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## Redox regulation of hepatitis C in non-alcoholic and alcoholic liver

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Running Title: Alcohol, oxidative stress, and hepatitis C virus infection

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**Abstract**

Hepatitis C virus (HCV) is an RNA virus of the *Flaviviridae* family that is estimated to have infected 170 million people worldwide. HCV can cause serious liver disease in humans such as cirrhosis, steatosis, and hepatocellular carcinoma. HCV induces a state of oxidative/nitrosative stress in patients through multiple mechanisms, and this redox perturbation has been recognized as a key player in the HCV-induced pathogenesis. Studies have shown that alcohol synergizes with HCV in the pathogenesis of liver disease, and part of these effects may be mediated by reactive species that are generated during hepatic metabolism of alcohol. Furthermore, reactive species and alcohol may influence HCV replication and the outcome of interferon therapy. Alcohol consumption has also been associated with increased sequence heterogeneity of the HCV RNA sequences, suggesting multiple modes of interaction between alcohol and HCV. This review summarizes current understanding of oxidative and nitrosative stress during HCV infection and possible combined effects of HCV, alcohol, and reactive species in the pathogenesis of liver disease.

**Keywords:** acetaldehyde, alcohol, evolution, antioxidant, hepatocellular carcinoma, hepatitis C virus, nitrosative stress, oxidative stress, pathogenesis, replication

## **I. Introduction**

Hepatitis C virus (HCV) was discovered in 1989 as an etiologic agent of non-A non-B hepatitis [1]. HCV is transmitted through blood. Currently, it is estimated that there are 170 million people worldwide who have been infected with HCV, including 4 million in the U.S. alone, of whom 2.7 million are viremic [2-4]. About 80% of HCV infections result in a persistent infection that can lead to serious health complications, such as cirrhosis and hepatocellular carcinoma (HCC) [5, 6]. HCC is the eighth most frequent cancer worldwide, and HCV-associated chronic liver disease is the leading cause for liver transplantation in the U.S [5-7]. HCV is responsible for at least 8,000 deaths and more than \$600 million spent annually in health care and work-loss in the U.S. It has been estimated that over the next twenty years, the proportion of infected patients with cirrhosis will increase from 16 to 32%, and other complications will also increase dramatically, including hepatic decompensation (increase by 106%), HCC (by 81%), and liver-related deaths (by 180%) [8]. Current anti-HCV therapy, which consists of pegylated interferon alpha and ribavirin, achieves sustained virological response (SVR) in only 50 – 60 % of individuals undergoing treatment [9, 10]. The SVR is as low as 30% in patients with HCV genotype 1 and a high viral load [10]. Antiviral drug resistance, undesirable side effects, the cost of these drugs, and incomplete understanding of the mechanism of pathogenesis continue to pose considerable challenges to the management of hepatitis C. There is currently no vaccine against HCV.

HCV is a single, positive-stranded RNA virus of the *Flaviviridae* Family [11]. The HCV genome is about 9.6 kb in length and consists of the 5' untranslated region (UTR), the structural (C, E1, E2) and nonstructural (p7, NS2, NS3, NS4A/B, NS5A/B)

protein-coding regions, and the 3' UTR (Figure 1). HCV enters the cell through clathrin-mediated endocytosis, and the virion is then uncoated, releasing the genome into the cytosol. The translation of the positive-stranded RNA genome, which occurs in a cap-independent manner, is mediated by an internal ribosomal entry site (IRES), located at the 5' end of the genome, and produces a polyprotein, that is cleavage by host and viral proteases to generate the individual viral proteins. The structural proteins are cleaved by host signal peptidases, whereas the nonstructural proteins are cleaved by the viral cysteine protease activity of NS2 and the serine protease activity of NS3/4 [12]. Some of the HCV proteins can also be synthesized from the alternate reading frames, potentially through multiple mechanisms that include translational frameshifting and internal initiation of translation [13-16]. HCV RNA replication, which occurs in the cytosol, is mediated by NS5B, an RNA-dependent RNA polymerase, and other proteins that comprise the replication complex. Virions are assembled by the formation of the capsid by the core protein and the internalization of the genome, which most likely then buds into the endoplasmic reticulum (ER) or an ER-derived compartment. Then, the virions, which are enveloped by lipid membranes plus viral proteins, are exported from the cell via normal host secretory pathway [12]. Recent reviews that summarize the HCV lifecycle and functions of the virally-encoded proteins are found in references [12] and [17].

Despite continued studies, the mechanism by which HCV induces these pathogenic changes in the liver remains largely unresolved. However, HCV-induced pathogenesis is affected by various host and environmental factors, suggesting complex interactions between HCV and these factors [18-20]. These host and environmental

factors include age, gender, co-infection with other viruses, and alcohol consumption [21-24]. In addition, HCV infection leads to severe oxidative/nitrosative stress in patients [25-35], and this has been recognized as an important component of HCV-induced liver disease. This review presents a comprehensive summary of current understanding of redox regulation of HCV in hepatopathogenesis, focusing on the interactions between HCV, reactive species, and alcohol.

## **II. Redox regulation of hepatitis C virus in the pathogenesis of liver disease**

HCV infection is associated with elevated levels of reactive oxygen/nitrogen species (ROS/RNS) and decreased antioxidant levels in patients [19, 25-33, 35, 36]. HCV patients have increased lipid peroxidation product levels in their serum, peripheral blood mononuclear cells, and liver specimens [25, 27, 29, 30, 32, 34, 37]. Other evidence of oxidative damage include elevated levels of 8-hydroxydeoxyguanosine and 4-hydroxynonenal (HNE) [28, 30, 36, 38, 39]. Glutathione (GSH) content is decreased in the liver, blood, and lymphatic system while the percentage of glutathione disulfide (GSSG) is increased, suggesting increased GSH turnover [25-27, 33, 35, 36, 40].

