Review

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Non-alcoholic steatohepatitis: The role of oxidized low-density lipoproteins

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Summary

Non-alcoholic steatohepatitis (NASH) is hallmarked by lipid accumulation in the liver (steatosis) along with inflammation (hepatitis). The transition from simple steatosis towards NASH represents a key step in pathogenesis, as it will set the stage for further severe liver damage. Yet, the pathogenesis behind hepatic inflammation is still poorly understood. It is of relevance to better understand the underlying mechanisms involved in NASH in order to apply new knowledge to potential novel therapeutic approaches. In the current review, we propose oxidized cholesterol as a novel risk factor for NASH. Here, we summarize mouse and human studies that provide possible mechanisms for the involvement of oxidized low-density lipoproteins in NASH and consequent potential novel diagnostic tools and treatment strategies for hepatic inflammation.

Introduction

Non-alcoholic fatty liver disease (NAFLD) involves a cluster of liver disease pathologies ranging from liver lipid accumulation (steatosis) through inflammation (non-alcoholic steatohepatitis) to fibrosis and finally, irreversible cirrhosis. Compared to simple steatosis, non-alcoholic steatohepatitis (NASH) is a more severe, but less common form of NAFLD. According to a prospective study, approximately 46% of a general patient population was classified with a fatty liver, of which 29% of ultrasound positive subjects were diagnosed with biopsy-proven NASH. Parallel to the increasing prevalence of obesity, there was a corresponding increase of body mass index (BMI) in this cohort [1]. Concomitantly, weight loss improved the histological disease activity of NASH [2]. Since obesity is a growing international epidemic both in adults and children, steatohepatitis is about to become the most common cause of liver cirrhosis and end-stage liver diseases, due to the complications of portal hypertension [3].

As of today, several mechanisms have been proposed for hepatic inflammation. Current interests implicate an important contribution of the adipose tissue, particularly visceral adipose tissue (VAT) and its secretory products [4]. Abnormal VAT function, primarily due to obesity, amplifies the release of adipocytokines from fatty tissue, which can lead to systemic effects, such as low-grade systemic inflammation and an altered metabolic state with insulin resistance. The increased lipid content in VAT, enhances free fatty acid (FFA) delivery from the adipocytes into the liver, impairing the hepatic lipid content and initiating hepatic insulin resistance. Whereas adipocytokines, including interleukin-8 and tumor necrosis factor-alpha (TNF-α), could contribute to hepatic inflammation via lipid peroxidation and modulating the inflammatory response, FFAs can induce NASH via hepatocyte apoptosis, lipotoxicity and increased production of reactive oxygen species (ROS) [5,6]. Recent evidence points toward another tissue, the gastrointestinal tract, as a source for liver inflammation. Apart from altered gut microbiota during obesity [7], studies showed increased intestinal permeability during NASH, which could lead to elevated levels of plasma lipopolysaccharide (LPS) [8,9]. This gut-derived LPS can activate the immune system via pro-inflammatory signaling pathways after binding to toll like receptors (TLRs), as those present on, for example, Kupffer cells [10]. Additionally, vascular abnormalities, as observed in atherosclerosis, have been strongly associated with NASH [11]. Thus, a potential interplay exists between metabolic tissues and inflammation, leading to the development of NASH (Fig. 1). At molecular level, increased FFA levels, among other factors, can initiate endoplasmic reticulum (ER) stress and mitochondrial dysfunction. Subsequently,

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Abbreviations: acLDL, acetylated low-density lipoprotein; AGEs, advanced glycation end products; ALP, alkaline phosphatase; ALT, alanine transaminase; AST, aspartate transaminase; BMI, body mass index; CCL23, chemokine C–C motif ligand 23; CVD, cardiovascular disease; DAMP, damage associated molecular pattern; ER, endoplasmic reticulum; FFA, free fatty acids; GGT, gamma-glutamyltransferase; HDL, high-density lipoprotein; HFD, high fat diet; HNE, 4-hydroxynonenal; KC, Kupffer cell; LDL(R), low-density lipoprotein (receptor); LPS, lipopolysaccharide; MCP-1, monocyte chemotactic protein 1; MDA, malondialdehyde; MetS, metabolic syndrome; MIP-2, macrophage inflammatory protein 2; MPO, myeloperoxidase; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; NF-κB, nuclear factor-kappaB; (Ox)LDL, (oxidized) low-density lipoprotein; PC, phosphorylcholine; PUFA, polyunsaturated fatty acids; ROS, reactive oxygen species; SEC, sinusoidial endothelial cell; SR(-A), scavenger receptor(-A); TBARs, thiobarbituric acid reactive substances; TLR, toll like receptor; TNF-α, tumor necrosis factor-alpha; VAT, visceral adipose tissue.

this will lead to excess ROS production and formation of lipotoxic molecules, hereby contributing to the hepatic inflammatory response [12]. Disturbed autophagic function, resulting from decreased removal of altered mitochondria and the ER, has been suggested to further aggravate hepatic inflammation [13]. Thus, several mechanisms play a role in the transition to NASH. Recently, increasing amounts of data show the involvement of oxidized low-density lipoproteins (oxLDL) in hepatic inflammation. While there is no evidence that the contribution of oxLDL to NASH is greater than other known mechanisms, oxLDL is emerging as a new risk factor for hepatic inflammation. Therefore, in this review, we will focus on oxLDL and its implications in NASH.

