

## 'Liver let die'

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1 "Liver let die: oxidative DNA damage and hepatotropic viruses"

2

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22 SUMMARY

23 Chronic infections by the hepatotropic viruses hepatitis B virus (HBV) and  
24 hepatitis C virus (HCV) are major risk factors for the development of  
25 hepatocellular carcinoma (HCC). It is estimated that more than 700,000  
26 individuals per year die from hepatocellular carcinoma, and around 80% of  
27 HCC is attributable to HBV or HCV infection. Despite the clear clinical  
28 importance of virus-associated HCC, the underlying molecular mechanisms  
29 remain largely elusive.

30

31 Oxidative stress, in particular DNA lesions associated with oxidative damage,  
32 play a major contributory role in carcinogenesis, and are strongly linked to the  
33 development of many cancers, including HCC. A large body of evidence  
34 demonstrates that both HBV and HCV induce hepatic oxidative stress, with  
35 increased oxidative DNA damage being observed both in infected individuals  
36 and in murine models of infection.

37

38 Here, we review the impact of HBV and HCV on the incidence and repair of  
39 oxidative DNA damage. We begin by giving a brief overview of oxidative  
40 stress and the repair of DNA lesions induced by oxidative stress. We then  
41 review in detail the evidence surrounding the mechanisms by which both  
42 viruses stimulate oxidative stress, before focusing on how the viral proteins  
43 themselves may perturb the cellular response to oxidative DNA damage,  
44 impacting upon genome stability and thus hepatocarcinogenesis.

45

46

47

48 INTRODUCTION

49 Hepatocellular carcinoma (HCC) is an increasing global health  
50 problem, accounting for more than 90% of primary liver tumours. Worldwide,  
51 HCC is the third cause of cancer-related death, responsible for 700,000  
52 deaths per year (Ferlay *et al.*, 2010), and the sixth most common cancer.  
53 Chronic infections with hepatitis B virus (HBV) and hepatitis C virus (HCV) are  
54 major risk factors for the onset and progression of HCC (El-Serag, 2002;  
55 Simonetti *et al.*, 1992). Globally, up to 80% of hepatocellular carcinoma is  
56 attributable to HBV or HCV infection (Perz *et al.*, 2006). The underlying  
57 mechanisms remain unclear, although an increasing body of evidence  
58 suggests that the viral proteins themselves may contribute directly to  
59 tumourigenesis.

60 Timely and precise repair of DNA lesions is crucial for the maintenance  
61 of genome stability. Since genomic instability is a characteristic of all tumours,  
62 DNA damage recognition and repair processes represent an important barrier  
63 to the initiation and progression of tumourigenesis. Cells in multicellular  
64 organisms are continuously exposed to DNA damage arising from a variety of  
65 endogenous and exogenous sources. These include reactive oxygen species,  
66 ultraviolet light, background radiation, and environmental mutagens. In  
67 particular, oxidative stress and elevated reactive oxygen species (ROS) levels  
68 are linked with number of human diseases. ROS that accumulate as a result  
69 of oxidative stress can directly react with DNA molecules to produce a variety  
70 of oxidative DNA lesions, whose repair by components of the base excision  
71 repair (BER) and nucleotide excision repair (NER) pathways is crucial in  
72 maintaining genome stability.

73           Oxidative DNA damage may play a pivotal role in  
74 hepatocarcinogenesis associated with chronic viral infection. A growing  
75 number of publications have concluded that chronic HBV and HCV infection  
76 correlates with an increased incidence of oxidative DNA damage (Bolukbas *et*  
77 *al.*, 2005; Demirdag *et al.*, 2003; Farinati *et al.*, 2007a; Fujita *et al.*, 2008;  
78 Machida *et al.*, 2006; Nishina *et al.*, 2008). Whilst it is clear that ROS are  
79 continuously generated during chronic inflammation over the course of  
80 chronic viral infection, increasing evidence suggests that the viral proteins of  
81 HBV and HCV may themselves contribute to a state of chronic oxidative  
82 stress in infected hepatocytes.

83           In this review, we will summarise the current knowledge surrounding  
84 oxidative stress and DNA damage during hepatotropic viral infection. We will  
85 compare and contrast the mechanisms by which the proteins encoded by  
86 HBV and HCV induce oxidative stress, before providing an overview of the  
87 impact of these viral proteins on oxidative DNA damage and repair, and briefly  
88 examining the ultimate consequences on the development of HCC.

89

## 90 OXIDATIVE STRESS

91           Reactive oxygen species may arise by exposure to exogenous agents  
92 such as ionizing radiation or drugs, or may be generated from endogenous  
93 sources such as metabolism, apoptosis or inflammation. Oxidative stress  
94 represents a shifting of the balance between oxidants (such as ROS) and the  
95 cellular antioxidant response, leading to potential cellular damage and  
96 contributing to disease. Cells are able to withstand a relatively low level of  
97 oxidative damage. However, sustained oxidative stress, arising through the

98 increased presence of radicals and ROS, or by a lack of antioxidant capacity  
99 within the cell, will engender increased damage to lipids, proteins and DNA. A  
100 detailed description of the repair of oxidative DNA damage is covered in a  
101 subsequent section. ROS are primarily generated in the mitochondria as by-  
102 products of cellular metabolism, through electron leakage from the  
103 mitochondrial electron transport chain. However, they also play a key role as  
104 second messengers in cellular signalling. ROS-induced damage may  
105 influence pathway signalling, gene expression, cell cycle, metabolism, and  
106 apoptosis. Oxidative stress may also activate oncogenic signalling pathways,  
107 ultimately contributing to cellular transformation (Hussain *et al.*, 2003).

108

#### 109 *Viral infection and oxidative stress: non-specific inflammation*

110 Although this review focuses on how the HBV and HCV viral proteins  
111 induce oxidative stress, it is clear that chronic infection by either virus triggers  
112 a non-specific immune-mediated inflammation (hepatitis), which is innately  
113 linked to oxidative stress. During acute liver injury and hepatic inflammation,  
114 ROS are generated by both neutrophils and Kupffer cells (reviewed by  
115 (Jaeschke, 2011)), as the principal toxic mediators to induce cell death. Since  
116 these cells exist in close proximity to hepatocytes, some ROS (i.e. H<sub>2</sub>O<sub>2</sub>) are  
117 able to diffuse into hepatocytes, although the plasma membrane represents a  
118 barrier to the free diffusion of O<sub>2</sub><sup>-</sup>. Membrane superoxide dismutases are able  
119 to convert these O<sub>2</sub><sup>-</sup> anions to H<sub>2</sub>O<sub>2</sub> in the extracellular space, which are then  
120 able to cross membranes and elicit intracellular signalling (Fisher, 2009).  
121 Thus, these ROS will enhance and amplify the intracellular effects  
122 engendered by the viral proteins themselves, and will also affect neighbouring

123 (uninfected) hepatocytes (Jaeschke, 2011), although they may also serve to  
124 activate intracellular antioxidant defences. Moreover, mitochondrial reactive  
125 oxygen species are able to drive proinflammatory cytokine production (Naik &  
126 Dixit, 2011), further exacerbating both inflammation and the production of  
127 ROS.

