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Mitochondrial Dysfunction and Oxidative Stress in the Pathogenesis of Alcohol and Obesity Induced Fatty Liver Diseases

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Abstract

Fatty liver disease associated with chronic alcohol consumption or obesity/type 2 diabetes has emerged as a serious public health problem. Steatosis, accumulation of triglyceride in hepatocytes, is now recognized as a critical “first-hit” in the pathogenesis of liver disease. It is proposed that steatosis “primes” the liver to progress to more severe liver pathologies when individuals are exposed to subsequent metabolic and/or environmental stressors or “second-hits”. Genetic risk factors can also influence the susceptibility and severity of fatty liver disease. Furthermore, oxidative stress, disrupted nitric oxide (NO) signaling, and mitochondrial dysfunctional are proposed to be key molecular events that accelerate or worsen steatosis and initiate progression to steatohepatitis and fibrosis. This review article will discuss the following topics regarding the pathobiology and molecular mechanisms responsible for fatty liver disease: 1) the “two-hit” or “multi-hit” hypothesis; 2) the role of mitochondrial bioenergetic defects and oxidant stress; 3) interplay between NO and mitochondria in fatty liver disease; 4) genetic risk factors and oxidative stress responsive genes; and 5) the feasibility of antioxidants for treatment.

Keywords

Liver; alcohol; obesity; steatosis; mitochondria; oxidative stress; nitric oxide; antioxidants; proteomics; AFLD; ASH; NAFLD; NASH

INTRODUCTION

Heavy, prolonged alcohol consumption is estimated to be the third leading cause of all preventable deaths in the US with up to 12,000 deaths each year specifically due to alcohol induced liver disease [1]. Moreover, with the increasing prevalence of obesity and type 2 diabetes, non-alcoholic fatty liver disease (NAFLD) has become a serious medical problem in many developed countries. In general, the spectrum of liver pathology in these circumstances runs the gamut from simple steatosis (triglyceride accumulation in hepatocytes) to the more serious conditions of steatohepatitis, fibrosis, and cirrhosis. Moreover, it should be pointed out

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that steatosis, while reversible, is not benign, and may actually "prime" the liver to progress to more serious pathologies including hepatocellular carcinoma. In the case of alcohol induced liver injury there is a longstanding belief that the severity of disease is dependent on the cumulative dose and duration of alcohol consumption, with decades of heavy drinking needed to induce pathology. However, studies show that only about 25% of heavy drinkers develop steatohepatitis with less than 10% developing alcoholic cirrhosis [2,3]. Based on this, it has been suggested that exposure to environmental toxicants like cigarette smoke or the presence of pre-existing conditions like type 2 diabetes, obesity, hypercholesterolemia, and/or hyperglycemia; conditions linked to the cardiometabolic syndrome, could worsen liver disease in the chronic alcohol consumer. Importantly, epidemiologic evidence demonstrates that alcohol, tobacco smoke, and obesity are synergistic risk factors for hepatocellular carcinoma [4]. Similarly, short-term binge alcohol exposure increases apoptosis and liver injury in obese rats compared to lean controls via increased oxidative stress [5]. Thus, there is clearly support for the concept that conditions of the cardiometabolic syndrome may exacerbate the hepatotoxic potential of alcohol in the liver and vice-versa.

Almost 30 years ago, Ludwig et al. [6] first coined the term non-alcoholic steatohepatitis (NASH) as a type of fatty liver disease that was histologically identical to alcoholic steatohepatitis (ASH), but occurred in overweight and/or type 2 diabetic patients who drank less than 20–40 g of ethanol/day (i.e. 1.5–3.5 drinks per day). Affected individuals typically present with moderate to severe steatosis and lobular inflammation with fibrosis in 70% and cirrhosis in 15% of patients. Since these early studies, it is currently estimated that the prevalence of NAFLD in the general US population is reaching 20% with the more severe form, NASH present in 3% [7,8]. Even more important, may be the fact that NAFLD is present in the pediatric population with prevalence estimated at 2–10% among children and adolescents in the US and Asia [9]. NASH is characterized histologically by macrosteatosis, lobular inflammation, hepatocyte ballooning, and pericellular fibrosis in the centri-lobular (zone 3) region of the liver lobule. Multiple factors have been associated with NAFLD including obesity, central adiposity, type 2 diabetes, and hyperlipidemia with insulin resistance postulated as an essential factor. Interestingly, the role of insulin resistance in alcohol-dependent liver has been invoked; however, the data in support of this mechanism is equivocal [10–12].

"Two-Hit" or "Multi-Hit" Hypothesis for Alcoholic and Non-Alcoholic Fatty Liver Disease

Mechanisms of alcoholic liver disease have been extensively studied; however, the events that cause progression to hepatitis and fibrosis/cirrhosis are still undefined. The same can also be said for the pathogenesis of NAFLD. Currently, the leading hypothesis for both alcohol and non-alcohol induced liver disease is the "two-hit" or "multi-hit" hypothesis with the "first-hit" being the accumulation of triglyceride in hepatocytes (steatosis) followed by "second-hits" which trigger the progression to more serious liver pathologies like steatohepatitis and fibrosis/cirrhosis (Figure 1). Examples of "second-hits" could include environmental stressors like dietary components, cigarette smoke, or pollutants and/or metabolic stressors such as hyperglycemia, hypertriglyceridemia, and hypercholesterolemia, which are components linked to the cardiometabolic syndrome. Interestingly, what these factors all have in common is the ability to induce oxidative stress arising largely in part via mitochondrial defects. It has been proposed that the elusive "second-hit" arises from the enhanced production of reactive oxygen and/or nitrogen species (ROS/RNS) in liver [13]. Once steatosis is present, the liver becomes more susceptible to the "second-hit"; oxidative/nitrosative/nitrative stress, which is thought to be one of several stimuli for the progression from simple fatty liver to ASH or NASH. The molecular mechanism underlying how steatosis predisposes liver to transition from simple fatty liver to steatohepatitis is not clear; however, several studies point to the possibility that when hepatocytes accumulate fat the fatty acids themselves are toxic and initiate a pathological

response called “lipotoxicity” [14]. Studies show that free fatty acids are toxic to hepatocytes via deregulation of lysosomal metabolism [15,16] and induction of endoplasmic reticulum stress [17,18] resulting in apoptosis. Similarly, palmitate induced lipid accumulation in hepatocytes stimulated production of the neutrophil chemoattractant interleukin-8, which could have the effect of initiating hepatic inflammation and injury [19]. The oxidation and release of reactive lipid species (i.e. lipid peroxidation products) present in fat deposits may also contribute to liver injury through the abilities of these molecules to adduct to cellular macromolecules thereby disrupting function. For example, reactive lipid species have been shown to bind to mitochondrial proteins, induce mitochondrial ROS production, and promote cytochrome *c* release [20,21]. Therefore, increased reactive lipid species may be a critical factor predisposing liver to more severe injury when exposed to other insults or “hits”. Finally, as both a site for fat metabolism and the main source of ROS/RNS in hepatocytes, the mitochondrion is postulated to play a central role in the development of alcoholic and non-alcoholic liver disease and this will be discussed in more detail later in the article.