Interestingly, oxidative/nitrosative stress is more pronounced with HCV than hepatitis B virus (HBV) [27], which also causes viral hepatitis. Possible mechanisms for severe increases in oxidative/nitrosative stress during HCV infection include chronic inflammation (i.e., activation of phagocytic NAD(P)H oxidase), which is rather non-specific, and iron overload, which appears to be more specific to HCV [26, 27, 30, 41-53]. In addition, ROS production within the hepatocytes can further result in the activation of neighboring Kupffer cells [54]. These cells express many pro-inflammatory

cytokines and, when ROS level increases, they begin to swell, until they burst and release their cellular content into the extracellular matrix of the liver. Cytokines like tumor necrosis factor-alpha and transforming growth factor-beta (TGF $\beta$ ) can increase ROS levels and play key roles in the mediation of liver diseases during states of increased oxidative stress [20, 55, 56], such as by promoting insulin resistance and fatty liver by inhibiting lipoprotein lipase and adiponectin, as well as promoting fibrosis by activating hepatic stellate cells [57-59].

Furthermore, HCV proteins may specifically increase oxidative/nitrosative stress in the infected cell (Figure 1). For example, HCV core protein has been shown to increase the levels of ROS/RNS, oxidized thioredoxin, lipid peroxidation products, and antioxidant gene expression, such as that of manganese superoxide dismutase (MnSOD) and metallothioneine family proteins, and to enhance the sensitivity to toxins such as ethanol and CCl<sub>4</sub> [38, 60-65]. In terms of a mechanism, HCV core gene expression decreases the intracellular/mitochondrial GSH levels and the mitochondrial NADPH levels, which are accompanied by increased Ca<sup>2+</sup> uptake as well as ROS generation at Complex I in mitochondria [60-62, 66]. Although whether the mitochondrial, cytosolic, and/or total intracellular GSH are decreased in a consistent and predictable manner is still unclear, only the mitochondrial, and not the total liver GSH, was altered with the HCV core protein in a recent study [60]. As GSH is transported from the cytosol into the mitochondria, and mitochondrial GSH is relatively resistant to depletion, for example, by *L*-buthionine *S,R*-sulfoximine (BSO), which inhibits GSH biosynthesis, a decreased transport or an increased utilization of GSH in the mitochondria, rather than decreased synthesis of GSH, are suspected. Indeed, a parallel decrease in the NADPH level is most

consistent with an increased consumption of GSH, as NADPH participates in a reaction that recycles GSSG back to GSH. In addition, core protein has also been shown to modulate the production of cytokines and host enzymes that can increase ROS/RNS, such as inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) [67-73]. These observations are consistent with the fact that HCV core protein, in addition to forming viral capsid, has many other functions in the modulation of host cell functions and pathogenesis [74-76].

In addition to the core protein, nonstructural proteins may play a role in the modulation of host redox status by HCV (Figure 1). For example, host antioxidant defense (e.g., MnSOD, heme oxygenase-1 (HO-1), catalase, and GSH) is elevated in the HCV subgenomic replicon cells, which only express the nonstructural genes of HCV, suggesting adaptation to oxidative/nitrosative stress [61, 77, 78]. These effects may be explained in part by increased synthesis of nitric oxide and increased gene expression of COX-2 with NS5A and/or NS3 [69, 70, 72, 73]. NS5A has also been suggested to induce ER stress and  $\text{Ca}^{2+}$  release from the ER, resulting in increased  $\text{Ca}^{2+}$  uptake and ROS generation by mitochondria [77]. Furthermore, HCV NS3 protein can stimulate ROS generation by activating NADPH oxidase of phagocytes [79, 80]. Importantly, HCV core and nonstructural proteins both induce oxidative stress; however, they may induce different antioxidant responses, indicating different mechanisms [61]. For instance, core has been reported to up-regulate MnSOD expression without elevating HO-1 and GSH levels [61] whereas nonstructural genes increase MnSOD, HO-1, and GSH [61, 77, 78]. HO-1 is also elevated in cells expressing HCV genes from core up to the N-terminal domain of NS3 [81]. These multiple interactions between HCV proteins and the host



immune system may thus act together to generate pro-oxidative environment in the HCV-infected liver. However, the biological significance of some of these seemingly opposing effects that the core and the nonstructural proteins have on the host antioxidant status is unclear. It should be noted that these proteins are produced simultaneously during the HCV lifecycle (see above). Therefore, whether the concurrent expression of the core and the nonstructural proteins up-regulates, down-regulates, or cancels out the effects of one another, in the natural context (i.e., with the entire HCV genome and the complete viral lifecycle) remains to be shown.

Oxidative stress plays critical roles in various liver diseases [18, 20, 46-48, 82-85]. Even among symptom-free HCV carriers, redox perturbation was correlated with increase in flare-ups of alanine aminotransferase (ALT) [86]. Therefore, increased ROS/RNS have been proposed to play an important role in the HCV-induced pathogenesis [18, 19, 46-48, 82-84]. Note that there are many factors that determine the biological effects of reactive species, which include the type of reactive species, their concentrations, proximity to cellular macromolecules/metals, and the cell's endogenous and inducible capacity to eliminate these molecules and/or reverse their effects. Both redox signaling and irreversible damage to cellular macromolecules have been implicated in pathogenesis [20, 87]. Therefore, ROS/RNS-induced pathogenesis during hepatitis C is likely to involve both irreversible cell damage and some components of redox signaling.

