXCL9 as compared to healthy controls (Grohmann et al. 2018). An additional link between oxidative stress and hepatic inflammation is represented by the capacity of ROS to stimulate inflammasome activation and therefore caspase 1-mediated release of active interleukin-1β (Szabo & Petrasek 2015). In detail, NASH associates with specific activation of NLR family pyrin domain containing 3 (NLRP3) inflammasome and NLRP3 blockade or interference with caspase 1 reduces liver inflammation and fibrosis in experimental NASH (Knorr et al. 2020). Finally, in the frame of the contribution of fructose in causing NASH (Jensen et al. 2018) a recent study has shown that oxidative stress contributes to gut leakiness of bacterial endotoxins by impairing the expression of tight junction proteins in intestinal epithelial cells (Cho et al. 2021).

An important aspect in the interplay between oxidative stress and the pro-inflammatory activation of liver macrophages relays on the fact that these cells can directly recognize lipid peroxidation products as well as their condensation adducts with different macromolecules through pattern recognition receptors (PRR) and scavenger receptors. For instance, oxidized cholesterol esters and oxidized phospholipids can stimulate macrophage activation through the interaction with members of the toll-like receptor (TLR) family of PRR such as TLR4 and TLR2 (Kadl et al. 2011, Hendrikk & Binder 2020) (Fig. 1). Scavenger receptors including CD36, scavenger receptor type A (SR-A1 and SR-A2), SR-B1, and lectin-like oxidized LDL receptor 1 are also very effective in mediating macrophage recognition and clearance of cellular structures modified by oxidative stress (Greaves & Gordon 2009 Hendrikk & Binder 2020). Oxidized phospholipids and aldehydes generated during lipid peroxidation are, in fact, highly reactive and can form a great variety of adducts with cellular macromolecules (Papac-Milicevic et al. 2016). These adducts also include the condensation products generated by the interaction between MDA and acetaldehyde, known as malonyl dialdehyde-acetaldehyde adducts (MAA) (Thiele et al. 2008). During NASH adducts containing lipid peroxidation products are detectable in dying fat-laden hepatocytes as well as in oxidized LDLs promoting their scavenging by macrophages (Busch et al. 2017). As a result of such a receptor-mediated uptake of lipid-rich structures liver macrophages become loaded with lipids assuming features comparable to that of foam cells present in atherosclerotic plaques (Kazankov et al. 2019). Foamy macrophages present in NASH livers have the phenotype of TREM-2-positive NAM (Seidman et al. 2020), release pro-inflammatory mediators (Jindal et al. 2015), and form clusters, known as hepatic crown-like structures or lipogranulomas (Itoh et al. 2013). Available evidence indicates that the presence of cholesterol crystals in macrophages sustains their pro-inflammatory activity (Ioannou et al. 2013). Nonetheless, these cells can also contribute to NASH evolution to fibrosis in view of their colocalization with regions of stellate cell expansion (Kazankov et al. 2019), and because of the production of pro-fibrogenic mediators such as galectin-3 and osteopontin (Seidman et al. 2020, Daemen et al. 2021). Consistently the lack of CD36 and SR-A1 in myeloid cells reduces foam cell formation and hepatic inflammatory responses in Ldlr<−/− mice, while NASH induced by feeding choline/methionine deficient (MCD) diet is less severe in TLR4 knockout mice (Busch et al. 2017). Bone marrow-derived macrophages from mice lacking CD36 and SR-A1 also show a decreased production of pro-inflammatory cytokines upon challenging with MDA (Busch et al. 2017).

Although these effects explain how oxidative stress can support hepatic inflammation during NAFLD/NASH progression, they do not fully clarify why only a fraction of the patients with simple steatosis develops hepatic inflammation and do not account for the increase in the risk of extrahepatic complications among NASH patients.