NAFLD corresponds to an altered lipid metabolism and is associated with the metabolic syndrome (MetS). One central feature is the elevation of triglycerides in plasma as well as in the liver. Sources of increased hepatic triglyceride content are due to excess dietary intake, elevated triglyceride synthesis in the liver from FFA formed during de novo lipogenesis, enhanced FFA influx into the liver from lipolysis of adipose tissue, and subsequent conversion into triglycerides, reduced lipid export from the liver via very low-density lipoprotein particles and diminished oxidation of fatty acids [14]. Other hallmarks associated with NAFLD are low plasma high-density lipoproteins (HDL), elevated low-density lipoproteins (LDL) and total cholesterol [15]. Currently, it has been postulated that different types of lipids mediate the disease spectrum of NAFLD. While hepatic accumulation of triglycerides is related to steatosis, it becomes more evident that cholesterol is implicated in the hepatic inflammatory response. For example, a high cholesterol diet induced liver inflammation in mice susceptible to NASH, while elimination of dietary cholesterol prevented steatohepatitis [16,17]. Although there is a clear association between obesity and NASH, dietary cholesterol was even found to be the main trigger of hepatic inflammation in non-obese rodents and humans [18,19]. Moreover, in livers of NASH patients, total plasma cholesterol as well as free cholesterol deposits were found to be increased compared to control subjects [20,21]. Altogether, these observations indicate that cholesterol is a key player in the onset of NASH.

Oxidative stress is another important and central mechanism in the progression towards NASH. Many cells, including macrophages, are capable of internalizing and accumulating excess amounts of plasma lipoprotein-derived cholesterol [22]. Mimicking this process *in vitro*, by loading macrophages with cholesterol, resulted in increased generation of ROS [23]. In turn, oxidative stress brings damage to cell structures such as membranes, proteins and DNA of liver cells, hereby triggering a hepatic inflammatory response, which can eventually lead to apoptosis [24]. Several sources of hepatic ROS have been determined regarding the development of NASH and include mitochondria, peroxisomes, the endoplasmic reticulum, and enzymes such as the cytochrome P450 superfamily, NAPDH oxidase and xanthine oxidase [24]. Recently, it has been reported that steatohepatitis may be caused by lipid-induced oxidative stress [25]. Thus, given that cholesterol and oxidative stress play a causal role in the pathogenesis of NASH, it is highly likely that not cholesterol alone, but consequent oxidation of cholesterol is the substantial risk factor for NASH. To support this hypothesis, we will evaluate current data that describe the involvement of oxLDL in inflammation and NASH. Additionally, potential clinical benefits of oxLDL in the field of NASH will be discussed.

Key Points

- Uptake of modified lipids by Kupffer cells, such as oxLDL, leads to the inflammatory response in NASH
- Disturbed intracellular trafficking of oxidized lipids within Kupffer cells is associated with hepatic inflammation
- Among other metabolic inflammatory diseases, oxLDL should be considered a substantial risk factor for NASH
- The pathogenesis of NASH in hyperlipidemic mice is associated with lysosomal storage defects
- Atherosclerosis and NASH are both metabolic diseases and share disease mechanisms
- Future therapy and diagnosis of NASH should focus on oxLDL

The inflammatory aspects of oxLDL

Recent studies show that oxLDL contributes to inflammatory processes through interaction with immune cells and disturbed intracellular cholesterol trafficking. To date, an increasing amount of evidence suggests an important role for oxLDL in obesity-related inflammatory disorders, such as atherosclerosis [26,27] and cardiovascular disease (CVD) [28,29].

So far, several mechanisms underlying LDL oxidation have been identified *in vivo*. Hyperglycemia, a pre-diabetic state prior to insulin resistance, has been shown to be strongly associated with oxidation of circulating LDL, as glucose decreases the antioxidant characteristics of serum albumin [30,31]. Chronic hyperglycemia has been implicated in the enhanced formation of advanced glycation end products (AGEs), eliciting alterations of the LDL particle [32]. Interestingly, feeding mice a high-AGE diet caused liver inflammation, suggesting that AGE-induced modified LDL plays an important role in inflammation [33]. The increase of FFA flux, primarily released from adipose tissue, into the liver, is strongly linked to insulin resistance and increased oxidative stress, possibly exacerbating oxidation of LDL [34].