In particular, oxidative/nitrosative stress has been strongly implicated in the HCV-induced carcinogenesis. For example, iron overload may increase oxidative DNA damage [53] and has been suggested to increase the risk of HCC in transgenic mice

expressing the HCV polyprotein [88]. In addition, HCV core-induced iNOS generates RNS that, like ROS, can cause DNA damage and increase mutations within the immunoglobulin and tumor suppressor genes [70, 89]. These genotoxic effects of oxidative/nitrosative stress are expected to contribute towards the development of HCC and B-cell lymphoma during HCV infection, and such association has also been documented *in vivo*, in HCV core-transgenic mice [62, 90]. Other mechanisms by which core protein enhances HCC include the modulation of tumor suppressor genes and proto-oncogenes as well as inhibition of apoptosis, although there is some evidence that core protein actually initiates apoptosis [38, 91-101][71]. In this regard, it should be noted that ROS/RNS can have diverse effects on cell growth and apoptosis [102, 103]. Reactive species may also facilitate the development of HCC by progressively damaging the liver and inducing cirrhosis that increases the risk of HCC [104, 105]. Consequently, antioxidants have been proposed as an adjunct therapy for chronic hepatitis C [106].

### **III. Synergistic effects of alcohol and HCV in liver disease**

Chronic alcohol consumption is a well-known risk factor for liver disease [23, 107, 108]. Over 12,000 deaths per year in the U.S. are attributed to alcohol-related liver disease [109]. The relationship between alcohol and HCV infection is not well understood, although the prevalence of HCV infection in patients with a history of alcohol abuse is significantly higher than in the general population, leading to the identification of alcohol abuse as a risk factor for hepatitis C [110-113]. Specifically, almost one-third of alcoholics with clinical symptoms of liver disease have been infected with HCV, which is four times the rate of HCV infection found in alcoholics who do not

have liver disease [114-116]. HCV-induced pathogenesis is exacerbated by alcohol consumption, suggesting a synergy between the virus and ethanol [24, 111-113, 117-134]. For instance, pro-inflammatory NF- $\kappa$ B signaling is induced by both HCV core protein and ethanol via acetaldehyde [135], and the core protein increases the secretion of TGF $\beta$  in the presence of alcohol [65]. Alcohol intake is associated with increased progression of fibrosis, higher likelihood of cirrhosis, and an elevated risk for HCC in individuals with chronic HCV infection [136, 137]. Likewise, HCV infection exacerbates alcohol-related liver damage [120, 138-142].

One obvious mechanism of synergy between ethanol and HCV infection in chronic liver disease involves the modulation of the host immune response by ethanol and HCV. The functions of antigen-presenting dendritic cells and other key immune cells are disrupted by both ethanol and HCV proteins [143]. Geissler and colleagues noted that chronic alcohol feeding of mice inhibited T-helper cell and cytotoxic T-lymphocyte functions that play a pivotal role in clearance of HCV from the body [144]. In addition, alcohol consumption is known to significantly decrease the efficacy of interferon therapy in chronic hepatitis C patients [145]. Oxidative stress, alcohol, and HCV core protein have each been proposed to inhibit the cellular interferon response by interfering with the JAK-STAT signaling pathway [146-148]. This inhibition of the immune response would allow for viral persistence and exacerbated pathogenesis via oxidative stress.

Ethanol also increases the generation of ROS, decreases GSH content, causes lipid peroxidation, and leads to selenium deficiency through multiple mechanisms [65, 108, 149-161]. The production of ROS/RNS and highly toxic by-products during ethanol

metabolism has been recognized as a key mechanism by which ethanol is hepatotoxic [83, 149, 150, 153, 155, 162, 163]. Hepatocytes have three mechanisms of metabolizing ethanol (Figure 2). In the cytosol, ethanol is metabolized to acetaldehyde by alcohol dehydrogenase (ADH) which leads to the production of ROS [7]. The corresponding build up of NADH can also interfere with the electron transfer system in the mitochondria, facilitating ROS generation [164-166]. Additionally, NADH promotes the synthesis of fatty acids and inhibits their oxidation, enhancing the development of steatosis [167]. The microsomal ethanol oxidizing system, located on the endoplasmic reticulum, which primarily consists of ethanol-inducible cytochrome P450 2E1 (CYP2E1), converts ethanol to acetaldehyde and generates ROS [151, 166, 168, 169]. Ethanol may also be metabolized by catalase in peroxisomes [170]. Once ethanol is metabolized to acetaldehyde, aldehyde dehydrogenase quickly converts it to acetate [170]. Acetaldehyde can react with mitochondrial GSH causing a decrease in the antioxidant defense of the mitochondria to increase susceptibility to oxidative stress [7, 167].