128

## 129 OXIDATIVE DNA DAMAGE AND REPAIR

130 ROS may directly interact with DNA to induce oxidative DNA damage.  
131 Oxidative DNA lesions may comprise abasic sites, deaminated or adducted  
132 bases, and single stranded DNA breaks (SSBs). These lesions include  
133 thymine and thymidine glycol, 5-hydroxymethyluracil, and 8-hydroxy-  
134 deoxyguanosine (8-OHdG). The latter represents the most widely studied of  
135 oxidative DNA lesions, and is used as a robust marker of oxidative DNA  
136 damage. The accumulation of oxidative DNA lesions is considered mutagenic,  
137 and a significant contributory factor to human disease (reviewed by  
138 (Sedelnikova *et al.*, 2010)).

139 The majority of oxidative DNA lesions are repaired by components of  
140 the base excision repair pathway (BER), and to a lesser extent, by nucleotide  
141 excision repair (NER). The rate of transient oxidative DNA damage is typically  
142 balanced by its rate of repair, but chronic oxidative stress may result in  
143 permanent genetic damage. Since there are multiple, overlapping pathways  
144 for the repair of oxidative DNA damage, perturbation of one component of  
145 these pathways is expected to slow but not abolish repair.

146 Base excision repair (Figure 1) is initiated by a DNA glycosylase that  
147 recognizes and removes the damaged base, generating either a SSB or an  
148 abasic site which requires further processing by an AP endonuclease (APE1).  
149 The resultant SSB may be repaired by two alternate pathways: short-patch  
150 BER (Figure 1, bottom right), involving Pol  $\beta$  and the DNA ligase III/XRCC1  
151 complex, which inserts a single nucleotide; or long-patch BER (Figure 1,  
152 bottom left), which replaces 2-12 nucleotides, and is dependent upon PCNA  
153 and Flap Endonuclease 1 (Fen1), and may involve several polymerases (Pol  
154  $\beta$ ,  $\delta$  and  $\epsilon$ ). The factors governing the choice of long- or short-patch BER  
155 remain unclear.

156 The nucleotide excision repair (NER) pathway (Figure 2), which is  
157 generally associated with repair of bulky, helix-distorting adducts, is also able  
158 to repair oxidative DNA lesions, albeit to a lesser extent. Detection of bulky  
159 lesions, whether in actively transcribed regions (right, panel) or not (left panel)  
160 (see Figure 2 for details), leads to recruitment of the TFIIH complex to sites of  
161 damage. Members of the TFIIH complex facilitates helix unwinding and  
162 excision of the strand containing the adduct, and repair is completed by  
163 several proteins which complete gap-filling/ligation. NER is capable of  
164 removing all of the oxidative lesions induced by ROS (Kuraoka *et al.*, 2000;  
165 Reardon *et al.*, 1997), as NER initiating factors (CSA, CSB, XPC and XPE)  
166 rapidly bind to oxidative lesions (Menoni *et al.*, 2012). However, the overall  
167 contribution of NER to the repair of oxidative lesions remains to be  
168 determined, and it is likely that NER acts as a backup pathway to BER.  
169 Interestingly, oxidative stress generated by activated neutrophils, and  
170 potentially by kupffer cells, results in a reduction in nucleotide excision repair



171 (NER) capacity, suggesting that inflammation may non-specifically reduce  
172 NER efficiency (Gungor *et al.*, 2007).

173 In some circumstances oxidative DNA damage may result in double  
174 strand break (DSB) formation, especially if lesions are clustered together on  
175 opposite strands. Although DSBs will arise at a lower frequency to SSBs, their  
176 rapid repair is crucial to maintaining genetic stability. Repair of DSBs involves  
177 either recombination using homologous sequences from undamaged sister  
178 chromatids (homologous recombination; HR), or non-homologous end-joining  
179 (NHEJ), which can result in small deletions. For a comprehensive view on  
180 DSB repair, the reader is directed to an excellent recent review by (Chapman  
181 *et al.*, 2012).

182 In addition, a growing body of literature suggests that cellular tumour  
183 suppressors, including those involved in repair of other types of DNA lesions,  
184 may play a vital role in regulating oxidative stress. p53 is activated in  
185 response to oxidative stress, and is thought to play an antioxidant role in such  
186 circumstances. Regulation of both pro- and anti-oxidant genes by p53 is  
187 thought to be crucial in regulating the cellular response and cell fate to acute  
188 oxidative stress (reviewed in (Vurusaner *et al.*, 2012)). Moreover, the breast  
189 cancer susceptibility genes BRCA1 and BRCA2, involved in DNA repair and  
190 cell cycle checkpoint progression, also play caretaker roles against oxidative  
191 stress. In particular, in response to oxidative stress, BRCA1 upregulates the  
192 expression of numerous antioxidant genes (e.g. glutathione-S-transferase,  
193 alcohol dehydrogenases) (Bae *et al.*, 2004). BRCA1 over-expression protects  
194 against oxidising agents, maintaining the cellular redox balance in the face of  
195 oxidative stress. Similarly, BRCA1 may enhance the activity of the antioxidant

196 transcription factor Nrf2 (Ishikawa *et al.*, 2005). In addition to its well-  
197 characterised role in the repair of DSBs by promoting HR, BRCA2 is also  
198 critical for the repair of oxidative lesions. Specifically, BRCA2 (as well as  
199 BRCA1) is required for the transcription-coupled repair of 8-OHdG lesions,  
200 crucial for the prevention of transcription stalling and subsequent mutagenesis  
201 (Bae *et al.*, 2004; Le Page *et al.*, 2000).

202 Thus, members of multiple, overlapping and functionally diverse  
203 pathways (namely BER, NER and DSB repair) are required for the effective  
204 repair of oxidative DNA lesions resulting from oxidative stress.