To understand the molecular mechanisms by which chronic alcohol consumption and the cardiometabolic syndrome cause liver disease, it is important to define the pathways leading to fat accumulation in liver. Excess fat accumulation in liver can result from one or a combination of the following metabolic alterations:

1. decreased β-oxidation of fatty acids;
2. increased fatty acid synthesis due to up-regulation of lipogenic pathways;
3. increased delivery of fatty acids from adipose and other organs due to lipolysis; and
4. inhibition of VLDL-triglyceride export.

In general, it is the metabolic consequence of ethanol oxidation; increase in the NADH/NAD^+ ratio, which disrupts fat metabolism leading to triglyceride accumulation in hepatocytes. Increased NADH leads to increased esterification of fatty acids into triglyceride and increased fatty acid synthesis, while inhibiting fatty acid β-oxidation and tricarboxylic (TCA) cycle activity. In the case of NAFLD, insulin resistance is thought to be responsible for the “first-hit”, steatosis, through the mobilization of peripheral fat to the liver through the development of hyperinsulinemia. Peripheral lipolysis and hyperinsulinemia lead to fatty liver through increased hepatic uptake of fatty acids and accumulation of fat via increased fatty acid synthesis, which again can be accompanied by decreased β-oxidation activity [22]. Recent data also suggests that peripheral lipolysis may contribute to alcohol-induced fatty liver in response to an alcohol-dependent suppression of insulin’s anti-lipolytic action in adipocytes [23]. While emerging data indicates that alcohol dependent insulin resistance may contribute to the accumulation of fat in hepatocytes, the evidence in support of this is less clear; primarily due to observations that alcohol has “dose-dependent” effects on insulin signaling [24]. Studies by Badger and colleagues have demonstrated that while high dose chronic alcohol consumption impairs hepatic insulin signaling, low dose ethanol exposure activates insulin signaling in hepatocytes [24,25]. Importantly, for the first time, these findings provide biochemical support for epidemiologic evidence suggesting that while low dose alcohol consumption may be cardio-protective and decrease the risk of developing type 2 diabetes; heavy, prolonged alcohol consumption increases the risk for type 2 diabetes.

It should also be mentioned that steatosis induced by chronic alcohol consumption and/or type 2 diabetes can be directly linked to two critical signaling pathways that increase lipogenesis in the liver; AMP kinase inhibition and SREBP-1 (sterol regulatory element binding protein-1) activation. AMP kinase, a protein kinase that inhibits lipogenesis and activates fatty acid oxidation, is inhibited by chronic alcohol feeding, which results in increased SREBP-1 activation [26,27]. As a consequence of the alcohol-dependent activation of SREBP-1, a

number of lipogenic genes are up-regulated in liver contributing to enhanced lipid synthesis. Similarly, the ethanol-dependent inhibition of AMP kinase leads to activation of acetyl CoA carboxylase; enhancing malonyl CoA levels, which inhibits fatty acid uptake and β-oxidation in mitochondria. Thus, alcohol-associated inhibition of AMP kinase contributes to fat accumulation via stimulation of lipogenesis and inhibition of fat oxidation. Additionally, it has been demonstrated that activation of AMP kinase by the adipocytokine adiponectin and other agents improves both alcohol and obesity-induced fatty liver disease in rodent models [28–30] demonstrating the key role of AMP kinase in the development of steatosis. Clearly, the question that still remains to be answered is, what is the mechanism through which ethanol inhibits AMP kinase activity? AMP kinase is activated by phosphorylation thus possible mechanisms of inactivation could include allosteric modification by AMP and ATP, activation of protein phosphatases, and modulation by other signaling pathways [31,32]. More recently, studies have suggested that the finely tuned mitochondrial ROS/RNS balance may also be a critical controlling factor in the activation of AMP kinase [33–35]. Therefore, it is possible that chronic alcohol and obesity mediated effects on mitochondrial functioning and redox signaling inhibit AMP kinase activity thereby contributing to the development and progression of fatty liver disease. Future studies are warranted to address this very interesting and provocative new question.

Before considering the role of mitochondrial dysfunction in the pathogenesis of alcohol and non-alcohol mediated fatty liver disease, it is important to highlight emerging evidence, which indicates that steatosis may not be an unvarying feature of ASH and NASH. Recent work by the Nagy [36] and Lindros [37] laboratories have revealed that activation of the complement pathway is necessary for the development of chronic alcohol induced steatosis. Indeed, both laboratories have independently shown that, unlike wild-type mice, complement component $C3^{-/-}$ mice do not develop fatty liver when fed an ethanol-containing diet for 6 weeks [36, 37]. More interesting, however, is the observation by Pritchard et al. showing that even though the $C3^{-/-}$ mice were protective against ethanol-mediated steatosis, mice still had elevated serum ALT and hepatic TNFα levels following alcohol exposure indicating hepatic inflammatory injury in the absence of steatosis [36]. In support of this, Diehl and colleagues demonstrated that inhibition of hepatocyte triglyceride synthesis and steatosis increased liver injury and fibrosis in obese mice with NASH [38]. Moreover, oleate- or palmitate-loaded HepG2 spheroids (hepatocyte-derived cells) were less susceptible to cytokine or peroxide induced cell death [39]. Taken together, these results are intriguing because they indicate that steatosis may not be the “first-hit” or a pre-requisite in the pathogenesis of ASH or NASH. In fact, steatosis may even be protective against hepatotoxicity [38]. Clearly, additional research is required to determine the role of steatosis in inflammatory and/or fibrotic liver diseases.

Mitochondria Dysfunction in Fatty Liver Diseases – Bioenergetic and Oxidative Stress

While the detrimental effects of chronic alcohol consumption on liver mitochondria have been known for many years [40], it has only been recently recognized that mitochondrial dysfunction may play a critical role in the development of NAFLD and progression to NASH [22]. Even though the molecular defects responsible for alcohol and non-alcohol dependent mitochondrial dysfunction remain to be defined, it is clear that under conditions of fatty liver disease one key functional change to mitochondria is the inability to maintain sufficient levels of ATP.