OxLDL-induced inflammation and apoptosis

Minimally oxidized forms of LDL contain lipid oxidation products without extensive protein modification. Since oxLDL particles stay longer in the plasma, they are more prone to further oxidation. As modification proceeds, the highly oxidized LDL particle turns into a structure similar to pathogen-related epitopes and therefore will be removed from plasma through binding and uptake by macrophages. This response is initially intended to be protective, however, an excessive amount of lipids will build up inside macrophages, leading to a phenomenon called foam cell formation [35]. This change in foamy appearance causes the swollen phenotype of the macrophage to activate the transcription factor nuclear factor-kappaB (NF-κB) [36], hereby inducing the production of inflammatory cytokines (Fig. 2) [36,37]. OxLDL has been shown to modulate inflammation by affecting several other cellular mechanisms, such as inducing transmigration of

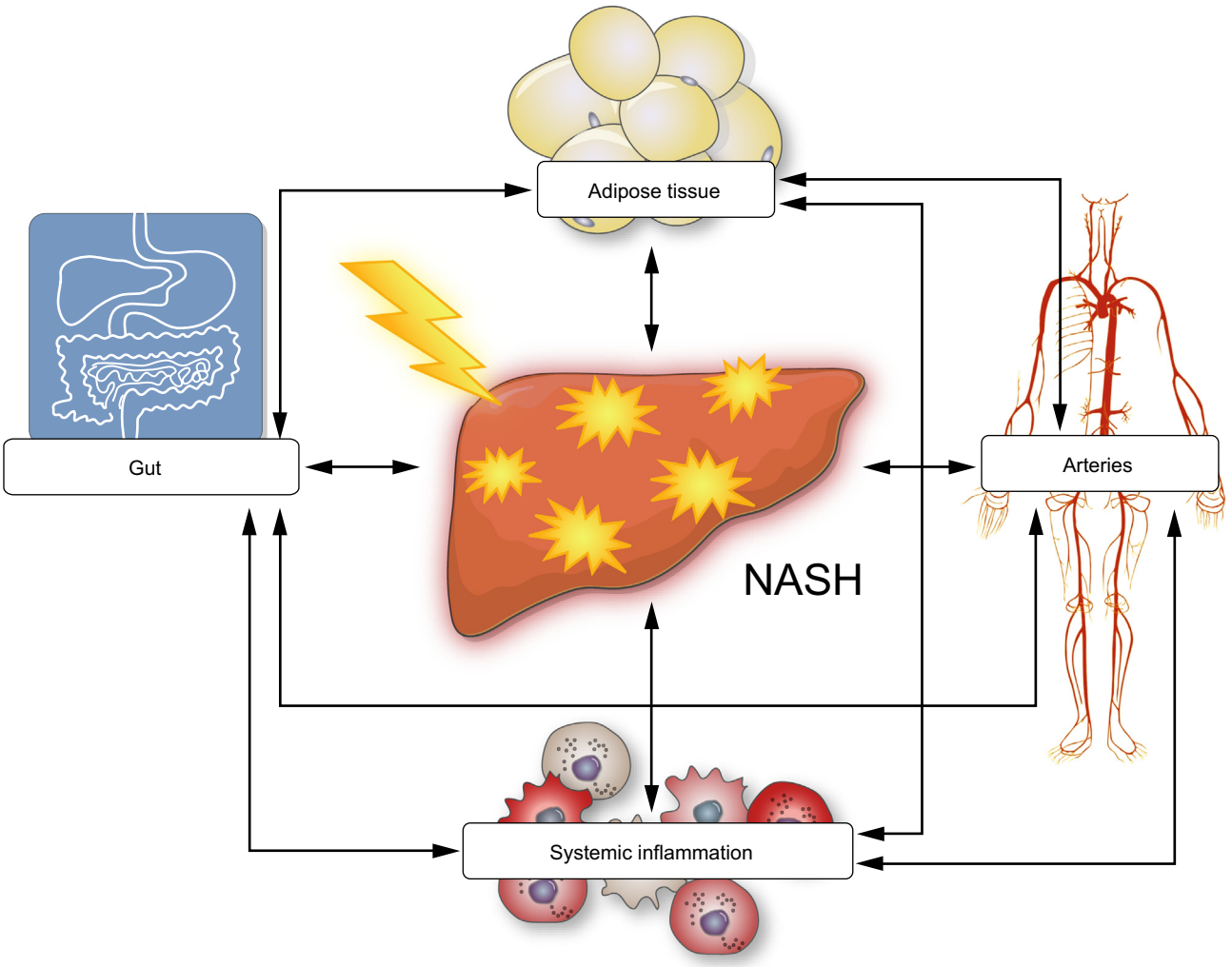

Fig. 1. Schematic diagram illustrating the metabolic crosstalk between liver, adipose tissue, gut, arteries and systemic inflammation. The development of NASH is dependent on underlying mechanisms related to the metabolic syndrome, such as disturbed intestinal permeability, gut microbiota, increased systemic inflammation, vascular abnormalities, and adipose tissue dysfunction as a result of increased macrophage infiltration and insulin resistance. In turn, NASH by itself can exacerbate inflammation in these metabolic tissues, retaining a positive feedback mechanism.

neutrophils [38], eosinophils [39], monocytes and T lymphocytes [40]; elevating several adhesion molecules [38,41–43], and recruiting immune cells through the release of the chemokine (C–C motif) ligand 23 (CCL23) [44]. Moreover, oxLDL induces inflammation through increased ROS generation [45] and elevated expression of metalloproteinases [46].