Therefore, alcohol-induced oxidative stress is likely to synergize with HCV-induced alterations of the host redox status to enhance the pathogenesis of liver disease in hepatitis C patients who drink alcohol. Indeed, ethanol has been reported to exacerbate oxidative stress in HCV core-transgenic mice and chronic hepatitis C patients [62, 65, 171]. Increased CYP2E1 expression during hepatitis C infection may also increase the ROS levels and increase the susceptibility to alcohol toxicity [67, 151, 172-174], as ethanol has been shown to induce significantly higher ROS/RNS levels in CYP2E1-overexpressing hepatoma cells that also express HCV core protein when compared to

cells expressing either CYP2E1 or core gene alone [172]. It may be speculated that such combined oxidative/nitrosative stress would accelerate DNA mutations and facilitate transformation during chronic hepatitis C [38, 62, 70, 89-101]. Unfortunately, despite the well-established role of oxidative/nitrosative stress in the pathogenesis of human diseases, not many studies have directly demonstrated the pathological effects of oxidative/nitrosative stress in the HCV-infected alcoholic and non-alcoholic liver to this date, and much work will be necessary to precisely delineate the molecular interactions between HCV, alcohol, and the redox status in the pathogenesis of HCV infection.

Besides oxidative/nitrosative stress, acetaldehyde can damage cells and potentially enhance pathogenesis, for example, by rapidly forming adducts with cell constituents, including DNA (Figure 2) [7, 167, 175-177]. Chemical modification of DNA by metabolite(s) of ethanol will increase mutations and thereby increase the risk of HCC [7]. Acetaldehyde also enhances the transcription of collagen genes and interferes with DNA repair mechanisms [165, 167, 176, 178]. Moreover, ethanol metabolism may increase the expression of pro-carcinogenic genes, such as galectin-1 [7, 179]. Therefore, it may be hypothesized that alcohol synergizes with HCV through combined oxidative/nitrosative stress as well as acetaldehyde formation, both of which can be pathogenic to liver.

#### **IV. Alcohol, reactive species, and HCV replication**

Alternatively, alcohol and reactive species may affect the virus itself and exert its pathogenic effects indirectly (Figure 3). In fact, hepatitis C patients who drink alcohol typically show a pattern of hepatic injury that is more characteristic of chronic viral

hepatitis than alcohol-induced injury [180, 181]. These findings suggest that alcohol enhances the pathogenic effects of HCV rather than exerting its independent effects on the liver. Interactions between alcohol, reactive species, and HCV itself involve at least three separate mechanisms: modulation of viral replication, modulation of the host antiviral response that in turn affects the viral titer, and modification of the viral genome (Figure 3).

### ***Alcohol, reactive species, and HCV replication***

Several clinical studies have correlated increased serum HCV titer with the amount of alcohol consumed [141, 182-186]. HCV titer is significantly greater in patients consuming greater than 10g of alcohol per day [187]. Habitual drinkers also showed higher levels of HCV RNA than non-habitual drinkers [182]. Abstinence or moderation of alcohol consumption could result in a substantial drop in the HCV RNA levels in some patients [141, 186, 187]. Whether heavy, moderate, and light alcohol intake leads to consistent elevations in serum as well as intrahepatic HCV RNA is still unclear [188-191]. Nevertheless, alcohol can increase intrahepatic HCV RNA titer in patients [185, 192] and subgenomic HCV RNA replication *in vitro*, in Huh7 human hepatoma cells, suggesting that alcohol may affect the viral life cycle itself [193]. Possible mechanisms include activation of the endogenous opioid system and nuclear factor kappa B (NF- $\kappa$ B) [193]. Products of ethanol metabolism, most likely downstream of aldehyde dehydrogenase, were implicated [193]. Notice that these subgenomic replicon cells support continuous HCV RNA genome replication without producing virus

particles. Whether alcohol is able to increase the production of infectious virus particles and/or its infectivity has not yet been determined.

Another possible mediator in the modulation of HCV replication by alcohol would be reactive species, generated during hepatic metabolism of alcohol (Figure 2). Indeed, the common location of HCV replication and CYP2E1 on the endoplasmic reticulum increases the likelihood of interaction between ROS and HCV during alcohol metabolism. Previously, high concentrations of iron (50 and 100  $\mu\text{M}$ ) were reported to enhance HCV replication [194]. Increased mitochondrial generation of ROS with HCV, secondary to the ER stress, has also been suggested to promote HCV replication through Stat-3 and antioxidant/antioxidant genes, to have the opposite effects [195-197]. These studies have since been cited as evidence that oxidative stress favors HCV replication, assuming that iron enhances HCV replication through oxidative stress.

However, a recent study that quantitatively monitored the enzyme kinetics of HCV NS5B, the RNA-dependent RNA polymerase, extensively demonstrated that  $\text{Fe}^{2+}$  and  $\text{Fe}^{3+}$  can directly bind to NS5B and inhibit its replicase activity [198]. The results are consistent with previous reports that, too, have demonstrated suppression of HCV replication by other divalent cations such as zinc [198-201]. In addition, Stat-3 participates in the anti-HCV activity of interferon [197, 202, 203], and the HCV-induced oxidative stress has recently been suggested to inhibit HCV RNA replication instead, by activating the gene expression of COX-2 and increasing the production of prostaglandin E2 [73].