### Oxidative stress as a trigger for adaptive immune responses in NASH

A key issue in understanding the interplay between oxidative stress and inflammation during NASH evolution concerns the role of oxidative mechanisms in triggering lymphocyte responses. A growing body of data indicates that the adducts originating from the interaction of proteins with oxidized phospholipids or lipid peroxidation-derived aldehydes are recognized as antigens by the immune system triggering the onset of both cellular and humoral immune responses (Thiele et al. 2008, Papac-Milicevic et al. 2016). These antigens, also called oxidative stress-derived epitopes (OSEs), are presently implicated in the stimulation of adaptive immune responses responsible for the evolution of atherosclerosis (Papac-Milicevic et al. 2016), in the breaking of self-tolerance in several autoimmune diseases (Smallwood et al. 2018) and together with MAAs in immune reactions associated with alcoholic liver disease (Sutti et al. 2016).
The observation that lipid peroxidation products accumulate within NASH livers has been the stimulus for investigating the involvement of OSEs in driving NAFLD/NASH-associated immune responses. Indeed, anti-OSE IgG are detectable in about 40% of two unrelated cohorts of adult NAFLD/NASH patients as well as in 60% of a group of children with histologically proven NASH (Albano et al. 2005, Nobili et al. 2010, Bruzzì et al. 2018). In the pediatric patients, high anti-OSE antibody titers associate with the severity of lobular inflammation (Nobili et al. 2010), while in the adult populations anti-OSE IgG positively correlates with the prevalence of intrahepatic B-/T-cell aggregates and is an independent predictor of fibrosis (Albano et al. 2005, Bruzzì et al. 2018). The contribution of oxidative stress in stimulating adaptive immunity in NASH is further supported by animal data demonstrating that humoral and cellular responses against OSEs are linked with hepatic inflammation and parenchymal injury in a dietary rat model of NAFLD/NASH as well as in mice with NASH induced by the MCD diet (Sutti et al. 2014). In these settings, the increase in anti-OSE IgG accompanies liver B-lymphocyte maturation to plasma cells, while reducing lipid peroxidation or B2-cell depletion prevents antibody responses (Sutti & Albano 2020). The experiments using rodent models of NASH have also shown that immunizing mice with MDA-protein adducts before the feeding with the MCD diet greatly enhances liver lymphocyte infiltration promoting parenchymal injury, lobular inflammation, and fibrosis (Sutti et al. 2014). Such an effect involves Th-1 activation of liver CD4+ T-lymphocytes, which, by releasing CD40 ligand (CD154) and IFN-γ, promote pro-inflammatory activation of hepatic macrophages (Sutti et al. 2014). These results are consistent with clinical observations showing a positive correlation between anti-OSE IgG and circulating IFN-γ levels in human NASH (Bruzzì et al. 2018) (Fig. 1).