Another important aspect during the pathogenesis of inflammation is apoptotic cell death, which has been shown to play an important role in NASH [47,48]. OxLDL has been found to increase apoptosis through activation of apoptotic signaling cascades including the Fas signaling pathway [49]. Additionally, biologically active oxidized lipids were found in apoptotic cells [50]. Thus, given that oxLDL induces apoptosis, oxLDL is not merely an inflammatory trigger, but also promotes subsequent cell damage.

Disturbed intracellular trafficking of oxLDL

OxLDL possibly exerts its inflammatory effects upon receptor-mediated macrophage endocytosis. Once internalized, it has been postulated that oxLDL is transported to the lysosomal compartment where it is poorly degraded or hydrolyzed and therefore accumulates in lysosomes. This is in contrast to native or acetylated LDL, which are normally degraded by lysosomal enzymes followed by relocation into the cytoplasm for further processing [51]. Lysosomal trapping of oxLDL, probably due to impaired cholesteryl ester hydrolysis or an alteration in lysosomal pH [52], has the potential to damage and disrupt the lysosomal membrane. Since lysosomes are involved in a wide variety of biological processes, cholesterol-induced lysosomal damage can lead to inflammation and apoptosis [53]. *In vitro* data demonstrated the appearance of cholesterol crystals inside lysosomes upon prolonged oxLDL incubation. It is speculated that these crystals represent an endogenous danger signal and trigger the activation of the NLRP3 inflammasome and subsequent pro-inflammatory interleukin-1 production [54]. In addition, it has been proposed that lysosomal cholesterol accumulation leads to disturbed autophagy, a process important in inflammation and apoptosis [55]. Taken together, lysosomal trapping of oxLDL inside

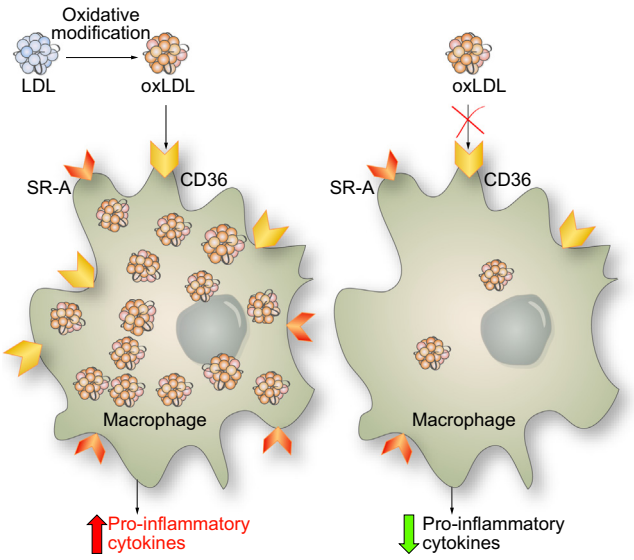

Fig. 2. Schematic illustration showing the involvement of oxLDL in the macrophage inflammatory response. Lipid-laden foamy macrophages express higher levels of the scavenger receptors CD36 and SR-A and produce more pro-inflammatory cytokines. Through interplay with surrounding cells, these cytokines further amplify the inflammatory response. Blocking macrophage uptake of oxLDL leads to macrophages smaller in size, less CD36 and SR-A expression and reduced inflammation. Modified from [135], reprinted by permission from Elsevier.

macrophages leads to cellular damage, possibly through mediating inflammation and apoptosis. Although the inflammatory effects of oxLDL are well-documented in the atherosclerosis field, the link between NASH and CVD has never been investigated directly by any study. Therefore, it is still questionable whether the inflammatory aspects of oxLDL summarized in this paragraph can be applied to NASH as well.

OxLDL and its implications in non-alcoholic steatohepatitis

An increasing amount of studies strongly emphasize the role of Kupffer cells (KCs), the liver's resident macrophage population, in the pathogenesis of NASH [56]. We now elucidate the effect of oxLDL on KCs and its contribution to hepatic inflammation.

Role of KCs

A growing body of evidence contradicts the "two-hit" model, in which is described that hepatic steatosis is considered to be the first critical 'hit' and a necessary prerequisite for further liver damage, such as inflammation [57]. Nowadays, it becomes increasingly clear that a multifactor etiology, with a central role for KCs, underlies the pathogenesis of NASH [58]. In contrast to the "two-hit" model, several papers describe the development of severe hepatic inflammation without the presence of hepatic steatosis [16,59]. For example, omitting cholesterol from hyperlipidemic mice prevented hepatic inflammation without affecting steatosis [16]. Furthermore, comparable to foam cell formation in atherosclerosis, hyperlipidemic mice showed bloated foamy KCs, which was correlated to hepatic inflammation. Consistently, a high-fat diet (HFD) without added cholesterol demonstrated reduced hepatic inflammation without swollen KCs [16]. During early steatohepatitis, isolated fat-laden KCs from HFD-fed mice predominantly contained cholesterol and displayed a pro-inflammatory phenotype [60]. Inflammation triggered by cholesterol-rich foam cells, is a well established hypothesis in the field of CVD and has been recognized as a significant parameter during atherosclerotic plaque formation [61]. Thus, cholesterol or its modified form, trapped inside KCs, is an actual trigger for NASH.