205

## 206 HBV, OXIDATIVE STRESS AND DNA DAMAGE

### 207 *HBV infection and oxidative stress*

208 HBV is one of several closely-related DNA viruses within the family  
209 *Hepadnaviridae*. The viral genome encodes a small number of gene  
210 products: a reverse transcriptase and DNA polymerase (pol); a capsid protein  
211 (core); envelope proteins L, M and S; and a multifunctional protein (X)  
212 involved in replication, oncogenesis, and a myriad of other cellular metabolic  
213 dysfunctions. Replication of HBV is complex and proceeds via the reverse  
214 transcription of genome-length RNA; thus, HBV is classified as a dsDNA  
215 retrovirus (group VII).

216 Chronic HBV infection is a major etiological factor for HCC: the risk of  
217 HCC development in chronic HBV carriers is more than 100-fold greater than  
218 in uninfected individuals (Ito *et al.*, 2010). The vast majority of new cases of  
219 HBV-associated HCC occur in developing countries, especially sub-Saharan

220 Africa and Southeast Asia. Although HCC generally occurs in cirrhotic livers,  
221 HBV is also able to transform hepatocytes in the absence of chronic  
222 inflammation and cirrhosis (Brechot, 2004). During chronic infection,  
223 fragments of HBV DNA may integrate into the host genome, preferentially into  
224 chromosome 17, creating mutations. These integrated fragments often  
225 encode the X protein of HBV (HBx) or truncated preS proteins, the integration  
226 of which often correlates with hepatocarcinogenesis (Ding *et al.*, 2012) .

227         Several groups have shown that HBV infection is associated with  
228 oxidative stress in chronically-infected individuals (Bolukbas *et al.*, 2005;  
229 Demirdag *et al.*, 2003). Both lipid peroxidation and oxidative DNA damage,  
230 markers of oxidative stress, are elevated in patients infected with HBV.  
231 Indeed, patients with chronic HBV infection exhibit increased 8-OHdG  
232 accumulation (Fujita *et al.*, 2008). Furthermore, *in vitro* HBV replication in  
233 hepatoma cell lines (specifically HepAD38 cells) induces oxidative stress  
234 (Severi *et al.*, 2006).

### 235 A direct role for HBV-encoded proteins in oxidative stress

236         In addition to non-specific oxidative stress generated by local  
237 inflammation in response to viral infection, increasing evidence suggests that  
238 the HBV proteins directly regulate cellular ROS production (Figure 3 upper  
239 panel), and deleteriously alter intracellular antioxidant defences in HBV-  
240 infected cells, causing apoptosis and extensive liver damage and thus  
241 engendering accelerated hepatocellular renewal. However, the consequences  
242 of these interactions and their impact are yet to be fully understood.

243 Over a decade ago, HBx was shown to localise to mitochondria,  
244 decreasing mitochondrial membrane potential, and increasing both  
245 cytochrome c release and apoptosis (Takada *et al.*, 1999). Moreover,  
246 transgenic mice expressing the HBV proteins (including HBx) also display  
247 elevated hepatic oxidative stress levels compared to nontransgenic controls,  
248 with a concurrent increase in oxidative DNA damage (Hagen *et al.*, 1994).  
249 These findings were extended to demonstrate that ROS scavengers were  
250 able to inhibit HBx-mediated mitochondrial membrane depolarisation and  
251 subsequent apoptosis (Shirakata & Koike, 2003). In agreement, Lee and  
252 colleagues demonstrated that HBx alters mitochondrial membrane potential,  
253 perturbs mitochondrial electron transport, affects hepatocyte metabolism and  
254 increases cellular ROS production (Lee *et al.*, 2004). HBx has accordingly  
255 been reported to sensitise cells to apoptosis induced by oxidative stress,  
256 mainly through loss of the anti-apoptotic protein Mcl-1 (Hu *et al.*, 2011). In  
257 general agreement, HBx was shown to induce apoptosis through regulation of  
258 Bcl-XL and Bax (Kim *et al.*, 2008; Miao *et al.*, 2006). It is therefore clear that  
259 expression of HBx stimulates intracellular ROS production and impacts upon  
260 apoptosis.

261 The HBV surface antigen (HBsAg) and associated PreS region have  
262 also been associated with oxidative stress. Expression of truncated mutants  
263 of the HBV PreS/S polypeptide in hepatoma (Huh7) cells induced production  
264 of ROS via ER-stress pathways, resulting in oxidative DNA damage (Hsieh *et al.*  
265 *et al.*, 2004; Wang *et al.*, 2005), although these results might be strongly  
266 influenced by non-specific ER stress triggered by high levels of HBV protein  
267 expression. Hsieh *et al.* (2004) also showed that transgenic mice expressing

268 these mutants exhibited elevated oxidative DNA damage and up-regulated  
269 expression of Ogg1, the DNA glycosylase mainly responsible for the repair of  
270 8-OHdG lesions. Such oxidative damage may play an important role in  
271 hepatocarcinogenesis associated with HBV infections, illustrated by elevated  
272 nodular proliferation and increased tumour development in mice expressing  
273 PreS mutants (Wang *et al.*, 2005). In contradiction, examination of a small  
274 cohort of patients (n=38) failed to reveal a link between PreS mutation and  
275 increased oxidative stress in HBV-infected patients, although HBV infection  
276 was again linked to elevated levels of 8-OHdG and Ogg1 expression (Gwak *et al.*, 2008).  
277

278         Given these data, one would expect that intracellular antioxidant  
279 defences would be activated in the presence of the HBV proteins.  
280 Accordingly, HBV upregulates the expression of cytoprotective genes  
281 containing antioxidant response elements (AREs), both *in vitro* and in HBV-  
282 infected liver tissues (Schaedler *et al.*, 2010). However, Schaedler and co-  
283 workers demonstrated that this upregulation was independent of ROS, and, in  
284 contrast to other studies, suggested that this upregulation might confer  
285 survival benefits upon HBV-infected cells, allowing them to survive the  
286 sustained oxidative stress found in the infected liver. The discrepancy of  
287 Schaedler's findings with other published data (described above) emphasises  
288 the difficulty of studying changes in the redox balance occurring during a  
289 natural HBV infection, and questions the relevance of using HBV models  
290 usually devoid of an immune response.