Using subgenomic and genomic replicons that continuously replicate in Huh7 human hepatoma cells, we also found that  $\text{H}_2\text{O}_2$ , at concentrations that did not deplete

intracellular GSH or induce cell death, rapidly suppressed HCV RNA replication in a dose-dependent manner [204, 205]. The suppressive effects of peroxide was comparable to those of interferon gamma and cyclosporine A, which are potent inhibitors of HCV replication [204, 206-209] (unpublished observation). The subgenomic replicon of Con1 sequence (genotype 1b) and a genomic hybrid derived from Con1/H77c sequences (genotype 1a/1b) were likewise affected, and the suppression could be partially reversed with *N*-acetylcysteine and completely blocked by buffering intracellular calcium, suggesting a signaling event [204]. Furthermore, the mechanism most likely involved a disruption of the HCV replication complex on cell membranes, and other agents that elevated cytosolic calcium concentration had similar suppressive effects on HCV replication, as reported by others [208]. Our findings were recently corroborated by Yano *et al.* who showed that vitamins A and E elevated HCV RNA content in a different HCV replicon model of genotype 1b [210]. These results suggest that the activation of Nox2 protein during inflammation, such as during chronic hepatitis C, functions to help decrease HCV replication. This conclusion is in line with clinical observations that individuals who have chronic granulomatous disease and do not have functional Nox2, tend to suffer from chronic infection [211]. In addition, H<sub>2</sub>O<sub>2</sub>, in contrast to its pro-viral effects on human immunodeficiency virus (HIV), has been shown to negatively regulate hepatitis B virus replication without affecting cell metabolism [212, 213]. These findings are consistent with the fact that reactive species can act as second messengers that participate in and modify signaling [87] and suggest that, during inflammation, reactive species do not have to reach high enough concentrations to directly damage viruses and virus-infected cells if they can induce antiviral redox signaling. Furthermore,



it may be concluded that whether reactive species up-regulate or down-regulate the replication of a particular virus would depend on the mechanics of viral replication and their interplay with the components of redox signaling.

Therefore, oxidative stress may be more likely to suppress than promote HCV RNA replication. Although it is difficult to reconcile the differences in these studies, part of the differences could have resulted from the choice of antioxidants/antioxidant genes that were used in the earlier studies. For example, antioxidants/antioxidant genes can display pro-oxidant as well as antioxidant functions [214-218], and such dual and, at times, conflicting findings are unfortunately not uncommon in the study of antioxidants. To clearly define the relationship between oxidative stress and HCV replication, one will need to take into consideration not only the kinetics, dosage-effects, and the mechanism of action of chemical/biological agents being studied but also, the cellular adaptive response to oxidative stress and its kinetics. In this regard, the oxidative suppression of HCV replication complex we found is highly consistent, occurring in response to H<sub>2</sub>O<sub>2</sub>, tertiary butyl hydroperoxide, extracellular generation of H<sub>2</sub>O<sub>2</sub> with glucose oxidase plus glucose, *tert*-butylhydroquinone, decreasing intracellular GSH content with BSO, as well as HNE [204, 205] (unpublished observation). The suppression starts at concentrations as low as 0.1 μM H<sub>2</sub>O<sub>2</sub> and starts within 15 – 30 min of exposure, progressively worsening by 6 hrs, and resulting in significant decreases in both the rate of HCV replication and the total viral RNA content over time. Therefore, oxidative stress is clearly capable of suppressing HCV, at least at the level of genome replication, through calcium elevation in cell culture.

### ***Antioxidants, alcohol, reactive species, and the host immune response***

The question of whether oxidants suppress HCV replication complex naturally raises another related question, which is whether antioxidants would then favor viral replication and have adverse effects on patients. Unfortunately, whether oxidants increase or inhibit the activity of the HCV replication complex *in vivo*, in patients, and whether antioxidants modulate this process has remained unclear. In one study, serum viral load was identified as a negative predictor of plasma antioxidant levels [219]. On the other hand, serum HCV RNA was positively correlated with erythrocyte malondialdehyde (MDA) but not with plasma MDA levels in another study [220]. Intrahepatic oxidative stress and HCV RNA levels were not measured. These studies demonstrate inherent difficulties in utilizing simple correlations to define the effect of oxidative stress on HCV replication, as HCV titer itself will affect the host redox status [62, 63, 70, 77, 79, 80] (Figure 3). A recent study, which examined the effects of an antioxidant cocktail, saw a reduction in the HCV RNA titer in 25% of patients. However, the study did not include a control group, and it is impossible to distinguish the effects of antioxidants from random fluctuations in the viral titer and other miscellaneous effects [106, 221].

Therefore, it remains to be clearly determined how oxidative stress and antioxidants affect HCV titer in patients and whether alcohol modulates HCV titer through oxidative stress. Nevertheless, antioxidants and removal of iron have now been shown in several studies to improve the clinical symptoms of hepatitis C and/or the outcome of anti-HCV therapy, albeit not without some controversies; importantly, however, no adverse effects have been reported [106, 222-226]. These results are

consistent with the well-known effects of oxidative stress on liver disease. If antioxidants do in fact help lower the viral titer *in vivo*, in hepatitis C patients, it is possible that this would be explained by the modulation of host immune response by reactive species and alcohol (Figure 3). As mentioned earlier, alcohol and reactive species can inhibit the antiviral activity of interferon alpha [7, 37, 40, 146, 148]. In one study, heavy drinkers who did not abstain from drinking before interferon treatment showed a total lack of HCV RNA clearance, whereas those who normally drank heavily but abstained from drinking before the interferon treatment showed some improvement in the HCV RNA clearance, and the virus completely disappeared in about 16 percent of heavy drinkers who abstained before treatment [145]. In this scenario, antioxidants, regardless of their independent effects on HCV genome replication, would help clear HCV by improving the host immune response. Antioxidants have also been proposed to reduce hemolytic anemia, a major side effect associated with oxidative membrane damage during ribavirin therapy, and thereby improve the tolerability of ribavirin therapy [227, 228]. Therefore, whether antioxidants/iron reduction therapy is beneficial to patients would depend on multiple variables, including effects on the HCV RNA replication, other steps of the viral replication cycle, oxidative stress-induced pathogenesis, and response to therapy. Again, whether antioxidants have beneficial or adverse effects on the HCV titer, in the absence of concurrent antiviral therapy, such as in the immune suppressed individuals, remains unclear. Choice of antioxidants is likely to be important as some antioxidants can have pro-oxidant effects, particularly in the presence of an iron overload [229].