Critical contributors for the uptake of modified lipids and cholesterol by macrophages are the scavenger receptors (SRs), scavenger receptor A (SR-A) and CD36 [62]. Literature describes a distinct affinity for binding of oxLDL between these two SRs. SR-A binds and mediates uptake of oxLDL to a lesser extent than CD36. Compared to incubation with LDL and acetylated LDL (acLDL), treatment with oxLDL elevated gene expression and protein levels of SR-A and CD36 in macrophages [63,64]. These data show that both scavenger receptors are involved in the uptake of oxLDL. Similar to typical macrophages, SRs were also identified on KCs [65]. Haematopoietic deletion of SR-A (Msr1) and/or Cd36 in hyperlipidemic mice resulted in decreased hepatic inflammation, indicating that SR-mediated uptake of modified cholesterol by KCs is the trigger for the development of steatohepatitis (Fig. 2) [59,66]. Loading bone-marrow derived macrophages of LDL receptor *(ldlr)-/-* mice with oxLDL, hereby mimicking foam cell formation, showed to be more inflammatory than macrophages without oxLDL loading [67]. Taken together, these data demonstrate the causal role of oxLDL as a driver of the inflammatory response.

Recently, a novel mouse model for NASH has been developed by using a combination of oxidized LDL and a HFD. Administration of oxLDL to wild type HFD-fed mice displayed the entire pathology of NASH, i.e., steatosis, hepatic inflammation, fibrosis, and also lipid-laden macrophages, dyslipidemia and aggravated hepatic lipid peroxidation [64]. This novel animal model shows the direct involvement of oxLDL in the development of NASH, however, the underlying intracellular pathway that contributes to hepatic inflammation has not been established. One proposed theory is a defective intrinsic mechanism of lipid trafficking inside KCs.

Macrophage-derived foam cells, as those present during atherosclerosis, predominantly contain enlarged lysosomes filled with cholesterol and cholesterol crystals, instead of cholesterol ester storage into the cytoplasm [54,68]. For the first time, our group demonstrated accumulation of cholesterol and cholesterol crystals inside lysosomes of KCs in a mouse model representing NASH [66,69]. In line with these data, hepatic inflammation was found to be associated with increased cholesterol storage inside lysosomes of KCs, providing evidence that lysosomal cholesterol accumulation in KCs is crucial for inflammation in the context of NASH [66,69]. Altogether, mounting evidence demonstrates that NASH exhibits similar characteristics to atherosclerosis, including foam cell formation and cholesterol-engorged lysosomes. Regarding the latter observation, it has been proposed that advanced stages of atherosclerosis are analogous to a modified form of lysosomal storage disorders [70]. Therefore, these results indicate that NASH can be considered likewise. Our novel hypothesis that NASH shares similarities with an acquired lysosomal storage disorder, opens up entirely new therapy possibilities for hepatic inflammation.

By interfering with the immune response, more evidence was provided for the relevant role of oxLDL in NASH. Oxidation structurally modifies the LDL particle, whereby the phosphorylcholine

(PC) headgroups, one of the so-called oxidation-specific epitopes, can be found on the outer surface [71]. Oxidation-specific epitopes are viewed as damage associated molecular patterns (DAMPs) and therefore serve as ligands for immune recognition [72]. Since these PC epitopes are also present on the capsular polysaccharide cell wall of *Streptococcus pneumoniae* [73], cross reactivity exists between PC epitopes from oxLDL and this bacterium. Therefore, a protective effect against NASH upon active immunization with heat-inactivated *S. pneumoniae* in *ldlr*−/− mice was found. Immunized mice fed a high fat cholesterol diet showed less foamy KCs, decreased hepatic inflammation and reduced cholesterol crystals inside lysosomes of KCs compared to mice without immunization [69]. More importantly, reduced inflammation was associated with lower cholesterol oxidation and an increase of IgM autoantibody levels against modified LDL in plasma [59]. These data strongly suggest that anti-oxLDL antibodies of the IgM subtype are protective against steatohepatitis (Fig. 2), supporting our hypothesis that oxLDL plays an important role in the development of NASH.

Crosstalk between KCs and other cell types in the liver

Activation of KCs leads to a rapid release of a wide range of inflammatory mediators and signaling molecules such as cytokines, ROS, proteases and lipid mediators [74]. One of the stimuli that has been shown to activate macrophages and to increase pro-inflammatory cytokines, is oxLDL [67]. Other than oxLDL, different stimuli can activate KCs, such as gut-derived endotoxins [75] and damaged hepatocytes. For example, due to intercellular communication between hepatocytes and KCs, hepatocyte stress and/or injury result in the excretion of inflammatory mediators, which in turn activate KCs hereby possibly inducing hepatic inflammation [76,77]. Furthermore, at a more advanced stage, the engulfment of apoptotic hepatocytes by KCs promotes their activation and could further contribute to hepatic inflammation [78].