291           These studies demonstrate that HBV infection induces extensive  
292 oxidative stress and activates intracellular oxidative repair pathways, and  
293 suggest that HBx expression (and perhaps that of PreS) is, in part,  
294 responsible. However, certain aspects require further clarification, and may be  
295 addressed by controlling the level of HBV protein expression to more closely  
296 resemble that observed in infected individuals and by validating the results in  
297 the context of the whole HBV genome. Although antioxidant treatments seem  
298 a promising avenue of therapeutic research for HBV infection, their efficacy is  
299 as yet unknown.

#### 300 HBV and oxidative DNA damage

301           As detailed above, HBV infection, HBx and PreS induce oxidative  
302 stress, which culminates in increased hepatic oxidative DNA damage, with  
303 increased levels of 8-OHdG found in HBV-infected patients, in transgenic  
304 mice expressing either pre-S mutants or HBx, and in hepatoma cells  
305 expressing HBx (Fujita *et al.*, 2008; Gwak *et al.*, 2008; Hagen *et al.*, 1994). A  
306 number of studies have suggested that the HBV-encoded proteins, in addition  
307 to their role in inducing oxidative stress, may also inhibit cellular DNA repair  
308 pathways. Despite the conflictual nature of some of these reports, we attempt  
309 to summarise the current state-of-knowledge below.

#### 310 *HBV and the repair of oxidative DNA lesions*

311           A growing body of literature suggests that the HBV proteins may alter  
312 the repair of DNA lesions, including oxidative DNA damage (Figure 4). There  
313 are, however, conflicting accounts of the effect of HBV on the base excision

314 repair pathway. Gwak and colleagues (2008) demonstrated that expression of  
315 Ogg1 was increased in hepatic tissues from HBV-infected patients, regardless  
316 of whether they originated from tumoural or non-tumoural regions (Gwak *et*  
317 *al.*, 2008). In agreement, expression of Pre-S mutants induced Ogg1  
318 expression *in vitro* and in murine models (Hsieh *et al.*, 2004). These studies  
319 suggest that infected cells respond to HBV-induced oxidative stress by  
320 upregulating cellular glycosylases involved in BER, activating DNA repair  
321 (Figure 4, left panel). However, recent studies have demonstrated that HBx  
322 inhibits BER initiated by Thymidine glycosylase (Tdg) (van de Klundert *et al.*,  
323 2012). Whilst this study examined the effects of HBx on BER induced by Tdg  
324 *in vitro*, the effect of HBx on accumulation of 8-OHdG remains unstudied, as  
325 Tdg acts on G/T mismatches and not on 8-OHdG.

326         A considerable number of studies report that HBV inhibits NER (Figure  
327 4, right panel). This has in large part been attributed to the HBx protein. Cells  
328 expressing HBx render hepatoma cells more sensitive to UV-C, and HBx  
329 inhibits global NER in a host-cell reactivation assay (Jia *et al.*, 1999; Lee *et*  
330 *al.*, 2005; Mathonnet *et al.*, 2004). Moreover, HBx inhibits the expression of  
331 the TFIIH subunits XPB and XPD, and interacts with and inhibits the function  
332 of TFIIH, leading to increased UV-C sensitivity of cells expressing HBx  
333 (Jaitovich-Groisman *et al.*, 2001; Qadri *et al.*, 2011). In addition to inhibiting  
334 global NER, HBx may also impede NER within transcriptionally active genes  
335 (transcription-coupled NER) (Mathonnet *et al.*, 2004). However, since the  
336 impact of NER on repair of oxidative DNA damage is unclear (as mentioned  
337 above), the impact of HBx-induced NER inhibition upon oxidative damage  
338 during HBV infection remains to be studied.

339 As outlined previously, the p53 tumour suppressor is also involved in  
340 the response to oxidative DNA damage. Intriguingly, HBV has been shown to  
341 perturb p53 function (Figure 4, lower panel). The interaction between HBx and  
342 p53 is well established (Chung *et al.*, 2003; Lin *et al.*, 1997; Wang *et al.*, 1994;  
343 Yun *et al.*, 2000), and it seems that this alters the binding of p53 to p53-  
344 responsive elements, resulting in aberrant gene expression (Chan *et al.*,  
345 2013). Given these data, one may imagine that interactions between HBx and  
346 p53 would negatively impact the repair of oxidative DNA lesions.

347 In addition to the specific mechanisms detailed above, the frequent  
348 random integration of HBV DNA into genes encoding DNA repair and  
349 checkpoint proteins (e.g. Wrm, hTERT, Rad17 (Toh *et al.*, 2013)) may also  
350 serve to perturb the cellular response to DNA damage. Furthermore, oxidative  
351 stress may increase the rate of HBV fragment integration *in vitro* (Dandri *et al.*,  
352 2002). In agreement with this latter remark, this study also suggests that  
353 the SSB repair factor PARP-1 may play a protective role against HBV DNA  
354 integration, presumably by rapidly repairing breaks which would otherwise  
355 favour integration of exogenous DNA.

356 From these studies, it is apparent that HBV proteins (notably HBx)  
357 interact with components of both BER and NER pathways, implying that the  
358 function of these pathways in the repair of oxidative lesions may be perturbed  
359 during HBV infection, ultimately contributing to the elevated levels of 8-OHdG  
360 observed in HBV-infected patients.

361

362 HCV, OXIDATIVE STRESS AND DNA DAMAGE



363 *HCV infection and oxidative stress*

364 HCV is a member of the Flaviviridae family of enveloped, positive-  
365 single strand RNA viruses. The positive-sense RNA genome acts as a  
366 template for viral genome replication, and is also translated into a polyprotein  
367 which is cleaved by both host and virally-encoded proteases to generate 10  
368 proteins: the capsid (core) protein; envelope glycoproteins E1 and E2; the p7  
369 ion channel; and 6 non-structural proteins (NS2-NS5B).

370 Chronic infection by HCV is a major risk factor for the onset and  
371 progression of HCC (El-Serag, 2002; Saito *et al.*, 1990). Chronic HCV  
372 infection is responsible for approximately a third of HCCs, and has become  
373 the principal cause of HCC in most industrialized areas. Cirrhosis appears to  
374 be an important nonspecific determinant of HCC occurrence in HCV-infected  
375 patients, and very few cases of HCC without cirrhosis have been reported in  
376 these individuals (Simonetti *et al.*, 1992).