Although these data strongly indicate that anti-OSE immunity contributes to the pathogenesis of NASH, the overall picture is complicated by the fact that antibodies targeting OSEs might also have a protective action. B-lymphocytes mainly consist of two main sub-sets known as B1 and B2-cells with no overlapping functions. In fact, upon antigen stimulation B1-cells mature in a T-cell independent manner to plasma cells producing natural antibodies of the IgM class (Barrow et al. 2021a). Natural antibodies are pre-existing germline-encoded antibodies with a broad specificity to target pathogens, but are also cross-reacting with endogenous antigens, such as oxidized phospholipids and protein adducted by end-products of lipid peroxidation (Barrow et al. 2021b). Conversely, the B2 sub-set requires Th-1 cells for proliferating and undergoing antibody affinity maturation and isotype class switching to generate plasma cells producing IgA, IgG, or IgE with high antigen specificity (Barrow et al. 2021b). The role of natural antibodies in NASH emerges from the observation that the induction of T15 IgM cross-reacting with oxidized phosphatidylcholine ameliorates steatohepatitis in Ldlr−/− mice receiving a high-fat diet containing 0.2% cholesterol (HFC diet) (Bieghs et al. 2012). Similarly, the presence of high circulating levels of IgM targeting oxidized LDL ameliorate steatohepatitis induced feeding the HFC diet to Ldlr−/− mice deficient for sialic acid-binding immunoglobulin-like lectin G, a negative regulator of B1-cells (Gruber et al. 2016). However, in WT mice liver B1-cells, as well as circulating anti-OSE IgM, are not appreciably modified during the evolution of experimental NASH (Baumgardner et al. 2008, Bruzzì et al. 2018). In these mice, anti-OSE IgG production involves instead the fraction of B2-cells and is accompanied by the upregulation in the hepatic expression of the B-cell cytokine B-cell Activating Factor (BAFF), one of the mediators driving B-cell survival and maturation (Bruzzì et al. 2018) (Fig. 1). Interestingly, circulating levels of BAFF are higher in patients with NASH than in those with simple steatosis and plasma BAFF correlates with the severity of steatohepatitis and fibrosis (Miyake et al. 2013). Moreover, selective B2-cell depletion in mice over-expressing a soluble form of the BAFF/APRIL receptor transmembrane activator and cyclophilin ligand interactor (TACI-Ig) prevents plasma cell maturation. TACI-Ig mice show milder steatohepatitis and a decreased progression to fibrosis (Bruzzì et al. 2018). These apparently contrasting results can be partially explained by the fact that in Ldlr−/− mice Kupffer cells engulfment by cholesterol-rich oxidized LDLs is an important factor in the formation of pro-inflammatory foamy macrophages. Thus scavenging oxidized LDL by anti-OSE IgM prevents liver macrophage activation (Bieghs et al. 2012). It is noteworthy that NAFLD patients have circulating IgM targeting OSE-modified LDLs lower than healthy controls and that IgM titers against one of the OSE structures (P1 mimotope) inversely correlate with markers of obesity, systemic inflammation, and liver damage (Hendriks et al. 2016). In this scenario, it is possible that during the transition from NAFLD to NASH, hepatocyte oxidative stress along with gut dysbiosis might selectively promote the activation of B2-lymphocytes and the production of anti-OSE IgG overcoming the OSEs scavenging by natural antibodies. Besides maturing to IgG-producing plasma cells, these B2-lymphocytes can contribute to NASH progression by releasing pro-inflammatory mediators, such as IL-6 and TNFα,
promote CD4 T-cell differentiation into Th1 cells (Barrow et al. 2021a) (Fig. 1). Indeed, B2 cell activation precedes the liver recruitment of CD4+ and CD8+ T-lymphocytes, whereas interfering with B2-cells by blocking BAFF or using anti-CD20 antibodies reduces Th-1 activation of liver CD4+ T-lymphocytes and IFN-γ production (Bruzzì et al. 2018, Barrow et al. 2021b). Although, in liver biopsies from NASH patients B2-cells co-localizes in T-lymphocyte rich aggregates (Bruzzì et al. 2018) and CD4+ and CD8+ T-lymphocytes recognizing OSEs are evident in the liver of mice immunized with MDA-adducts (Sutti et al. 2014), at present little is known about OSE contribution in supporting cytotoxic T-cell responses that according to recent data play a major role in driving parenchymal damage and inflammation in the advanced phase of NASH (Ramadori et al. 2022). Clarifying this aspect is critical to fully understand the role of OSEs in supporting adaptive immunity in NASH.

Mechanisms possibly involved in promoting oxidative stress-mediated immunity in NASH

A still open issue concerning the interplay between hepatic oxidative stress and adaptive immunity in NAFLD/NASH involves the mechanisms responsible for subverting the liver immunotolerant milieu, thus favoring OSE presentation by antigen-presenting cells to B- and T-lymphocytes. It is known, in fact, that at homeostasis the liver plays important immunosuppressive functions toward antigens from food or commensal bacteria (Horst et al. 2016). These actions are mediated by the combined action of Kupffer cells, dendritic cells, and non-professional APC including hepatic sinusoidal endothelial cells and HSCs which present antigens to T-cells in combination with signals leading to T-cell apoptosis, anergy, or differentiation into CD4+CD25+/Foxp3+ regulatory T-cells (Tregs) (Horst et al. 2016). Studies in rodents indicate that chronic liver inflammation abolishes the tolerogenic capacity of Kupffer cells favoring instead the immunogenic stimulation of antigen-specific CD4+ T-cells (Heymann et al. 2015) (Fig. 2). Furthermore, recent data have evidenced that Kupffer cells are lost during NASH evolution becoming replaced by TREM-2+ NAMs highly efficient in antigen presentation (Daemen et al. 2021). Interestingly, TREM-2 appears involved in the recognition and clearance of OxPL (Dong et al. 2021). This suggests the possibility that TREM-2+ NAMs might have the specific capability of triggering immune responses toward OSEs. Besides that, the analysis of mice models of NASH has revealed that myeloid hepatic dendritic cells (HDCs) expand in the early phases of steatohepatitis

Figure 2
Factors involved in the development of immune responses against oxidative stress-derived antigens (OSE) in nonalcoholic steatohepatitis. During non-alcoholic fatty liver disease evolution, the combined action of damage-associated molecular patterns released by damaged hepatocytes, dysbiosis, and obesity-related changes in the adipokine network stimulates hepatic inflammation impairing the liver tolerogenic environment. Concomitantly the expansion and activation of hepatic dendritic cells along with the enhanced expression of lymphocyte co-stimulatory molecules favor the presentation of OSEs to lymphocytes leading to the development of both cellular and humoral immune responses.