### ***Modification of viral genome***

Another potential variable in the complex interactions between alcohol and HCV titer involves the mutagenic potential of alcohol and reactive species. RNA viruses, such as HCV, exist as a population of closely related, but distinct genetic variants, referred to as “quasispecies” [230]. High rates of mutation allow these viruses to maximize adaptability, while conserving essential genetic information [231]. The high mutation rates are generally attributed to the lack of proofreading activity of viral replicases as well as genetic recombination, and these have been suggested to provide the viruses with a greater repertoire of genetic sequences to escape the host immune surveillance and to facilitate the development of antiviral resistance [232].

Alcohol intake is associated with increased heterogeneity of HCV RNA in patients [233, 234]. Some of these changes to the viral genetic sequences are likely to stem from the many effects that alcohol has on the host immune system. It is also possible; however, that alcohol or reactive species induce chemical modifications of the viral RNA which would lead to an increased mutation rate of the HCV genome. In fact, although chemical modification of nucleic acids is mostly studied in the context of DNA damage in chemically-induced carcinogenesis, these agents can also modify RNA [235], which would be important to RNA viruses that rely on RNA genomes. Here, alcohol and ROS/RNS-induced chemical modifications of the viral RNA would amplify the error rates of the already error-prone viral replicase and enhance the antigenic drift, promoting the generation of quasispecies, immune escape, and the development of antiviral drug resistance by HCV. Thus, alcohol may also affect the HCV titer indirectly, by driving viral evolution (Figure 3).

## **V. Alcohol, Oxidative Stress, and HCV Infection – Conclusions:**

Therefore, HCV is likely to interact with alcohol, reactive species, and antioxidants through multiple mechanisms. As oxidative stress is a key factor in HCV and alcohol-induced liver disease, alcohol is likely to synergize with HCV-induced oxidative/nitrosative stress to enhance the pathogenesis of liver disease. How alcohol, reactive species, and antioxidants ultimately affect the production of infectious HCV particles as well as the infectivity, persistence, and transmissibility of HCV *in vivo* remains to be fully characterized. The synergistic hepatopathogenesis and high prevalence of alcohol abuse and HCV infection signify the importance of research into the mechanisms behind their combined effects.

It should be noted that HCV produces hepatic as well as extrahepatic complications [236]. Many hepatitis C patients are also co-infected with HIV and other viruses, showing accelerated disease progression. Furthermore, whether all HCV sequences produce oxidative/nitrosative stress in a consistent and predictable manner remains to be determined [25, 26, 237]. Much study, using both *in vitro* and *in vivo* models of HCV, therefore, will be necessary to fully understand the mechanism and the role of redox regulation of hepatitis C in alcoholic as well as nonalcoholic liver, and how it ultimately affects patient care.

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## **List of Abbreviations**

ADH, alcohol dehydrogenase; BSO, *L*-buthionine *S,R*-sulfoximine; COX-2, cyclooxygenase-2; CYP2E1, cytochrome P450 2E1; ER, endoplasmic reticulum; GO, glucose oxidase; GSH, glutathione; HCV, hepatitis C virus; HIV, human immunodeficiency virus; HNE, 4-hydroxynonenal; HO-1, hemeoxygenase-1; iNOS, inducible nitric oxide synthase; IRES, internal ribosomal entry site; MDA, malondialdehyde; MnSOD, manganese superoxide dismutase; NAC, *N*-acetylcysteine; NF- $\kappa$ B, nuclear factor kappa B; RNS, reactive nitrogen species; ROS, reactive oxygen species; TGF $\beta$ , transforming growth factor beta; UTR, untranslated region

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## **Figure Legends**

**Figure 1. Role of HCV proteins in oxidative/nitrosative stress.** Genome organization of HCV showing the role of individual HCV proteins in the induction of oxidative/nitrosative stress. Superscript numbers correspond to references.

**Figure 2. HCV and alcohol metabolism in chronic hepatitis C**

\* Refer to Figure 1 and the accompanying text for the mechanism of increased oxidative/nitrosative stress during HCV infection.