377 Oxidative stress and elevated reactive oxygen species (ROS)  
378 production are frequently observed during chronic HCV infection. As with  
379 HBV, they are thought to play a central role in HCV-associated HCC. Elevated  
380 levels of oxidative DNA damage (namely 8-OHdG), 4-hydroxynoneal, and  
381 increased lipid peroxidation have been observed in HCV-infected patients  
382 (Farinati *et al.*, 2007b; Kato *et al.*, 2001; Konishi *et al.*, 2006; Mahmood *et al.*,  
383 2004; Shackel *et al.*, 2002). HCV infection has been associated with an  
384 almost fourfold increase in 8-OHdG levels compared to uninfected controls,  
385 and HCV infection induces higher levels of oxidative stress than does HBV  
386 (Farinati *et al.*, 2007b). In line with this, antioxidant therapies are able to

387 alleviate to some extent the level of hepatic damage in chronic HCV infection,  
388 although there is no clear evidence that antioxidants alone are useful  
389 therapeutic agents in these patients (reviewed by (Singal *et al.*, 2011)).

#### 390 HCV proteins directly induce oxidative stress

391 A considerable body of experimental evidence demonstrates a direct  
392 role for the HCV proteins in inducing oxidative stress (Figure 3 lower panel),  
393 from a variety of different models (reviewed in (Ivanov *et al.*, 2013; Simula &  
394 De Re, 2010)). Indeed, FL-N/35 mice transgenic for the entire ORF of HCV  
395 (thus expressing the entire complement of viral proteins) exhibit elevated ROS  
396 levels (Higgs *et al.*, 2012; Nishina *et al.*, 2008), and expression of the HCV  
397 polyprotein induced ROS production (Piccoli *et al.*, 2007). Expression of  
398 several individual HCV proteins have also been linked with overproduction of  
399 ROS, as detailed below. Although a causative role of HCV-associated  
400 oxidative stress in the development of HCC in murine models has yet to be  
401 shown, several studies suggest that oxidative stress induced by the HCV  
402 proteins may trigger genomic instability, eventually leading to HCC.

#### 403 *HCV core and oxidative stress*

404 Although core (and other HCV proteins) primarily localises to the  
405 endoplasmic reticulum, it also associates with mitochondria (Korenaga *et al.*,  
406 2005). Several studies have demonstrated that expression of the HCV core  
407 protein induces oxidative stress, in a variety of experimental systems. Mice  
408 transgenic for core, or for core, E1 and E2, demonstrate increased oxidative  
409 stress and enhanced ROS production (reviewed in (Wang & Weinman, 2006,

410 2013)). Moriya and colleagues showed that core induced a shift in the hepatic  
411 oxidant/antioxidant state, leading to mitochondrial damage and possibly  
412 contributing to the onset of HCC in their core-transgenic mice (Moriya *et al.*,  
413 2001). Korenaga *et al.* demonstrated that core protein associates with  
414 mitochondria and remains associated with the mitochondrial outer membrane  
415 in core-E1–E2 transgenic mice, leading to a disruption of mitochondrial  
416 electron transport complex 1, the generation of ROS and oxidation of the  
417 glutathione pool (Korenaga *et al.*, 2005). Thus it seems that expression of the  
418 HCV core protein provokes mitochondrial dysfunction, leading to oxidative  
419 stress, coupled with activation of components of the intracellular superoxide  
420 scavenging system, including catalase and glutathione (Koike, 2007).

421         Similar results have been obtained from study of HCV-infected cell  
422 cultures, or of tumoural cells expressing core. Expression of core under the  
423 control of a tetracycline-regulated promoter induced oxidative stress and lipid  
424 peroxidation in HeLa and Huh7 cells (Okuda *et al.*, 2002). Such expression  
425 efficiently induced a cellular antioxidant response, and increased expression  
426 of antioxidant genes (Li *et al.*, 2002). Expression of core (as well as E1 and  
427 NS3) in Huh7 cells leads to an increase in reactive oxygen species (ROS),  
428 and a decrease in mitochondrial permeability (Machida *et al.*, 2006; Pal *et al.*,  
429 2010). Overexpression of core in Huh7 hepatoma cells also increased  
430 mitochondrial  $\text{Ca}^{2+}$  uptake, perhaps explaining the increased cytochrome c  
431 release by mitochondria in response to  $\text{Ca}^{2+}$  in the presence of core (Li *et al.*,  
432 2002). Similar observations have also been made *in vivo* in core-transgenic  
433 mice (Korenaga *et al.*, 2005). It is therefore clear that increased mitochondrial  
434 uptake of  $\text{Ca}^{2+}$  induced by HCV core stimulates ROS production, leading to

435 the modification of electron transport components, and inducing cellular  
436 oxidative stress.

#### 437 *The HCV non-structural proteins and oxidative stress*

438 Numerous studies have linked the HCV non-structural proteins,  
439 especially NS3 and NS5A, to oxidative stress. The vast majority of these have  
440 involved expression of a single viral protein in isolation, although expression  
441 of the non-structural HCV proteins also induces ROS (Boudreau *et al.*, 2009;  
442 Rivas-Estilla *et al.*, 2012). Expression of the NS3 protease enhances ROS  
443 production in hepatoma cells (Machida *et al.*, 2006; Pal *et al.*, 2010). Similarly,  
444 NS5A expression in hepatoma cells induces mitochondrial ROS production,  
445 Ca<sup>2+</sup> release and activates downstream kinases (Gong *et al.*, 2001; Machida  
446 *et al.*, 2006; Pal *et al.*, 2010). Overproduction of ROS has also been observed  
447 in NS5A-transgenic mice (Wang *et al.*, 2009).

448 Expression of both NS4B and NS5A also induce ER stress (Gong *et al.*  
449 *et al.*, 2001; Li *et al.*, 2009). In agreement, Asselah *et al.* (2010) reported that ER  
450 stress markers were activated in biopsies from HCV-infected patients.  
451 However, transgenic mice expressing low levels of the HCV proteins do not  
452 exhibit ER stress (Lerat *et al.*, 2009). Taken together, these data suggest that  
453 ROS production through HCV-induced ER stress might be linked to HCV  
454 infection rather than HCV protein expression (Asselah *et al.*, 2010).

455 Recently, we demonstrated both in HCV patients' biopsies and in the  
456 FL-N/35 mouse lineage (transgenic for the entire ORF of an HCV genotype 1b  
457 isolate, and expressing the full repertoire of HCV proteins at a low level in the

458 liver), that hepatic c-Myc expression is elevated, increasing ROS production,  
459 and that NS5A is involved in this process (Higgs *et al.*, 2012). It is well  
460 established that expression of the proto-oncogene c-Myc can induce ROS  
461 production (Dang *et al.*, 2005; Graves *et al.*, 2009; Karlsson *et al.*, 2003; Ray  
462 *et al.*, 2006; Vafa *et al.*, 2002). We demonstrated that the increased ROS  
463 production induced by c-Myc is, at least in part, associated with transcriptional  
464 deregulation of cytochrome P2C9 (CYP2C9), a component of the  
465 mitochondrial respiratory chain cytochrome P450 (CYP450).