As discussed earlier, oxLDL trapping inside lysosomes triggers inflammation, most likely due to its activation of KCs. Once inflammation is elicited, KCs can further spread hepatic injury by amplification of the inflammatory response through interactions with neighboring hepatocytes, sinusoidal endothelial cells (SECs) and hepatic stellate cells [77]. Upon activation, KCs primarily release TNF-α and interleukins [79], hereby influencing hepatocyte function and viability or indirectly by activating other cells, including SECs. Activation of SECs can indirectly lead to neutrophil-mediated damage to the hepatocytes or even cell death [74]. Additionally, inflammatory signaling initiated by KCs can be further amplified by the secretion of chemokines, followed by recruitment of infiltrating macrophages and neutrophils [80]. KC-derived TNF-α contributes to elevated secretion of the chemokine macrophage inflammatory protein 2 (MIP-2) and monocyte chemotactic protein 1 (MCP-1), facilitating activation and infiltration of neutrophils and macrophages into the liver [74,81]. The hepatic accumulation of neutrophils in turn can lead to hepatotoxicity.

In summary, oxLDL is a harmful lipid that causes cellular injury and activation of macrophages and endothelial cells, particularly. OxLDL-induced KC activation enhances cytokine-driven hepatocellular signaling pathways, hereby inducing KCs to further augment inflammation through interaction with other cell types in the liver.

Oxidative stress

Oxidative stress, the primary risk factor for LDL oxidation, is believed to be a central mechanism in the pathogenesis of NASH. Therefore, in mouse models, as well as in human studies, markers for oxidative stress were measured as potential surrogate markers for NASH.

Neutrophils are a potent source of the oxidant-generating enzyme myeloperoxidase (MPO) and are abundantly present in the liver [82,83]. *In vitro* data demonstrated that uptake of MPO-induced oxidation of LDL leads to foam cell formation [84]. In line with this finding, Rensen *et al.* detected increased MPO-positive KCs in the livers of obese NASH patients, which was accompanied by elevated plasma MPO levels [85].

During oxidative modification of LDL, a variety of reactive aldehydes on apoB lysine residues are generated by decomposition of lipid peroxidation products, such as 4-hydroxynonenal (HNE) and malondialdehyde (MDA) [35]. While HNE has shown to contribute to foam cell formation, MDA modification of lysine residues contributes to functional properties of oxLDL [86]. Consistently, increased hepatic MDA and HNE levels in rodent models of NASH were identified [47,87]. Other pivotal contributors to oxidative stress are microsomal cytochrome P450 enzymes, such as P450 2E1, which are mainly located in the liver. Deletion of P450 CYP2E1 in mice resulted in less susceptibility for NASH, decreased oxidized proteins, as well as MDA and HNE levels, and protection against insulin resistance compared to their wild type littermates [88]. While mouse studies show straightforward results about the role of oxidative stress in NASH, less outspoken data are represented by human studies. Koruk *et al.* demonstrated an increase of serum MDA in patients with biopsy proven NASH, while the antioxidants glutathione peroxidase and glutathione reductase showed no difference compared to the control group [89]. Moreover, an increase of serum thioredoxin, thiobarbituric acid reactive substances (TBARS) and plasma oxLDL was detected in NASH patients in comparison to control subjects [90,91]. Although the data was statistically significant, a small sample size was used and there were large standard deviations between the groups, considering these studies as being underpowered. Additionally, cohorts were poorly controlled regarding overlapping risk factors for NASH, such as the MetS and/or diabetes. Yet, although a small cohort was used, increased hepatic CYP2E1 activity in non-diabetic NASH patients was demonstrated compared to BMI-matched controls [92]. Evidence implicates other pro-oxidant enzymes, such as 15-lipoxygenase and ceruloplasmin, to be involved in the oxidation of LDL [93,94]. Therefore, clinical data showed a concomitant increase of enzymatic sources of ROS during hepatic inflammation, in parallel to the progression of NASH [15,95]. Of note, the changes observed in ceruloplasmin levels and P450 liver enzymes are not specifically related to NASH, but also to other aspects of the MetS, including obesity and diabetes mellitus.

In general, most of the human studies presented in this paragraph do not show a causal link between oxidative stress and NASH.

Anti-oxidants

Oxidative stress represents an oxidant/anti-oxidant imbalance, which is shifted towards greater oxidant activity and/or decreased anti-oxidant levels. Enzymatic and non-enzymatic anti-oxidant

defense mechanisms play a role in protecting lipids, such as LDL from oxidation. Observations described above clearly indicate a pro-oxidant state for NASH patients, suggesting there is a diminished anti-oxidant defense status in this population. Indeed, a diversity of anti-oxidant enzymes was found to be reduced in the plasma of NASH patients [89,96]. Moreover, total anti-oxidant capacity and anti-oxidant enzymes were specifically decreased in the livers of patients with steatohepatitis compared to healthy controls [97]. Consistent with a decreased activity of anti-oxidant enzymes, non-enzymatic anti-oxidants, such as glutathione content and vitamin E, were also diminished in NASH subjects [97,98]. In parallel with the disease progression of human NAFLD, a decline of glutathione transferase enzyme activity was detected in liver specimens [99]. In striking contrast, extreme low anti-oxidant levels alleviated the progression towards NASH, as observed in glutathione-deficient mice, indicating the activation of a protective compensatory mechanism under severe low anti-oxidant conditions [100]. Altogether, NASH patients reflect a pro-oxidant state and a reduced anti-oxidant capacity, implying limited ability to counteract oxidation. Thus, these data point towards an important role for oxidation, most likely of LDL, in the development of NASH. However, the decreased level of anti-oxidants as observed in NASH subjects could also be a consequence of other related disorders or risk factors, such as the MetS, obesity and diabetes mellitus.