Interestingly though, this event may occur by different types of biochemical and molecular changes to mitochondria. In general, respiration in the presence of ADP (state 3 respiration) with NADH-linked substrates, fatty acids, or succinate is decreased as a consequence of chronic alcohol consumption, whereas respiration in the absence of ADP (state 4 respiration) is largely unaffected [41]. Moreover, this decrease in ADP-stimulated respiration is linked to decreased electron transport in all segments of the respiratory chain as chronic alcohol consumption

decreases the activities of all the respiratory complexes, except complex II [41,42]. Several laboratories have presented strong evidence that inhibition of mitochondrial protein synthesis [43] linked to mtDNA damage [44–46] and ribosomal defects [47,48] contribute, in part, to decreased functioning of the oxidative phosphorylation system following chronic alcohol consumption. These alterations translate into profound modifications to the mitochondrial proteome that encompass not only losses in the 13 mitochondrial encoded polypeptides, but also decreases in numerous nuclear encoded proteins that make up the oxidative phosphorylation complexes [46]. Proteomic analyses have also shown alcohol-dependent changes in mitochondrial matrix enzymes both at the level of abundance and post-translational modifications [46,49,50].

Like chronic alcohol induced liver injury, emerging evidence strongly implicates mitochondrial dysfunction contributing to obesity induced fatty liver disease (i.e. NAFLD). Early studies by Diehl and colleagues reported that obesity induced steatosis was associated with increased expression of uncoupling protein-2 (UCP-2) in liver, which promoted ATP depletion [51]. This results from increased H^+ leak across the inner mitochondrial membrane, which dissipates the membrane potential and decreases ATP synthesis. Decreased activity of all five oxidative phosphorylation complexes was found in liver biopsies from human patients with NASH as compared to normal liver [52]. Similarly, decreased respiratory complex activities were also measured in the livers of *ob/ob* mice [53]. While these studies clearly point to a defect in mitochondrial bioenergetics, additional studies are required to identify and better understand the molecular mechanisms and targets responsible for mitochondrial dysfunction in obesity induced fatty liver disease. This is particularly important because studies have shown that state 3 respiration is not decreased in liver mitochondria isolated from *ob/ob* mice and that *ob/ob* mitochondria maintain a similar level of respiratory control compared to lean control mitochondria [51]. It is only when substrate is limited (i.e. decreased supply of electrons) to the respiratory chain that the membrane potential declines and ATP production is inhibited in mitochondria isolated from the severely steatotic liver of *ob/ob* mice. Thus, it has been proposed that up-regulation of UCPs negatively impacts cellular energy conservation only when the availability of oxidizable substrates becomes limited, e.g. under conditions of acute stress like ischemia, partial hepatectomy, and lipopolysaccharide exposure [51,54,55]. Based on this information, further studies are required to elucidate the impact of obesity on hepatic mitochondrial physiology *in vivo* particularly in the context of whether mitochondria are coupled or un-coupled as a consequence of steatosis.

Undeniably, one question that continues to come up in the field is whether mitochondrial dysfunction, i.e. the inability to maintain hepatic ATP levels, contributes to, or is simply a consequence of, fatty liver disease. While a difficult question to “prove” a few studies have shed light on this. As mentioned above, up-regulation of UCPs as an adaptive response to obesity results in hepatocyte ATP depletion when energy needs are acutely increased. Importantly, these studies demonstrated that hepatic necrosis occurred only when mitochondrial electron transport was inhibited [51,54]. Similarly, Diehl and colleagues demonstrated using ^{31}P NMR spectroscopy that recovery from hepatic ATP depletion is severely impaired in NASH patients [56] suggesting a NASH dependent bioenergetic defect. However, what was more revealing from this small pilot study was the observation that body mass index was inversely correlated with ATP recovery even in the healthy lean control subjects. This finding indicates that defects in energy conservation (i.e. mitochondrial dysfunction) may occur before fatty liver disease is present [56]. Studies have also shown that alcohol mediated liver damage accompanies defects in mitochondrial function and the inability to maintain hepatic ATP concentrations [57,58]. While these findings are not unequivocal, they do support the hypothesis that defects in hepatic energy metabolism may indeed increase the susceptibility and risk for disease when the liver experiences other insults or “hits” like increased ROS/RNS.

Alcohol dependent disturbances in structure and function of the electron transport chain have also been proposed to be associated with increases in mitochondrial ROS production and oxidative injury in steatotic liver. Mitochondrial ROS production occurs as a by-product of electron transport; electrons "leak" from Complexes I and III to oxygen to form superoxide anion radicals ($O_2^{\bullet -}$), which can be metabolized to hydrogen peroxide, interact with other free radicals like nitric oxide to form more toxic RNS, and initiate reactions leading to the generation of reactive lipid species (i.e. RLS). While mitochondria are known to be a significant source of oxidants in response to alcohol consumption [40,59], fewer studies have linked mitochondrial oxidant production to the development of NAFLD and NASH. More importantly, the molecular mechanisms responsible for oxidant production in obesity induced fatty liver are not known, especially because mitochondrial un-coupling has been shown in experimental models of NAFLD [51]. Studies using liver mitochondria from *ob/ob* mice have however demonstrated increased $O_2^{\bullet -}$ compared to mitochondria from lean mice [60,61]. Possible mechanisms for enhanced mitochondrial ROS production include molecular defects within Complexes I and III, as well as increases in RLS, and TNFα mediated ceramide [22, 59,62,63]. See review [64] for a detailed description of the mechanisms responsible for alcohol induced increases in mitochondrial ROS.

The mechanisms discussed in the previous paragraph are proposed to function by blocking or inhibiting the transfer of electrons along the respiratory chain, which results in increased reduction of the redox active centers of the respiratory complexes. These "overly" reduced complexes (I and III) then transfer electrons one at a time to oxygen to increase $O_2^{\bullet -}$ generation, which can initiate a cycle of more lipid peroxidation, mitochondrial damage, and further ROS/RNS production. RLS (i.e. lipid peroxidation products), ROS, and RNS can also damage mtDNA. Studies have suggested that Complex II-linked respiration may lead to increased production of $O_2^{\bullet -}$ within Complex I as a consequence of reverse electron transfer [65,66]. It is proposed that reverse electron transfer may contribute to increased mitochondrial production of $O_2^{\bullet -}$ due to chronic alcohol-mediated defects within Complex I [43,46]. This would have the effect to compromise the functioning of the oxidative phosphorylation system as a result of the loss of mitochondrial gene products that make up segments of the respiratory chain; subsequently leading to more ROS production. This concept is supported by observations of significant mtDNA mutations and abnormalities in NASH patients [67,68] and in an animal model of fatty liver disease [60]. Whether these mtDNA defects translate into decreased activity and content of the respiratory complexes in obesity induced fatty liver disease is not known. Thus, future studies should be directed at determining whether alterations to the mitochondrial genome and proteome accompany oxidant damage and pathology of NAFLD.