466 Limited evidence also suggests that the HCV non-structural proteins,  
467 together with core, repress hepcidin expression in a ROS-dependent manner,  
468 altering iron metabolism (Miura *et al.*, 2008). Importantly, NS5A-induced ROS  
469 production may also impact on glucose production, since an NS5A-dependent  
470 decrease in the phosphorylation of the transcription factor Foxo1 and  
471 subsequently increased glucose production was decreased by *N*-acetyl  
472 cysteine (Deng *et al.*, 2011). It is probable, therefore, that oxidative stress  
473 induced by HCV impacts on several other HCV-associated pathologies,  
474 including diabetes.

#### 475 *HCV and ROS detoxification*

476 Since oxidative stress is a hallmark of HCV infected cells, the excess  
477 ROS produced are clearly inefficiently detoxified. In an attempt to examine the  
478 impact of HCV on ROS detoxification pathways, several publications have  
479 examined the impact of core and the other HCV proteins on the Nuclear  
480 factor-erythroid 2-related factor 2 (Nrf2) pathway, which is of crucial  
481 importance in the regulation of intracellular oxidation. When associated with

482 small proteins (sMaf), Nrf2 positively regulates the transcription of genes  
483 containing antioxidant response elements (ARE) in their promoters. (Hirotsu  
484 *et al.*, 2012). Recently, Carvajal-Yepes *et al.* showed that, in hepatoma cells  
485 harbouring JFH1 replicons, HCV core triggers the delocalization of sMaf  
486 proteins from the nucleus to the endoplasmic reticulum where they bind HCV  
487 NS3. This ultimately restrains Nrf2 from entering into the nucleus and thereby  
488 inhibits the induction of Nrf2/ARE-regulated genes, thus resulting in lower  
489 expression of cytoprotective genes (Carvajal-Yepes *et al.*, 2011). In  
490 contradiction, other published results showed that the overexpression of core,  
491 NS3 and NS5A enhances Nrf2 expression (Ivanov *et al.*, 2011), and that the  
492 Nrf2/ARE antioxidant pathway is activated in cells infected with HCV *in vitro*,  
493 providing an anti-apoptotic protection mechanism (Burdette *et al.*, 2010).  
494 Further study is therefore necessary to determine how HCV impacts on the  
495 Nrf2/ARE pathway.

496 Collectively, these studies clearly demonstrate that several of the HCV  
497 proteins (notably core, NS3 and NS5A) induce oxidative stress through a  
498 variety of pathways, thus increasing the likelihood of oxidative DNA damage.  
499 ROS-induced apoptosis and oxidative DNA damage may both contribute to  
500 carcinogenesis, by on the one hand increasing compensatory hepatocellular  
501 proliferation to create a mitogenic and mutagenic environment, whilst on the  
502 other hand inducing further heritable genetic damage.

### 503 HCV and oxidative DNA damage

504 As described above, the role of HCV and the viral proteins in inducing  
505 oxidative stress is well established, and results in the increased 8-OHdG

506 levels found both in HCV-infected patients, and in HCV-infected cells *in vitro*.  
507 We have also observed increased levels of single-stranded DNA damage in  
508 mice transgenic for the entire complement of HCV proteins (Higgs *et al.*,  
509 2012). In a manner similar to HBx, several studies have indicated that, in  
510 addition to their role in inducing oxidative stress, HCV core, NS3 and NS5A  
511 may also have deleterious consequences on the repair of oxidative lesions  
512 (amongst other types of DNA damage) (Figure 4).

### 513 *HCV and the repair of oxidative DNA lesions*

514         There is a relative paucity of studies examining the impact of HCV on  
515 the DNA damage response. Moreover, given the importance of HCV in  
516 inducing oxidative DNA damage, there are surprisingly few reports concerning  
517 HCV and BER. HCV core has been suggested to inhibit the DNA glycosylase  
518 activity responsible for excision of 8-OHdG, although the mechanisms remain  
519 unclear, since core fails to interact with or perturb the expression of the BER  
520 components (Machida *et al.*, 2010a). Pal and colleagues found that  
521 expression of the Neil1 DNA glycosylase (which has marginal activity towards  
522 8-OHdG) was perturbed in HCV-infected cell cultures and biopsies from HCV-  
523 infected patients, with a concomitant reduction in Neil1-specific glycosylase  
524 activity, and increased 8-OHdG levels (Pal *et al.*, 2010). Together, these two  
525 reports provide the only preliminary evidence that HCV may have deleterious  
526 consequences on BER.

527         In a similar manner, there are limited reports that HCV deregulates  
528 NER. We have shown that hepatocytes of mice expressing the HCV proteins  
529 exhibit reduced NER repair by means of a plasmid reactivation assay (Higgs

530 *et al.*, 2010). Although unconfirmed, it is possible that the reduced expression  
531 of Gadd45 $\beta$ , a p53-response gene linked to NER, plays a contributory role.  
532 Intriguingly, these mice also develop hepatic steatosis (as do many HCV-  
533 infected individuals), which, since NER is diminished in steatotic livers  
534 (Schults *et al.*, 2012), may suggest that HCV-induced steatosis contributes to  
535 the observed decrease in NER.

536         The Ataxia telangiectasia mutated (ATM) kinase plays a key role in the  
537 cellular response to DSBs, and also plays an important role in the response to  
538 oxidative DNA damage (reviewed in (Chen *et al.*, 2012)). ATM is necessary  
539 for repair of ROS-induced DSBs in non-replicating cells (Guo *et al.*, 2010;  
540 Woodbine *et al.*, 2011). Previous reports have suggested that HCV interacts  
541 with ATM, and perturbs its function (Ariumi *et al.*, 2008; Machida *et al.*,  
542 2010b). Thus, it is tempting to speculate that HCV may also disrupt the repair  
543 of DSBs arising from both clustered oxidative lesions as well as exogenous  
544 sources.