**Figure 3. Proposed mechanisms of changes to HCV replication/viral titer by alcohol and reactive species.**



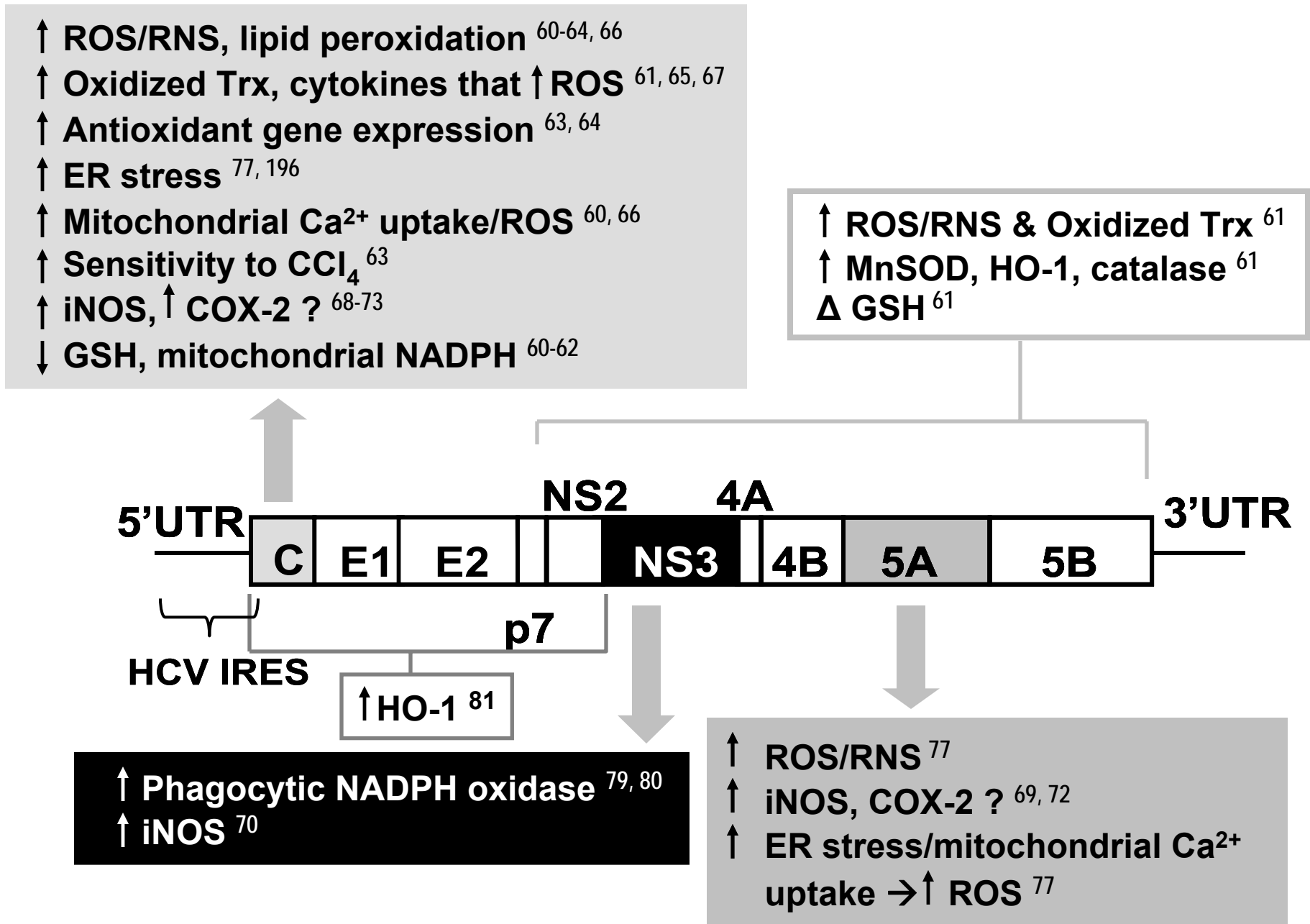