In addition to the respiratory chain being a significant source of mitochondrial ROS production, work by Fromenty and colleagues demonstrated an alcohol dependent increase in both microsomal and mitochondrial cytochrome P450 2E1 (CYP450 2E1) in cultured hepatocytes and liver of wild-type mice [69] suggesting an additional source of oxidants in alcohol exposed mitochondria. Indeed, CYP450 2E1 is known to be a significant source of ROS as it generates high amounts of hydrogen peroxide in the absence or presence of oxidizable co-substrates due to the "un-coupled" nature of this P450 isoform [70]. In contrast, short-term alcohol exposure did not enhance mitochondrial CYP450 2E1 levels in liver of *ob/ob* mice. It is important to note that there was no difference in the levels and activity of CYP450 2E1 in liver from lean and ethanol-naïve *ob/ob* mice [69]. Mitochondrial ROS generation has also been ascribed to several matrix enzymes including α-ketoglutarate dehydrogenase [71,72] and α-glycerophosphate dehydrogenase [73]. Whether these enzymes contribute to increased ROS production during the conditions of alcohol and obesity induced fatty liver disease is not known, however it is predicted that higher rates of ROS in mitochondria during these pathologies will negatively affect mitochondrial and cellular function by oxidative modification and alteration in redox-sensitive signaling pathways.

Depletion of mitochondrial glutathione (GSH) has also been implicated as a contributor to the development of alcoholic liver disease as GSH functions in pathways responsible for ROS detoxification [74]. Studies by Fernandez-Checa and colleagues demonstrated that chronic alcohol consumption depletes mitochondrial GSH [75] and treatments that prevent the loss in inner membrane fluidity maintain GSH uptake and preserve mitochondrial GSH levels in liver of alcohol-fed animals [76,77]. Importantly, it is proposed that loss of mitochondrial GSH sensitizes hepatocytes from alcohol-fed animals to TNFα induced cell death [78,79], specifically when mitochondria are "loaded" with free cholesterol [80]. In contrast, several studies have shown that mitochondrial GSH depletion may not be a consistent outcome following chronic alcohol consumption as studies have shown increased mitochondrial GSH in response to alcohol consumption [81–84]. Similarly, Cederbaum and colleagues have shown increased GSH in HepG2 cells over-expressing CYP450 2E1 due to upregulation of glutamate cysteine ligase [85]. Based on this, increases in mitochondrial GSH in response to mild-moderate alcohol consumption may represent an adaptive response to counteract oxidative stress induced by chronic alcohol exposure. Indeed, adaptive increases in GSH have been demonstrated in multiple models of mild oxidative stress [86,87]. Conflicting effects of obesity and NAFLD on mitochondrial GSH levels have also been reported. For example, decreased [53] and increased [61] levels of mitochondrial GSH have been reported in liver of *ob/ob* mice. In addition, two glutathione-replenishing agents, S-adenosylmethionine and 2(*RS*)-*n*-propylthiazolidine-4(*R*)-carboxylic acid (i.e. an *L*-cysteine pro-drug), were shown to protect against steatohepatitis induced by the methionine-choline deficient diet; however, these agents did not prevent alterations in GSH and GSSG levels in response to steatohepatitis [88]. Nonetheless, increased GSSG concentrations were found in the blood of NASH patients suggesting defects in glutathione metabolism [89]. Taken together, these findings demonstrate that while a potential player in the pathogenesis of fatty liver disease, additional studies are necessary to delineate the precise role of mitochondrial GSH depletion in the pathogenesis of alcohol and non-alcohol mediated mitochondrial dysfunction and liver injury.

Interplay Between Nitric Oxide and Mitochondria in Fatty Liver Disease

Nitric oxide (NO) and other RNS are increased in response to chronic alcohol consumption through induction of inducible nitric oxide synthase (iNOS) [82,90]. In normal healthy liver iNOS levels are very low or undetectable [91], whereas during inflammation and other disease states iNOS levels are increased in liver due to infiltrating inflammatory cells and via induction in Kupffer cells (resident liver macrophage), hepatocytes, and biliary epithelial cells [92–94]. Studies also report increased iNOS expression in liver of *ob/ob* mice [53], CCl_4-induced steatotic liver following normothermic ischemia [95], and in the obese *fa/fa* rat exposed to binge alcohol [5]. This is important for hepatotoxicity because NO and peroxynitrite ($ONOO^-$) are known to mediate mitochondrial dysfunction [96,97] and increases in iNOS expression in fatty liver are correlated with nitration of mitochondrial proteins [53,98]. Moreover, NO regulates mitochondrial respiration through reversible binding at the redox-active heme site in cytochrome *c* oxidase [99–101] and affects mitochondrial biogenesis through interactions with soluble guanylate cyclase [102]. Studies have shown that mitochondria from chronic alcohol-fed animals are more sensitive to the inhibitory effect of NO on respiration [90,103]. While it is known that chronic alcohol consumption results in cytochrome *c* oxidase defects [82,90,104], it is likely that this alteration in NO responsiveness may be due to alcohol-dependent changes in interactions of NO with sites other than Complex IV. It is postulated that this disruption in NO signaling contributes to alcohol hepatotoxicity through inhibition of ATP synthesis, increased ROS, and inability to adapt to hypoxic stress [105]. This concept is supported because increased sensitivity to NO is absent in $iNOS^{-/-}$ mice exposed to chronic alcohol [90]. Similarly, alcohol-associated inflammation and steatosis is abrogated in $iNOS^{-/-}$ mice [90,106]. Taken together, these studies demonstrate that iNOS induction is linked to alterations in NO-dependent control of mitochondrial respiration, which

potentially contributes to the development of alcoholic steatohepatitis. A scheme summarizing the effects of alcohol and obesity to disrupt mitochondrial function is provided in Figure 2.