Clinical implications

At present, the most accurate diagnostic tool to determine NASH is the histological assessment of a liver biopsy. Due to its invasive procedure, patients experience discomfort and there is a risk for complications including pain, hemorrhage, bile peritonitis and pneumothorax [101]. The existing non-invasive biomarkers for NASH used in the clinic, i.e., transaminases (ALT, AST), alkaline phosphatase (ALP) and gamma-glutamyl-transpeptidase (GGT), lack specificity and sensitivity to distinguish NASH from steatosis and have been reported as unreliable [102]. Instead of inflammation, these plasma liver enzymes represent liver damage, of which a novel potential biomarker, plasma cytokeratin 18, is a marker specifically for hepatocyte apoptosis [103]. Concerning therapeutics against NASH, there is no proven effective treatment available that specifically reduces hepatic inflammation. Although not all patients fit the following description, NASH patients typically meet the criteria for the MetS, i.e., being obese, insulin resistant and hyperlipidemic [104]. Therefore, the most adequate recommendation for reducing hepatic inflammation focuses on lifestyle alterations, such as changing nutritional habits and increasing physical activity [104]. Additional to lifestyle modifications, pharmacological interventions against NASH target hyperlipidemia, insulin resistance and oxidative stress and are therefore similar to that of the MetS. Altogether, non-invasive tests are warranted to diagnose NASH at early stages of the disease process, to allow opportunities to prevent further progression towards severe and irreversible liver damage, such as fibrosis and cirrhosis. Moreover, there is a need for novel and safe therapeutic strategies against NASH that lead to a pronounced reduction in hepatic inflammation.

Plasma OxLDL

Higher circulating oxLDL levels were detected in CVD patients compared to healthy subjects [105]. Generally, the important role of plasma oxLDL has been reviewed extensively for atherosclerosis [26,35]. In line with this, Binder *et al.* have shown to reduce atherosclerosis by inducing protective plasma anti-oxLDL antibodies in mice [106]. Similarly, we have recently shown that these antibodies are also effective against NASH [69]. Thus, these data point towards oxLDL as a potential target for the prevention of both atherosclerosis and NASH. However, clinical studies are at their infancy and comparative studies of testing various assays to monitor oxLDL are needed to assess which assays have enhanced clinical utility for detecting CVD and NASH. So far, none of the tested assays are approved for routine clinical use [29].

As for diagnosis, oxLDL is not used as a marker to detect atherosclerosis. Similarly, while we found an association between antibodies against oxLDL and NASH, there is no sufficient evidence to suggest that plasma oxLDL can be used as a non-invasive marker to detect hepatic inflammation. To evaluate the prognostic value of plasma oxLDL for the detection of NASH, several bigger cohort studies are necessary.

Anti-oxLDL antibodies

The finding that oxidation-specific epitopes are not merely present on oxLDL, but also on apoptotic cells [107], reflects the link between oxLDL and tissue damage. Therefore, anti-oxLDL antibodies have been shown to be predictors of inflammatory diseases, such as atherosclerosis and CVD [108,109]. In line with these findings, we have found that plasma IgM anti-oxLDL antibodies correlate negatively with hepatic inflammation in mice [69]. In this view, anti-oxLDL antibodies can potentially be used as a diagnostic tool for the detection of NASH. However, it is important to note that the amount of anti-oxLDL antibodies may differ naturally between people and can vary over time [110,111]. Additionally, molecular mimicry exists between oxidation-specific epitopes of oxLDL and epitopes located on infectious agents, suggesting that exposure to pathogens influences the production of anti-oxLDL antibodies [110]. This argument may not be beneficial for the use of anti-oxLDL antibodies for the diagnosis of NASH, yet it opens up promising therapeutic strategies against liver inflammation. Boosting the production of anti-oxLDL antibodies via immunization approaches ameliorated atherosclerosis [106,112]. Since atherosclerosis shares features with NASH, i.e., foam cell formation and inflammation, these immunization approaches hold promise as treatment against NASH and should be tested clinically in the future.

Cholesterol lowering medication

Hypertriglyceridemia and hypercholesterolemia are commonly found in NASH patients, suggesting that NASH is strongly associated with hyperlipidemia [113]. Therefore, lipid-lowering agents, such as polyunsaturated fatty acids (PUFAs), fibrates and statins, have been tested in patients with NASH. Recent work reported a positive effect of PUFAs on lobular inflammation and ballooning of the liver in mice, as well as in human NASH, although the human study lacked a control group[114,115]. Therefore, it has been proposed that randomized controlled trials of adequate size are needed in the future to propose such PUFA treatment to NASH patients[2].