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acquiring an immune-stimulating phenotype capable to promote T-lymphocyte (Henning et al. 2013) (Fig. 2). These HDCs co-express monocyte/macrophage markers and have an increased TNF-α production, suggesting their differentiation from inflammatory monocytes (Sutti et al. 2015). However, the actual role of HDCs in NASH pathogenesis deserves further investigations since experiments interfering with HDCs functions have failed to improve hepatic inflammation (Sutti & Albano 2020). The APC activity of macrophages and HDCs can be favored by an increased hepatic expression of lymphocyte co-stimulatory molecules such as OX40 (CD134) and its ligand OX40L (CD252). In fact, soluble OX40 is elevated in the plasma of NASH patients positively correlating with the severity of steatohepatitis and OX40 deficiency selectively lowers hepatic CD4+ T-cell recruitment and their Th-1 and Th-17 differentiation ameliorating lobular inflammation in mice fed with a high-fat diet (Sun et al. 2018). Additional mechanisms contributing to the impairment of liver immune tolerance might involve alterations of gut dysbiosis that are evident in NAFLD/NASH patients (Aron-Wisnensky et al. 2020). In this scenario, recent reports show that intestinal bacterial overgrowth and increased endotoxin reabsorption into the portal circulation can stimulate hepatic inflammation and B2 cell activation (Barrow et al. 2021b). Furthermore, dysbiosis may contribute to NASH-associated immune responses by altering gut tolerance to autoantigens and by modifying the production of short-chain fatty acids. Indeed, NAFLD/NASH associates with a decreased production of β-hydroxybutyrate which is an anti-inflammatory action, and an increased generation of acetate and propionate which instead promote the lowering of circulating resting Tregs (He et al. 2021). Altogether these data indicate the possibility that the combination of dysbiosis, hepatic inflammation, and oxidative stress can damp hepatic immune tolerance and promote APC presentation of OSEs to lymphocytes triggering both B- and T-cell activation (Fig. 2). Nonetheless, more data are still awaited to better characterize the interplay between B- or T-lymphocytes, macrophages, and NKT cells as well as the possible interactions with γδ T-cells, innate lymphoid, and mucosal-associated invariant T cells (Huby & Gautier 2021).

Oxidative stress and extra-hepatic complications of NASH

As mentioned in the introduction, the presence of NAFLD and even more NASH is associated with an increased risk of extra-hepatic diseases and particularly type 2 diabetes mellitus and cardiovascular diseases (Mantovani et al. 2020). Such an association is not surprising considering that obesity, insulin resistance, dyslipidemia, oxidative stress, and inflammation are all common pathogenetic factors in these diseases (Mantovani et al. 2020). However, a more specific link between NAFLD/NASH and cardiovascular diseases might reside in the fact that the pathogenesis of both atherosclerosis and NASH involves the formation of OSEs and their capacity of stimulating adaptive immunity. It is well known in fact that the oxidation of LDL and OSE accumulation are key factors in sustaining inflammatory reactions in atherosclerotic plaques, while the activation of either T- or B-lymphocytes significantly contributes to plaque evolution (Papac-Milicevic et al. 2016). In line with these notions, Ldlr−/− mice feed with a high-fat/cholesterol diet, an established rodent model of atherosclerosis, also develop NASH (Hendriks & Binder 2020), while the scavenging OxPL or OSE-modified LDLs in these animals ameliorates the evolution of both diseases (Hoebinger et al. 2021). Along with that, elevated titers of IgG targeting OSEs are associated with more severe atherosclerosis in humans and mice (Hoebinger et al. 2021). Although there are some inconsistencies about the actual significance of high anti-OSE IgG as a predictor of CDV (Hoebinger et al. 2021), the fact these antibodies are detectable in about one-third of NASH patients suggests the possibility that anti-OSE immunity might speed up the evolution of atherosclerosis in these subjects. At present, it is unknown whether high anti-OSE IgG might identify NAFLD/NASH patients with specific risks for cardiovascular complications. Characterizing this association would be important considering that cardiovascular diseases are the main cause of mortality among patients with NAFLD/NASH (Kasper et al. 2021).