It is also important to highlight the beneficial effects of NO in the context of liver diseases. Studies implicate that decreased production of NO from endothelial NOS (eNOS) contributes to liver pathology via dysregulation of blood flow and oxygen delivery [107]. In support of this, NO-donor administration and eNOS overexpression have been shown to prevent liver injury in animal models of hepatotoxicity [108,109]. For example, the oral administration of *S*-nitroso-*N*-acetylcysteine prevented the onset of NAFLD in response to a methionine-choline deficient diet [110,111]; however, it is unclear whether the benefit was due to NO or *N*-acetylcysteine exposure. Recently, inhaled NO administration was shown to accelerate the restoration of liver function in transplantation patients presumably through increased circulating levels of nitrite [112], a newly recognized vascular endocrine transporter of NO [113] that protects against heart and liver ischemia-reperfusion injury [114]. In models of chronic alcohol consumption eNOS activity is decreased in liver through increased expression of the inhibitory protein caveolin-1 and/or decreased eNOS phosphorylation [115,116]. Similarly, Nanji and colleagues have demonstrated *L*-arginine supplementation prevents and reverses chronic alcohol mediated liver injury [117,118]. Based on this, they propose that decreased NO from non-parenchymal cells, i.e. endothelial cells and eNOS, and increased NO from hepatocyte iNOS work in concert to exacerbate chronic alcohol mediated liver injury [117,118]. While these data support the hypothesis that the source, site, and concentration of NO produced are critical for determining the functional consequence of NO, i.e. good or bad NO, the complex interplay between NO and mitochondria remains to be defined in the context of fatty liver disease from alcohol and obesity. Moreover, the critical importance of understanding the regulation of the mitochondrial respiratory chain by NO in hepatic physiology and pathophysiology is supported by several studies demonstrating that localized NO production can occur within the organelle through a specific mitochondrial NOS isoform or via metabolism of nitrite to NO [119–121].

Genetic Factors Determining the Pathobiology of Fatty Liver Diseases – “Oxidative Stress Responsive Genes”

Emerging evidence indicates that genetic factors contribute to increase the risk and severity of alcohol and non-alcohol induced liver disease [3,122]. Indeed, several genes have been identified as influencing the impact of oxidative stress in the pathogenesis of fatty liver disease. Studies have reported genetic polymorphisms in the genes encoding enzymes responsible for alcohol metabolism; alcohol dehydrogenase, CYP450 2E1, and the mitochondrial low K_m aldehyde dehydrogenase [123]. Unfortunately, only weak associations between these polymorphisms and increased alcohol hepatotoxicity have been shown [124].

In contrast to these findings, a polymorphism responsible for a valine to alanine substitution in the mitochondrial targeting sequence for manganese superoxide dismutase (*SOD2*) was found to be a major risk factor for severe alcoholic liver disease in two separate studies [125, 126]. The alanine-*SOD2* variant, whose pre-sequence is an α-helix, is readily imported and achieves high activity in mitochondria, whereas the valine-*SOD2* variant, whose pre-sequence is a partial β-sheet, is poorly imported resulting in low SOD2 activity [127,128]. It is proposed that the presence of the high-activity, alanine-*SOD2* variant will increase hydrogen peroxide production in mitochondria, which might have the effect to disrupt cellular redox signaling pathways and initiate oxidative damage [129]. This is supported by studies demonstrating an interaction of the high-activity, alanine-*SOD2* variant with a low activity glutathione peroxidase-1 variant (leucine-*GPx1*) to increase hepatic iron accumulation and risk for hepatocellular carcinoma in alcoholic cirrhotics [129]. Indeed, cirrhotics possessing two copies of the low-activity, valine-*SOD2* variant and high-activity, proline-*GPx1* variant are protected

from cancer. Furthermore, chronic alcohol consumption is known to increase SOD2 activity [130,131] and decrease mitochondria GPx1 activity [81]. Thus, an imbalance resulting in increased hydrogen peroxide formation and decreased detoxification may be a critical event for the initiation and progression of alcohol induced liver injury. With this said, however, studies performed in the UK with a larger cohort of alcoholic patients showed no association among the valine-alanine *SOD2* polymorphism, alcohol-dependent markers of oxidative stress, and liver fibrosis [132]. Preliminary studies indicate that the *SOD2* polymorphism may be correlated to fibrosis in NAFLD patients [133].

Other studies have reported associations between genetic polymorphisms in endotoxin receptors, cytokines, and fibrosis genes with the pathogenesis of fatty liver diseases. The role of endotoxin mediated cytokine release in the etiology of alcohol hepatotoxicity was established by Thurman and colleagues in the 1990's through an elegant series of experimental animal studies [134,135]. Likewise, some genetic studies have shown an association between alcoholic liver disease [136] and NASH [137] with a polymorphism present in the CD14 promoter that results in increased CD14 levels [138]. The CD14 receptor binds endotoxin and enhances signaling through the toll-like receptor-4 (TLR4). However, other investigations have reported no association between the CD14 and TLR4 polymorphisms and established alcoholic liver disease [139,140]. Similar conflicting results have been reported for the cytokines TNF-α and interleukin-10 [140]. Moreover, while fibrogenesis/fibrinolysis genes are predicted to be strong candidates to influence liver fibrosis, only one report has shown a link between transforming growth factor-β 1 (TGF-β 1) and angiotensinogen polymorphisms and obesity induced liver fibrosis [141]. Lastly, it should be mentioned that an association between naturally occurring variations in the mitochondrial genome and conditions of the cardiometabolic syndrome has been proposed [142,143]. Recently, Pravenec et al. [142] using conplastic rat strains with the same nuclear genome but dissimilar mitochondrial genomes encoding oxidative phosphorylation proteins of differing amino acid composition showed differences in glucose and glycogen metabolism; two risk factors for type 2 diabetes. Whether mtDNA variants are linked to risk factors associated with steatosis, hepatitis, and fibrosis/cirrhosis is a stimulating concept and one that clearly requires further study. Taken together, these findings indicate that genetic factors may contribute to the pathobiology of alcohol and non-alcohol induced steatohepatitis and/or fibrosis, however additional investigations with larger populations are needed to solidify these linkages.

Are Antioxidants Feasible Treatments for Fatty Liver Disease?

While abstinence and weight loss are critically important for ensuring successful outcomes from alcohol and obesity induced liver diseases, respectively, numerous therapeutic agents have been investigated for treatment of these liver diseases (Figure 3). Unfortunately, no proven drug therapies have emerged as being highly effective. This section will provide a brief overview of some of the newer agents that show promise in treatment of fatty liver diseases.