545         A substantial body of evidence demonstrates that various HCV proteins  
546 alter p53 signalling and function *in vitro* (Alisi *et al.*, 2003; Deng *et al.*, 2006;  
547 Kao *et al.*, 2004; Kwun & Jang, 2003; Lan *et al.*, 2002; Smirnova *et al.*, 2006;  
548 Yamanaka *et al.*, 2002). From these studies, it is apparent that core, NS3 and  
549 NS5A interact with p53, and this interaction seems to inhibit p53 activity,  
550 although the reported consequences are sometimes contradictory. However,  
551 these findings are yet to be repeated *in vivo* in the various transgenic or  
552 humanised murine models currently available, and these observations may be  
553 a consequence of the use of hepatoma cells and/or of over-expressed



554 proteins in the majority of these studies. However, it is clear that the impact of  
555 the HCV proteins on p53 function, especially during DNA repair, is worth  
556 further investigation.

557 From these data, it is apparent that HCV proteins interact with several  
558 DNA repair factors, and may therefore contribute to the elevated levels of 8-  
559 OHdG and SSBs observed in HCV-infected individuals by negatively  
560 regulating the repair of oxidative DNA lesions. Clearly future work must focus  
561 on whether HCV perturbs these processes, and on the precise mechanisms  
562 involved.

563

#### 564 CONCLUSIONS AND FUTURE PERSPECTIVES

565 The causative link between HBV and HCV infection and oxidative DNA  
566 damage is well established. Although a number of mechanistic insights  
567 concerning the ability of virus-infected cells to repair such damage have been  
568 provided from *in vitro* and *in vivo* models, it is still not possible to present a  
569 final picture of the effect of the HBV or HCV viral proteins on such repair  
570 processes. Therefore, at present it is probably easier to list the areas  
571 surrounding these viruses and DNA damage which remain to be studied,  
572 rather than those that have been studied.

573 Clearly, further detailed studies need to be carried out on the ability of  
574 cells expressing the viral HBV or HCV proteins to repair oxidative DNA  
575 lesions, since sustained oxidative damage probably plays a contributory role  
576 in the development of virus-associated HCC. Given the importance of both  
577 viruses in inducing oxidative DNA damage, there is currently a relative lack of

578 literature on the impact of the viral proteins on BER. On the other hand,  
579 despite a number of publications describing the ability of both viruses to  
580 perturb NER, it is unclear whether this impacts significantly upon oxidative  
581 repair. The eventual impact on virally-associated pathogenesis, including  
582 hepatocellular carcinoma, must also be studied, although this work is  
583 hampered by the lack of current suitable *in vivo* models for both HBV and  
584 HCV (reviewed by (Lerat *et al.*, 2011)). Increased future understanding of  
585 how these viruses regulate host cellular DNA repair pathways in response to  
586 oxidative stress will be invaluable in the development of new strategies for the  
587 treatment and prevention of chronic liver diseases.

588

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595 FIGURE LEGENDS

596 Figure 1: Repair of ROS-induced DNA lesions. The majority of oxidative  
597 lesions are repaired by the BER pathway. Oxidative damage is recognised by  
598 a glycosylase (Ogg1 or Neil1 in the case of 8-OHdG), which removes the  
599 altered base. Depending upon the glycosylase involved, this will result in  
600 either a single-stranded break (SSB) or an abasic (AP) site. The latter is then  
601 processed by the Ape1 endonuclease, giving an SSB. These breaks are then  
602 repaired by two subpathways of the BER pathway: short-patch (bottom right)  
603 or long-patch (bottom left). During short-patch BER, DNA polymerase  $\beta$  fills  
604 the gap with a single nucleotide, and the nicked DNA is ligated by XRCC1 and  
605 LigIII. In the alternative long-patch repair, SSBs repair involves the  
606 replacement of 2-12 nucleotides. This is dependent upon synthesis of a new  
607 2-12bp fragment by either Pol  $\beta$ , Pol  $\delta$  or Pol  $\epsilon$ , and the concomitant  
608 displacement of a 5' DNA flap. This flap is then removed by the enzyme Flap  
609 Endonuclease 1 (FEN1). Ligation of the nick involves PCNA, LigI and LigIII.  
610 The choice between short-patch repair and long-patch repair (dotted arrows)  
611 is yet to be understood, and is complicated by the high degree of redundancy  
612 between the two pathways.

613

614 Figure 2: Alternative repair of oxidative damage by NER. An unknown  
615 proportion of oxidative lesions may be repaired by nucleotide excision repair  
616 (NER). Two NER subpathways exist, which differ in their ability to detect helix-  
617 distorting regions arising anywhere within the genome (global genome NER;  
618 left), or those lesions which arise in the transcribed strands of expressed  
619 genes (transcription coupled NER; right). In both cases, repair is effected

620 through the actions of detector proteins (XPC and XPE or CSA and CSB)  
621 which recruit TFIIH and its components to the site of damage. TFIIH, XPB and  
622 XPD act in conjunction with XPF and XPG to unwind the DNA surrounding the  
623 lesion and to excise one strand of the unwound bubble. The final stages of  
624 repair involve gap filling by Pol  $\delta$  or Pol  $\epsilon$ , and DNA ligation involving LigIII and  
625 XRCC1.

626

627 Figure 3: Sources of oxidative stress induced by the HBV (upper panel) and  
628 HCV (lower panel) viral proteins. As a result of the innate immune response,  
629 chronic infection with HBV or HCV will induce chronic inflammation, eliciting  
630 ROS production and creating oxidative DNA lesions. Both viruses, and their  
631 proteins, may cause ER stress or induce lipid accumulation, which in turn  
632 leads to oxidative stress and oxidative DNA lesions. In addition, HBx and HCV  
633 Core and NS5A stimulate ROS by perturbing mitochondria function. HCV  
634 NS5A also stimulates c-Myc transcription, leading to perturbation of  
635 cytochrome function and thus mitochondrial ROS, and directly increasing  
636 oxidative stress. In the case of HBV, the resultant oxidative DNA lesions may  
637 contribute to HBV genome integration if left unrepaired. The impact of either  
638 virus on the cellular detoxification of ROS remains unclear (denoted by a '?').