Targeting oxidative stress in NAFLD/NASH therapy

Despite the worldwide growing diffusion of NAFLD/NASH, so far effective treatments for preventing the disease evolution to liver fibrosis/cirrhosis are still lacking. As a result of the evidence concerning the dysregulation of redox homeostasis in NAFLD and the involvement of oxidative stress in NASH pathogenesis, many studies have investigated the therapeutic potential of antioxidant compounds in NAFLD/NASH treatment. Data from animal experiments show that the supplementation with antioxidant compounds such as α-tocopherol (vitamin E) or defined polyphenols is effective in improving hepatic inflammation and, in some instance, the development
of fibrosis (Ma et al. 2021). However, the translation potential of these studies to clinical practice has been modest, since, so far, only few clinical trials have evaluated the possible use of antioxidant compounds in NASH patients. Among these, the PIVENS phase III trial has evidenced that the administration of 800 IU/day vitamin E (RRR-α-tocopherol enantiomer) for 96 weeks is effective in improving steatosis, hepatocyte ballooning, and lobular inflammation in approximately half of adult NASH patients without diabetes. However, vitamin E does not affect the extent of fibrosis (Sanyal et al. 2010) and fails to improve NASH in children and adolescents (Lavine et al. 2011). A subsequent phase IIb multicenter randomized double-dummy placebo-controlled trial has evaluated the action in pediatric NAFLD/NASH of cysteamine, an aminothiol that can act ROS scavenger and a GSH precursor. The results indicate that 1-year treatment with a delayed-release cysteamine bitartrate preparation significantly reduces serum aminotransferase levels and lobular inflammation, although the overall histological severity of the disease is not significantly modified (Schwimmer et al. 2016). Although these data show some potential of antioxidants in the treatment of NASH, they do not allow to draw definitive conclusions. Possible reasons for the inconsistent efficacy of these therapies might be related to the complexity of oxidative mechanisms in the pathogenesis of NASH and the fact the patients involved in the trials have not been randomized according to the presence of oxidative stress markers.

A different approach to target oxidative stress-related mechanisms in NASH might involve the scavenging of OSEs. As recently discussed by Hoebinger and coworkers, the direct administration of anti-OSE IgM or procedures capable to induce natural IgM cross reacting with OSEs appear an effective strategy to improve NASH in rodents (Hoebinger et al. 2021). Interestingly this approach is currently under investigation for the treatment of atherosclerosis (Hoebinger et al. 2021). However, the still incomplete understanding of the role of OSEs in supporting hepatic inflammation does not allow to predict whether OSE scavenging can have a translational impact on NASH.

**Conclusions and perspectives**

Although solid evidence indicates that oxidative stress is involved in the pathogenesis of NAFLD/NASH the complexity of the mechanisms involved has so far hampered a complete identification of the role played in the disease development and progression. An emerging aspect in the contribution of oxidative stress to NAFLD/NASH evolution concerns the involvement in supporting hepatic inflammation. In this setting, growing data indicate that by acting as DAMPs oxidized lipids and lipid peroxidation products are involved in triggering the activation of innate immune cells. Furthermore, the capacity of OSEs to act as antigens suggests their possible implication in sustaining lymphocyte-mediated adaptive immune responses which represent a novel inside of the mechanisms responsible of sustain steatohepatitis. However, several unresolved questions remain in understanding the mechanisms by which oxidative stress can contribute to NASH. For instance, in view of the involvement of gut dysbiosis in obesity and NAFLD/NASH, an important issue is represented by the possible reciprocal influences between oxidative mechanisms and alteration of intestinal microbiota and its products. Moreover, additional data are required to fully appreciate the contribution of OSE-mediated immunity in the development of liver damage and inflammation. This issue is important also in relation to emerging evidence linking OSEs to the high risk of cardiovascular complications of NAFLD/NASH. New insights into these issues will ultimately lead to develop effective strategies for controlling oxidative stress-derived effects to apply to the treatment of NAFLD/NASH.

**Declaration of interest**

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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