Corticosteriods, pentoxifylline, propylthiouracil, and anti-TNFα have all been used to treat patients with alcoholic hepatitis with some success; however, issues regarding timing of administration in relation to alcohol exposure and efficacy need to be addressed through more randomized clinical trials [144]. Similarly, insulin sensitizing drugs such as metformin and the thiazolidinediones show promise in treating fatty liver disease. Metformin was shown to prevent alcohol induced liver injury in mice [145]. Interestingly, the protective effect of metformin in this study did not correlate to AMP kinase activation, but via prevention of plasminogen activator inhibitor (PAI)-I upregulation by alcohol [145]. Rosiglitazone and pioglitazone, thiazolidinedione peroxisomal proliferator-activated receptor (PPAR)-γ agonists, decrease insulin resistance and have been shown to decrease TNF-α levels and inflammation in rodent models of NASH [146–150]. Similarly, the PPAR-γ agonist

pioglitazone prevents alcohol induced liver injury through interactions with the c-Met signaling pathway resulting in decreased triglyceride synthesis [151]. It should also be mentioned that treatment with the PPAR-α agonist WY14,643 prevented alcohol induced steatosis via up-regulation of β-oxidation activity [152]. However caution should be exercised when using these medications because recent reports have demonstrated mitochondrial abnormalities associated with rosiglitazone therapy in both experimental [153] and human patients [154] with NAFLD.

Both traditional and newly recognized antioxidants have been proposed to slow the progression, decrease the severity, and/or reverse pathology from fatty liver diseases presumably through their ability to decrease oxidative stress in tissues. And, like the therapeutic agents described above, results using antioxidants have been varied with some antioxidants showing positive effects and others not. In both adults and children with NASH, treatment with α-tocopherol (vitamin E) has been shown to improve serum liver enzymes [155–157]. Similarly, NASH patients treated with vitamin E (400 IU/day) and pioglitazone for 6 months showed significant improvement in liver histology over vitamin E treatment alone [158]. While results with α-tocopherol are promising, recent reports have suggested that γ-tocopherol may be a more effective therapeutic agent in treating diseases involving oxidant stress and inflammation due to its potent anti-inflammatory activity [159,160]. Furthermore, Murphy and colleagues have developed novel strategies to “target” traditional antioxidants, like vitamin E, ubiquinone, and lipoic acid to the mitochondrion by attaching them to a lipophilic triphenylphosphonium moiety [161–163]. This modification allows the antioxidant to accumulate several-hundred fold in the mitochondrion in response to the mitochondrial membrane potential where it can exert maximum benefit. Indeed, mitochondria-targeted ubiquinone (a.k.a. MitoQ) was shown to decrease cardiac ischemia-reperfusion injury [164]. In contrast, mitochondria-targeted vitamin E (a.k.a. MitoE) caused neurotoxicity in a hypoxic-ischemia model of striatal injury [165]. Thus, additional studies are required to demonstrate the safety and efficacy of these novel mitochondrially targeted antioxidants in diseases where mitochondrial damage and oxidant stress are proposed to play a role in pathology.

Numerous other antioxidants show efficacy in treating fatty liver diseases and include *N*-acetylcysteine [166], zinc [167,168], silymarin [169], and ursodeoxycholic acid [170], just to name a few. Please refer to the following excellent review articles for additional information on antioxidant therapy for treatment of fatty liver diseases from multiple causes [144,171–174].

Defects in methionine metabolism occur in response to both alcohol and non-alcohol induced liver disease and are postulated to contribute to the disease process. One key metabolite of the methionine metabolic pathway is S-adenosylmethionine (SAM), which is the main methyl donor for biological reactions and serves as a precursor in glutathione and polyamine synthesis [175].

In mammalian cells SAM is synthesized by the enzyme methionine adenosylmethytransferase (MAT). Alcohol and other hepatotoxicants decrease hepatic levels of SAM due to decreased activity of the hepatocyte specific form of MAT [176] and methionine synthase [177]. Studies have shown that NO inactivates rat liver MAT *in vivo* by *S*-nitrosation of cysteine 121, which leads to decreased synthesis and depletion of hepatic SAM [178,179]. SAM depletion correlates with DNA hypomethylation and strand breaks [176] and several parameters of oxidative stress including decreased GSH and increased lipid peroxidation [180]. Accordingly, exogenous administration of SAM in animal models of alcohol toxicity has been shown to be protective [82,181,182]. SAM supplementation was also shown to significantly improve the outcome of alcoholic cirrhotic patients [183]. Emerging evidence suggests that the mitochondrion may be an important target for the benefits afforded by SAM in the treatment of fatty liver disease.

Studies by Mato and colleagues showed that *MAT1A*$^{-/-}$ mice, which are deficient in SAM, spontaneously develop steatohepatitis and hepatocellular carcinoma demonstrating that SAM depletion primes the liver for progression to more severe injury [184,185]. Mitochondria isolated from liver of *MAT1A*$^{-/-}$ mice also have decreased levels of the mitochondrial encoded subunits for cytochrome *c* oxidase and reduced membrane potential [186]. This finding is consistent with the concept that SAM plays an essential role in the maintenance and proper function of mitochondria and the oxidative phosphorylation system. Early studies showed that co-administration of SAM during alcohol feeding maintained mitochondrial GSH transport and GSH levels, which preserved ATP levels and membrane potential [76,77]. Recently, we have extended these earlier studies and shown that SAM supplementation prevents alcohol-dependent decreases in mitochondrial respiration, increases in superoxide production, mtDNA damage, and iNOS induction [82]. Similarly, SAM prevented alcohol-associated losses in both nuclear and mitochondrial encoded subunits of cytochrome *c* oxidase, which preserved oxidase activity [82]. These results are supported by observations that depletion of mitochondrial SAM levels sensitizes HepG2 cells to TNF-α cytotoxicity [187].

In addition to SAM, the methionine pathway metabolite betaine has been shown to prevent hepatotoxicity in animal models of alcohol [188,189] and diet [190] induced steatosis, as well as improve serum liver enzymes and liver histology in NASH patients [191]. Betaine, a natural antioxidant, functions as an osmolyte and a methyl donor for the remethylation of homocysteine, “the bad thiol” [192], back to methionine [193]. Hyperhomocysteinemia is associated with steatosis presumably through induction of endoplasmic reticulum stress and activation of SREBPs leading to increased hepatic synthesis and uptake of triglycerides and cholesterol [194]. Indeed, hyperhomocysteinemia may be a suitable predictor of NASH [195]. Preliminary data demonstrates that betaine supplementation prevented chronic alcohol-induced steatosis and losses in the respiratory chain complexes, particularly subunits of Complex I and IV [196]. Taken together, these data reveal a novel molecular mechanism through which SAM and betaine may work to prevent one key component of alcohol and obesity-induced liver disease; mitochondria dysfunction.