Figure 1

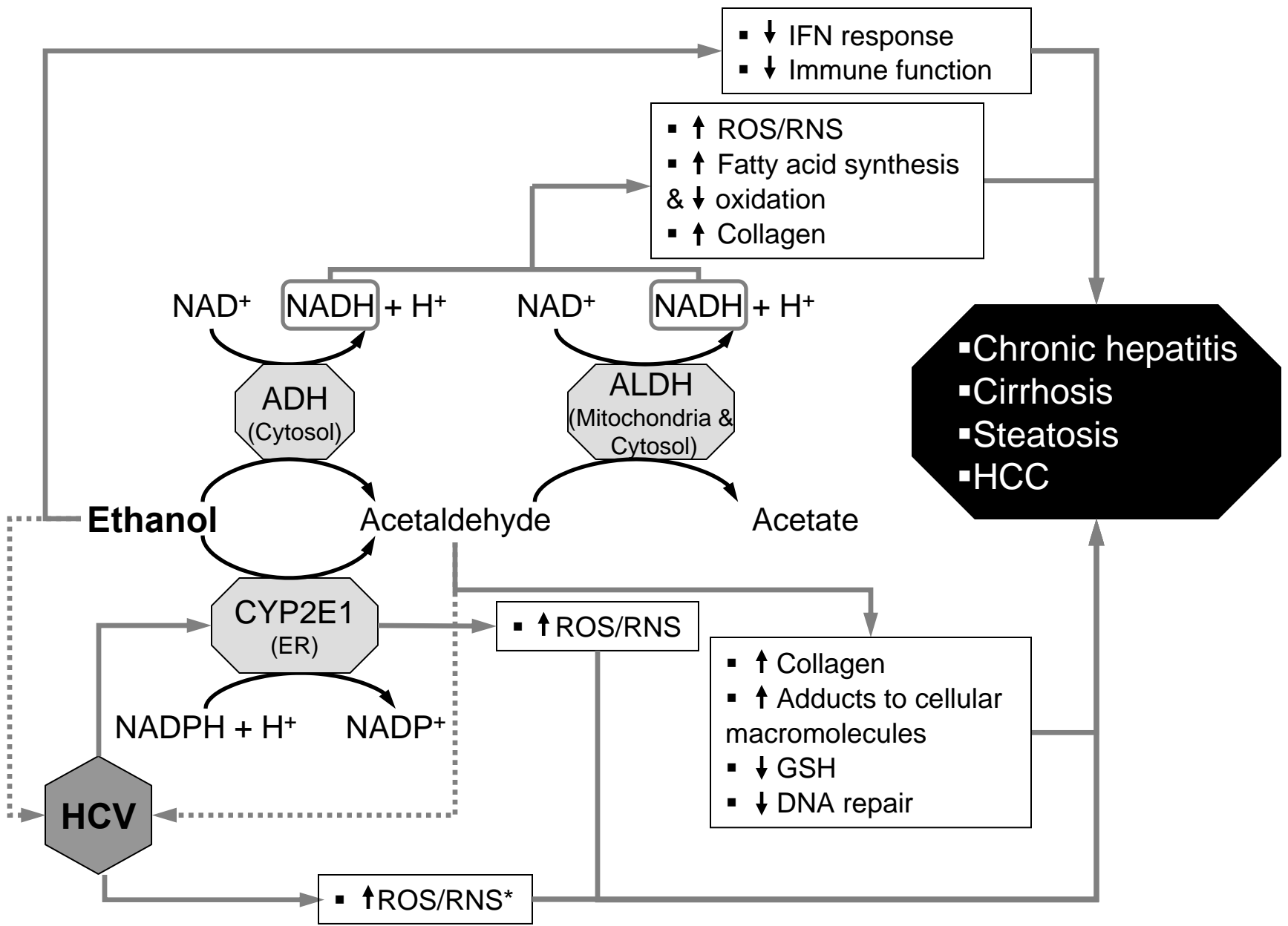


Figure 2

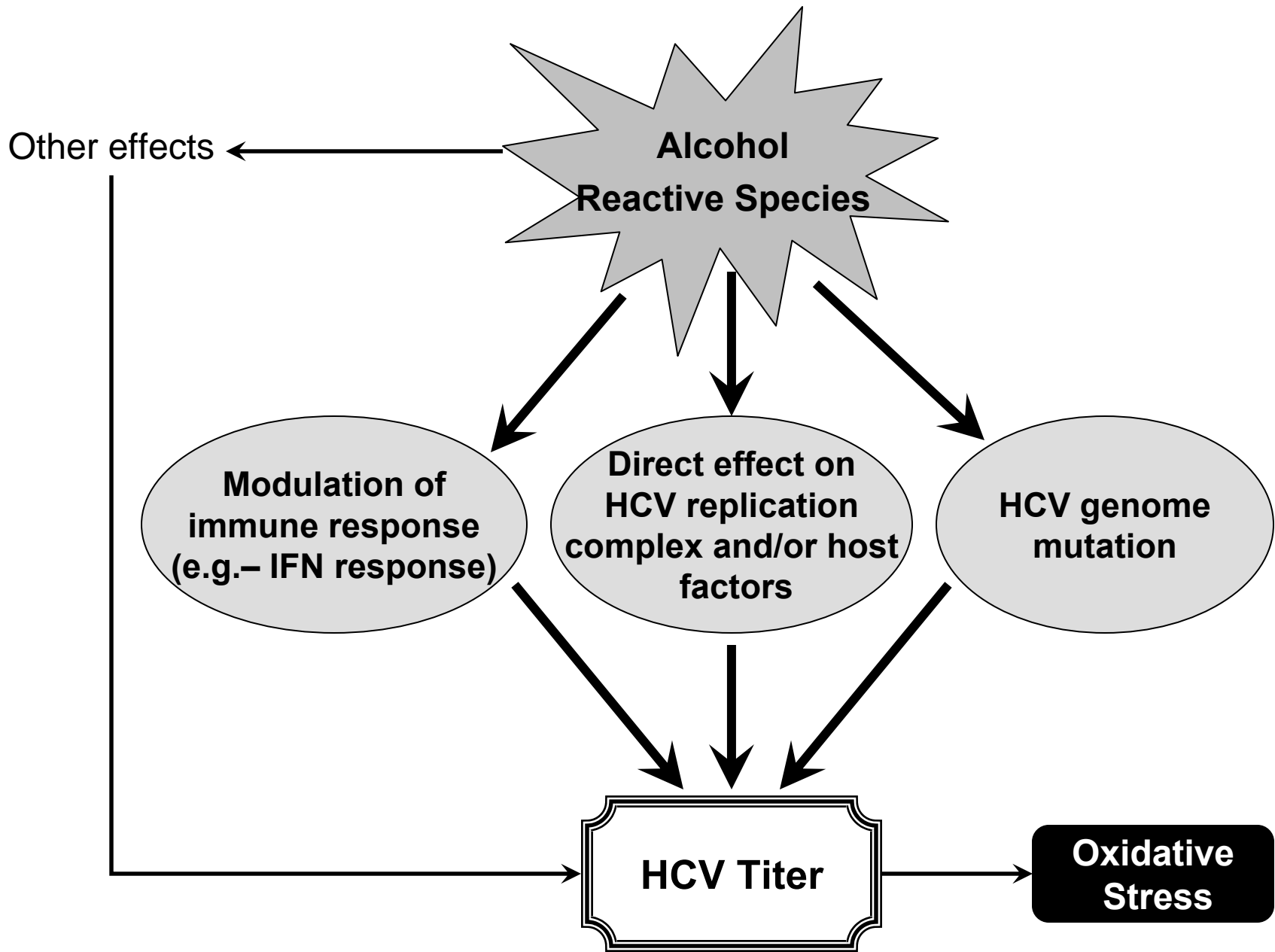


Figure 3