The use of fibrates, which are ligands of the peroxisome proliferator-activated receptor, and statins are still controversial. Fenofibrate administered to mice has been shown to ameliorate

hepatic inflammation, while human studies demonstrated no difference in plasma liver enzymes or without changes in histological end points for NASH [116–118]. Statin therapy was investigated by human pilot studies, but only in a limited number of patients [119–121]. Short-term outcomes show promising results on liver inflammation, as proven by serum aminotransferase activities and liver histology [119–121]. In addition to their anti-inflammatory properties, statins are generally targeted at lowering lipids. Interestingly, patients who received statins even demonstrated reduced oxLDL, which could be relevant for NASH patients with increased plasma oxLDL levels [122]. Still, statin-treated NAFLD patients developed advanced fibrosis based on liver histology after a long-term follow-up period [119,123]. In conclusion, the beneficial effects of statins and fibrates on NASH are still debatable, due to clear limitations to monitor NASH. While some human studies use unspecific plasma liver enzymes, other studies assess liver histology for the development of NASH. Furthermore, there is a clear lack in repeated measurements to monitor NASH progression. Moreover, the difference in beneficial outcome after statin therapy could be explained by the fact that statins are directed at lipid lowering in general and are not directly related to oxLDL. Therefore, future adequate and well-designed human intervention studies examining the effect of statins or fibrates on NAFLD/NASH should be conducted. To monitor long-term statin or fibrate therapy on the development of NASH in human studies, liver histology assessment is critical.

Anti-oxidant therapy

A pivotal contributor to the pathophysiology of NASH includes oxidative stress. As pro-oxidant activity is paralleled with oxidation of lipids, including LDL, anti-oxidants have the potential to treat NASH. Promising results were obtained during a clinical trial where non-diabetic NASH patients were randomly assigned to receive the anti-oxidant, vitamin E, or placebo for 96 weeks. Vitamin E treatment improved individual features of NASH, such as lobular inflammation and hepatocellular ballooning, as well as the overall NAFLD activity score [124]. A similar positive outcome of the NASH phenotype was demonstrated in a clinical trial where NASH patients received the anti-oxidant pentoxifylline [125]. Vitamin E has been shown to inhibit CD36-mediated uptake of oxLDL, hereby preventing foam cell formation, whereas pentoxifylline reduced oxLDL-induced leukocyte adhesion to the endothelium and downregulated the integrin receptor CD11b/CD18 [43,126]. Additional clinical studies could not attribute a favorable effect to vitamin E and pentoxifylline treatment in the development of NASH [127,128]. Nevertheless, this could be due to the variable disease course of NAFLD/NASH, sampling error during liver biopsy [129] and the use of plasma transaminases as a non-specific predictor for NASH [102]. Although further investigations are needed, other anti-oxidants have also shown to be effective against NASH and include ursodeoxycholic acid with or without vitamin E [130,131], betaine and other dietary supplements [87,132–134]. In summary, anti-oxidant therapy, either via supplementation of anti-oxidants or agents that increase the generation of anti-oxidant enzymes, seems to be effective in reducing NASH. Even though anti-oxidant therapy counteracts oxidative stress and thereby inflammation, anti-oxidants might serve as a useful adjunct therapy to support targeted therapies.

Concluding remarks

A number of studies demonstrated a close relationship between the MetS and increased plasma oxLDL levels. In recent years, a greater amount of evidence therefore linked oxLDL to the pathogenesis of NASH, the hepatic manifestation of the MetS. It has been known for a long time that oxLDL is cytotoxic and induces cellular damage. However, until recently, oxLDL has also been found to exert its harmful effects on KCs, followed by KC-derived interplay with other hepatic cells. The reviewed data suggest, for the first time, that oxLDL is an important trigger for NASH development. Since cholesterol and its oxidized form play a crucial role in the progression of NAFLD, most therapeutic strategies against NASH should aim at lowering plasma cholesterol, prevention of (oxidized) cholesterol uptake by macrophages and enhancement of the whole-body anti-oxidant status. The finding that NASH can be viewed as an acquired lysosomal storage disorder has significant implications for the development of novel therapeutics against liver inflammation. Higher oxLDL levels in the plasma does not necessarily discriminate NASH from its overlapping risk factors, obesity, diabetes or atherosclerosis. On the one hand, lowering plasma oxLDL has therefore additional beneficial effects on metabolic related disorders. On the other hand such an overlap puts the diagnostic value of plasma oxLDL, and its specificity to detect NASH, at risk. Therefore, we suggest that studies in mice and large human cohorts should be used in the future to test the clinical utility of plasma oxLDL as a non-invasive marker for NASH. All in all, these diagnostic and therapeutic strategies provide a basis for the amelioration of NASH and related metabolic risk factors that can lead to CVD, diabetes mellitus and its associated complications.

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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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