Summary

Strong evidence indicates that the pathogenesis of chronic alcohol and obesity/type 2 diabetes induced fatty liver disease is linked to mitochondrial dysfunction. The initial formation of an inflammatory response and increased production of ROS/RNS associated with the metabolic alterations induced by these conditions leads to modifications of the mitochondrial genome and proteome. These alterations result in loss of mitochondrial respiration, the inability to maintain sufficient ATP concentrations, and a further enhancement of ROS/RNS production. Indeed, the role of oxidative stress in these disease processes has been demonstrated in numerous experimental models. Thus, the goal of current and future investigations will be to provide a thorough characterization of the molecular mechanisms involved in the pathogenesis of fatty liver diseases with particular emphasis on defining the role of mitochondrial oxidative stress in the disease process. A more complete understanding of the ROS/RNS mediated effects on the spectrum of alcohol and obesity induced liver disease will facilitate the development of new mitochondrially targeted molecular medicines for treatment.

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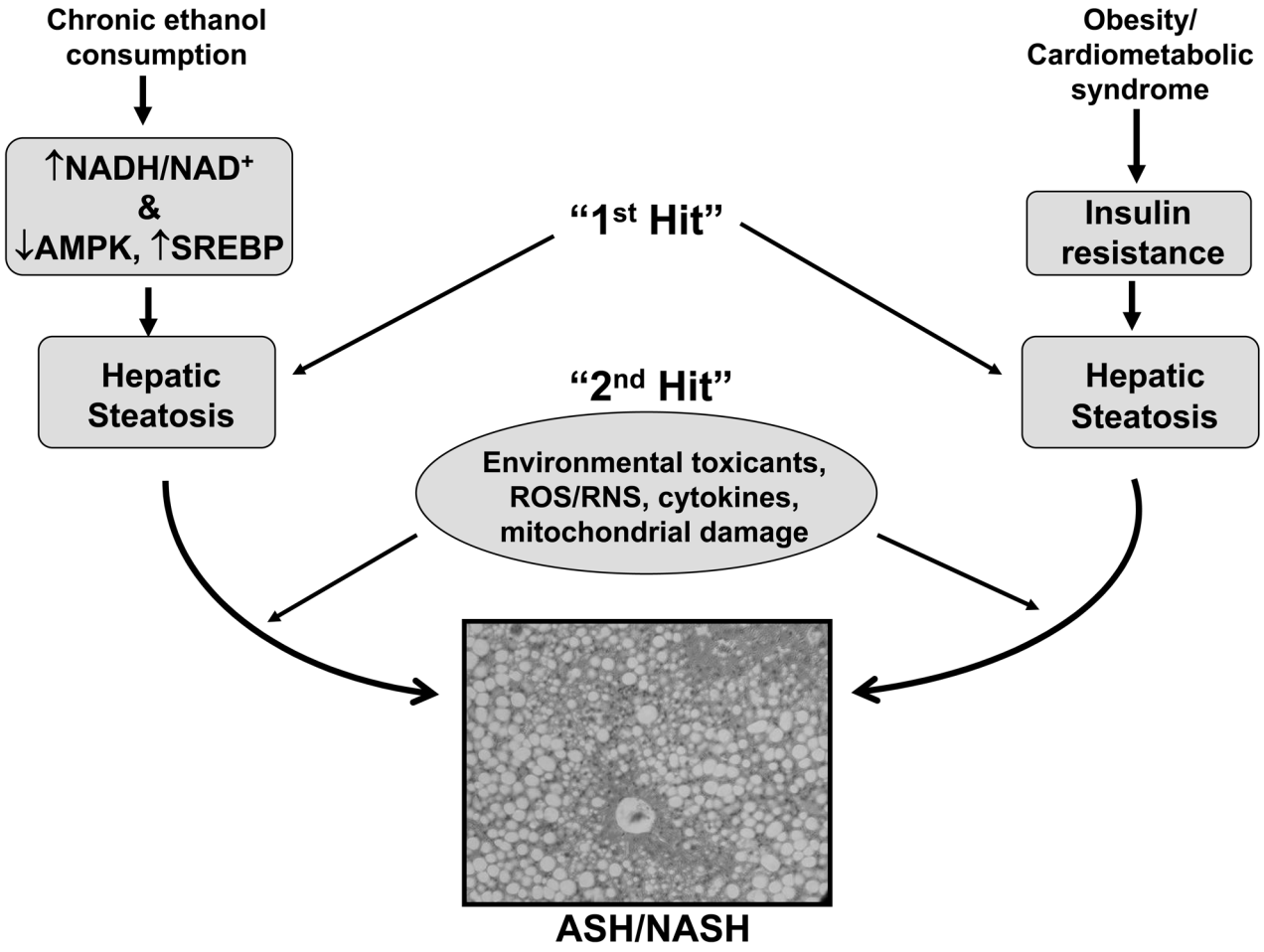

Figure 1. Pathophysiology of alcoholic steatohepatitis (ASH) and non-alcoholic steatohepatitis (NASH)

