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# 'Liver let die'

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1 "Liver let die: oxidative DNA damage and hepatotropic viruses" 2 Martin R. Higgs<sup>1\*</sup>, Philippe Chouteau<sup>2</sup> and Hervé Lerat<sup>2</sup> 3 <sup>1</sup>School of Cancer Sciences, University of Birmingham, Birmingham, UK 4 <sup>2</sup>INSERM U955, Université Paris-Est, Créteil, France. 5 6 7 \*Corresponding author: Dr Martin Higgs, School of Cancer Sciences, 8 University of Birmingham, Birmingham, UK 9 Email: m.r.higgs@bham.ac.uk 10 Tel: +44 121 414 4033 Fax: +44 121 414 4486 11 12 13 14 Word count (main text): 6328 15 Word count (summary): 207 16 Number of figures: 4 17 18 Running title: Oxidative DNA damage and hepatotropic viruses 19 20 Contents Category: Other viruses 21

# 22 SUMMARY

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

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

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

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### 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 (EI-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 play pivotal role in may а 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.

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

89

# 90 OXIDATIVE STRESS

Reactive oxygen species may arise by exposure to exogenous agents such as ionizing radiation or drugs, or may be generated from endogenous sources such as metabolism, apoptosis or inflammation. Oxidative stress represents a shifting of the balance between oxidants (such as ROS) and the cellular antioxidant response, leading to potential cellular damage and contributing to disease. Cells are able to withstand a relatively low level of 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.  $H_2O_2$ ) are 117 able to diffuse into hepatocytes, although the plasma membrane represents a 118 barrier to the free diffusion of  $O_2^{-}$ . Membrane superoxide dismutases are able 119 to convert these  $O_2^-$  anions to  $H_2O_2$  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 (uninfected) hepatocytes (Jaeschke, 2011), although they may also serve to
activate intracellular antioxidant defences. Moreover, mitochondrial reactive
oxygen species are able to drive proinflammatory cytokine production (Naik &
Dixit, 2011), further exacerbating both inflammation and the production of
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-hydroxylmethyluracil, 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)).

The majority of oxidative DNA lesions are repaired by components of the base excision repair pathway (BER), and to a lesser extent, by nucleotide excision repair (NER). The rate of transient oxidative DNA damage is typically balanced by its rate of repair, but chronic oxidative stress may result in permanent genetic damage. Since there are multiple, overlapping pathways for the repair of oxidative DNA damage, perturbation of one component of 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 kuppfer 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).

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

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# 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 Hepadnaviridiae. 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 Africa and Southeast Asia. Although HCC generally occurs in cirrhotic livers, HBV is also able to transform hepatocytes in the absence of chronic inflammation and cirrhosis (Brechot, 2004). During chronic infection, fragments of HBV DNA may integrate into the host genome, preferentially into chromosome 17, creating mutations. These integrated fragments often encode the X protein of HBV (HBx) or truncated preS proteins, the integration 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 <u>A direct role for HBV-encoded proteins in oxidative stress</u>

In addition to non-specific oxidative stress generated by local inflammation in response to viral infection, increasing evidence suggests that the HBV proteins directly regulate cellular ROS production (Figure 3 upper panel), and deleteriously alter intracellular antioxidant defences in HBVinfected cells, causing apoptosis and extensive liver damage and thus engendering accelerated hepatocellular renewal. However, the consequences 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.

The HBV surface antigen (HBsAg) and associated PreS region have also been associated with oxidative stress. Expression of truncated mutants of the HBV PreS/S polypeptide in hepatoma (Huh7) cells induced production of ROS via ER-stress pathways, resulting in oxidative DNA damage (Hsieh *et al.*, 2004; Wang *et al.*, 2005), although these results might be strongly influenced by non-specific ER stress triggered by high levels of HBV protein 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 profileration 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 277 *al.*, 2008).

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 the rapeutic 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

A growing body of literature suggests that the HBV proteins may alter the repair of DNA lesions, including oxidative DNA damage (Figure 4). There 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 glycolsylase (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. Wrn, 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 352 al., 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.

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

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# 362 HCV, OXIDATIVE STRESS AND DNA DAMAGE

#### 363 HCV infection and oxidative stress

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

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

alleviate to some extent the level of hepatic damage in chronic HCV infection,
although there is no clear evidence that antioxidants alone are useful
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

Although core (and other HCV proteins) primarily localises to the endoplasmic reticulum, it also associates with mitochondria (Korenaga *et al.*, 2005). Several studies have demonstrated that expression of the HCV core protein induces oxidative stress, in a variety of experimental systems. Mice transgenic for core, or for core, E1 and E2, demonstrate increased oxidative stress and enhanced ROS production (reviewed in (Wang & Weinman, 2006, 410 2013)). Moriva 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 mitochondrial Ca<sup>2+</sup> uptake, perhaps explaining the increased cytochrome c 430 release by mitochondria in response to Ca<sup>2+</sup> in the presence of core (Li et al., 431 432 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 uptake of Ca<sup>2+</sup> induced by HCV core stimulates ROS production, leading to 434

the modification of electron transport components, and inducing cellularoxidative 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).

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

Since oxidative stress is a hallmark of HCV infected cells, the excess ROS produced are clearly inefficiently detoxified. In an attempt to examine the impact of HCV on ROS detoxification pathways, several publications have examined the impact of core and the other HCV proteins on the Nuclear factor-erythroid 2-related factor 2 (Nrf2) pathway, which is of crucial 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.

Collectively, these studies clearly demonstrate that several of the HCV proteins (notably core, NS3 and NS5A) induce oxidative stress through a variety of pathways, thus increasing the likelihood of oxidative DNA damage. ROS-induced apoptosis and oxidative DNA damage may both contribute to carcinogenesis, by on the one hand increasing compensatory hepatocellular proliferation to create a mitogenic and mutagenic environment, whilst on the 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 levels found both in HCV-infected patients, and in HCV-infected cells *in vitro*.
We have also observed increased levels of single-stranded DNA damage in
mice transgenic for the entire complement of HCV proteins (Higgs *et al.*,
2012). In a manner similar to HBx, several studies have indicated that, in
addition to their role in inducing oxidative stress, HCV core, NS3 and NS5A
may also have deleterious consequences on the repair of oxidative lesions
(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 *et al.*, 2010). Although unconfirmed, it is possible that the reduced expression
of Gadd45β, a p53-response gene linked to NER, plays a contributory role.
Intriguingly, these mice also develop hepatic steatosis (as do many HCVinfected individuals), which, since NER is diminished in steatotic livers
(Schults *et al.*, 2012), may suggest that HCV-induced steatosis contributes to
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

Figure 2: Alternative repair of oxidative damage by NER. An unknown proportion of oxidative lesions may be repaired by nucleotide excision repair (NER). Two NER subpathways exist, which differ in their ability to detect helixdistorting regions arising anywhere within the genome (global genome NER; left), or those lesions which arise in the transcribed strands of expressed genes (transcription coupled NER; right). In both cases, repair is effected through the actions of detector proteins (XPC and XPE or CSA and CSB) which recruit TFIIH and its components to the site of damage. TFIIH, XPB and XPD act in conjunction with XPF and XPG to unwind the DNA surrounding the lesion and to excise one strand of the unwound bubble. The final stages of repair involve gap filling by Pol  $\delta$  or Pol  $\varepsilon$ , and DNA ligation involving LigIII and 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

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

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