639

640 Figure 4: Interactions between HBV and HCV proteins and the cellular actors  
641 of oxidative DNA repair. (Upper panel): HBV infection upregulates the activity  
642 of the BER glycosylases Neil1 and Ogg1 (green arrows), probably as a  
643 consequence of increased oxidative stress. In contrast, the HCV proteins  
644 inhibit the activity of the Neil1 glycosylase, and HCV core decreases the

645 repair of oxidative DNA lesions (red lines). HBx also inhibits the activity of a  
646 third glycosylase, Tdg, although the impact of Tdg on oxidative DNA lesions is  
647 unclear. HBx also inhibits NER by reducing expression of the NER proteins  
648 XPB and XPD, as well as inhibiting transcription-coupled NER. In addition, the  
649 HCV proteins also seem to inhibit NER, perhaps as a consequence of  
650 reduced Gadd45 $\beta$  expression. (Lower panel): A subset of oxidative lesions  
651 are converted into DSBs. HCV non-structural proteins NS3, NS4A and NS5B  
652 are reported to interact with the DSB sensor ATM and inhibit its function. Both  
653 HBx and the HCV core, NS3 and NS5A proteins also inhibit p53 (red lines),  
654 although there are suggestions that Core and NS5A may also stimulate p53  
655 activity (green arrows).  
656

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Figure 1

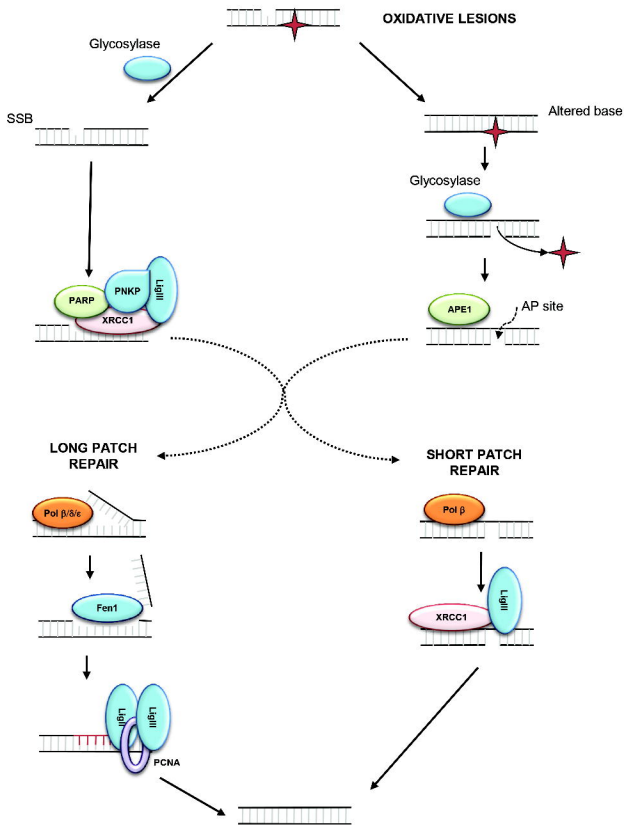


Figure 2

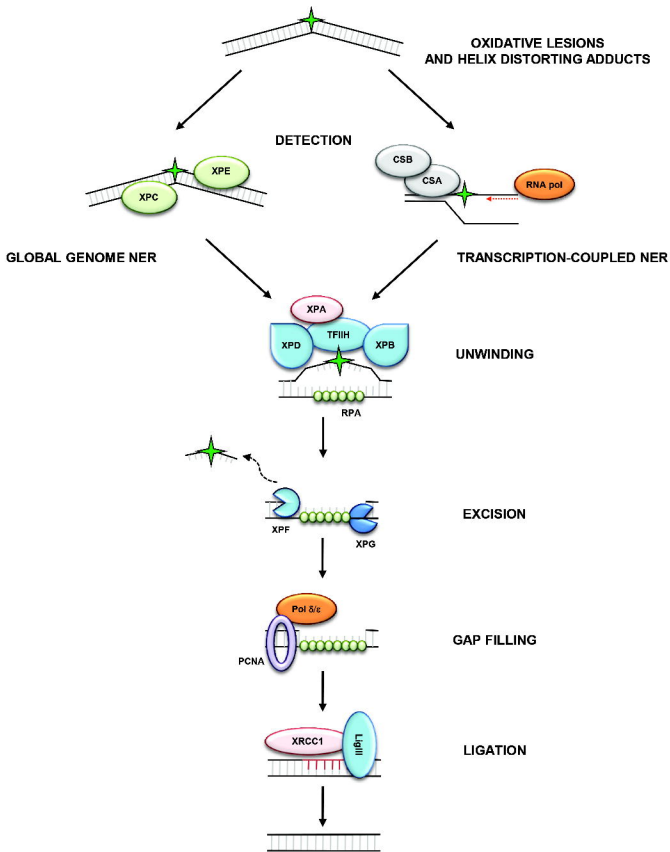




Figure 3

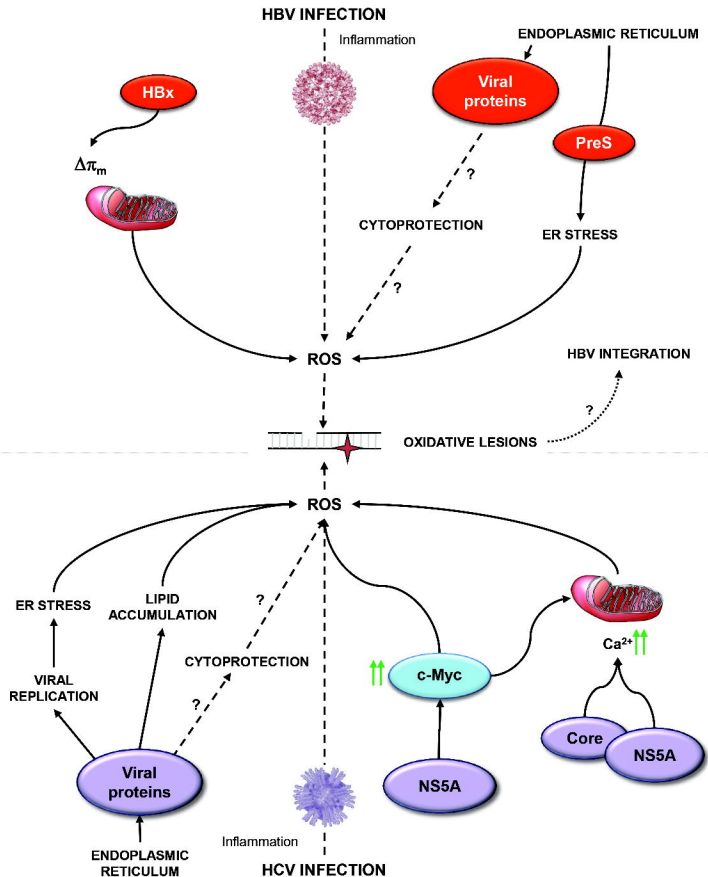


Figure 4