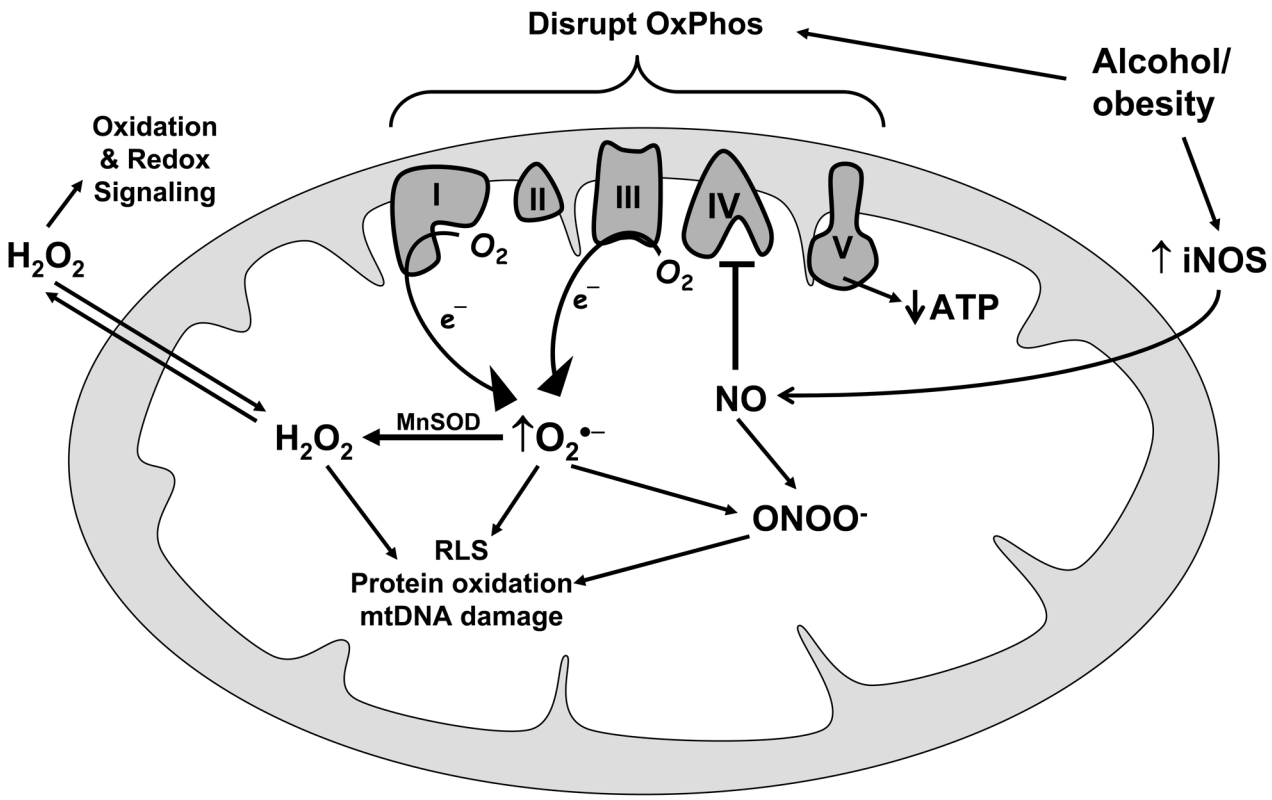

Figure 2. Alcohol and obesity induced mitochondrial dysfunction and oxidative stress

Alcohol and obesity disrupt the functioning of the oxidative phosphorylation (OxPhos) system which results in the generation of superoxide ($O_2^{\bullet -}$) within Complexes I and III. The $O_2^{\bullet -}$ is dismutated to hydrogen peroxide (H_2O_2) by manganese superoxide dismutase (MnSOD) that can then diffuse from the mitochondrion into the cytoplasm to affect cellular redox signaling pathways. Alcohol and obesity also cause an increase in iNOS expression that leads to diffusion of nitric oxide (NO) into mitochondria where it inhibits cytochrome *c* oxidase; reacts with $O_2^{\bullet -}$ to form highly reactive peroxynitrite ($ONOO^-$). Hydrogen peroxide, $O_2^{\bullet -}$, and $ONOO^-$ can initiate reactions that lead to increased generation of reactive lipid species (RLS), induce post-translational modification of proteins, and damage mitochondrial DNA (mtDNA).

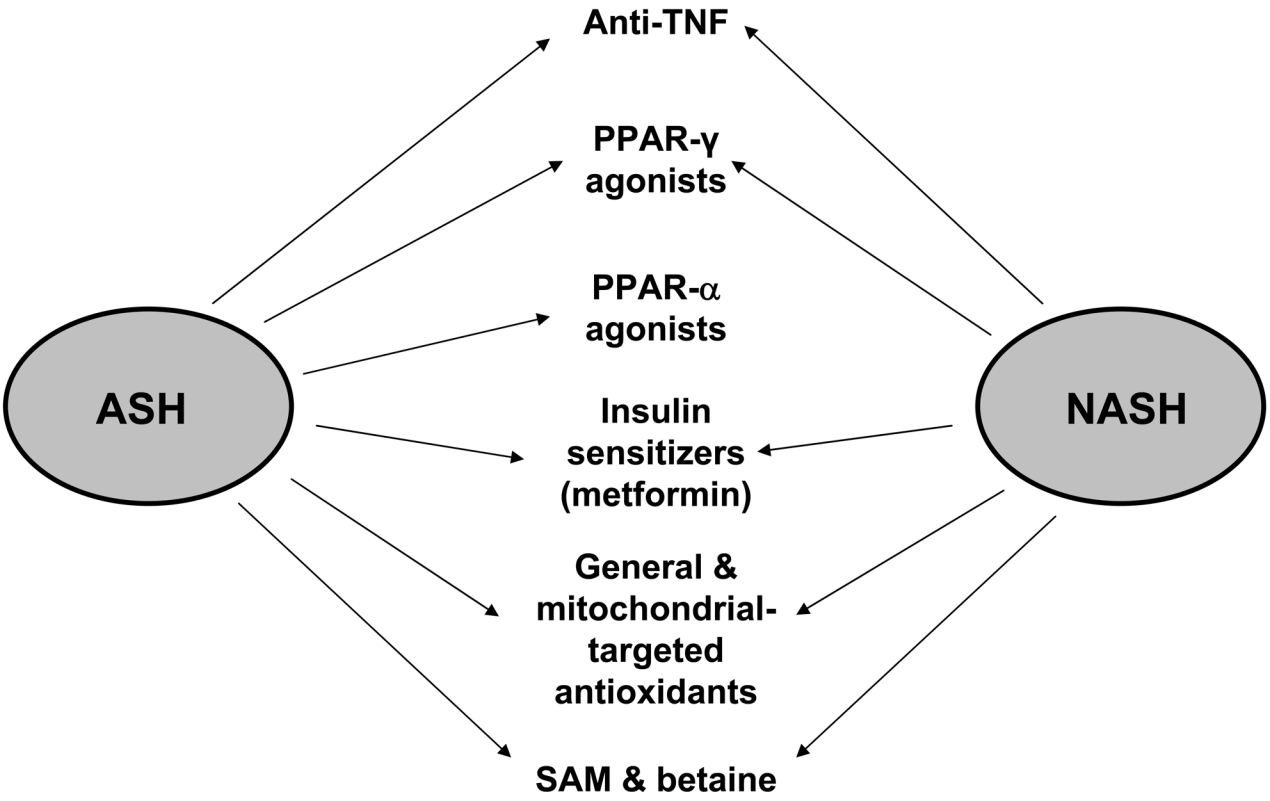

Figure 3. Proposed therapeutic agents for the treatment of fatty liver diseases