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## **Impact of hepatitis C virus and alcohol, alone and combined, on the unfolded protein response in primary human hepatocytes**

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## **Abstract**

Hepatitis C virus (HCV) infection and alcohol abuse are leading causes of chronic liver disease and frequently coexist in patients. The unfolded protein response (UPR), a cellular stress response ranging along a spectrum from cytoprotection to apoptosis commitment, has emerged as a major contributor to human diseases including liver injuries. However, the literature contains conflicting reports as to whether HCV and ethanol activate the UPR and which UPR genes are involved. Here we have used primary human hepatocytes (PHH) to reassess this issue and address combined impacts. In this physiologically relevant model, either stressor activated a chronic complete UPR. However, the levels of UPR gene induction were only modest in the case of HCV infection. Moreover, when combined to the strong stressor thapsigargin, ethanol exacerbated the activation of pro-apoptotic genes whereas HCV tended to limit the induction of key UPR genes. The UPR resulting from HCV plus ethanol was comparable to that induced by ethanol alone with the notable exception of three pro-survival genes the expressions of which were selectively enhanced by HCV. Interestingly, HCV genome replication was maintained at similar levels in PHH exposed to ethanol. In conclusion, while both HCV and alcohol activate the hepatocellular UPR, only HCV manipulates UPR signalling in the direction of a cytoprotective response, which appears as a viral strategy to spare its own replication.

### *Keywords:*

Hepatitis C virus

Alcohol

Unfolded protein response

Primary human hepatocytes

*Abbreviations:* HCV, hepatitis C virus; UPR, unfolded protein response; ER, endoplasmic reticulum; BiP, immunoglobulin heavy-chain-binding protein; GRP94, 94 kDa glucose-regulated protein; ERAD, ER-associated degradation; EDEM, ER degradation-enhancing  $\alpha$ -mannosidase-like protein; HERP, homocysteine-induced ER protein; ISR, integrated stress response; eIF2 $\alpha$ , eukaryotic initiation factor 2 $\alpha$ ; ATF, activating transcription factor; GADD34, growth arrest and DNA damage-inducible protein 34; CHAC, cation transport regulator-like protein 1; CHOP, C/EBP homologous protein; PHH, primary human adult hepatocytes; HCVcc, HCV derived from Huh-7.5.1 cell culture; MOI, multiplicity of infection; ffu, focus-forming units; RT-qPCR, reverse-transcription quantitative PCR; RPL13A, 60S ribosomal protein L13A; gDNA, genomic DNA; XBP1, X-box binding protein 1; LDH, lactate dehydrogenase; CYP2E1, cytochrome P450 2E1; ADHs, alcohol dehydrogenases; IRE1, inositol-requiring enzyme 1.

## 1. Introduction

Hepatitis C virus (HCV) infection and excessive alcohol consumption are leading causes of chronic liver disease which can progress from steatosis to fibrosis, cirrhosis and hepatocellular carcinoma. In addition, these two factors often coexist in the same individual, their association leading to more severe liver injury, accelerated fibrosis progression and increased risk for the development of cirrhosis and hepatocellular carcinoma [1–3]. Accordingly, chronic liver disease caused by HCV infection and alcohol abuse alone and in combination represents a major health problem, yet the pathogenesis of hepatic injury remains largely elusive partly due to the multifactorial and intricate nature of the underlying mechanisms. Moreover, research in this field has long been hampered by the lack of a relevant experimental model to study the impact of HCV and alcohol on their primary target cell, the normal human adult hepatocyte.

Among the cellular responses to various stresses, the unfolded protein response (UPR) has emerged as an important contributor for a number of human diseases including, in particular, hepatic steatosis [4–6], which is often the first symptom of chronic liver disease in patients with HCV infection and/or alcohol abuse. The UPR is a cellular program which is triggered upon disruption of homeostasis of the endoplasmic reticulum (ER), the cellular organelle responsible for folding and maturation of membrane-bound and secreted proteins, a situation termed ER stress [7]. The UPR aims to restore ER homeostasis (i) by increasing ER folding capacity through induction of ER-resident molecular chaperones such as immunoglobulin heavy-chain-binding protein (BiP) and 94 kDa glucose-regulated protein (GRP94) and (ii) by activating ER-associated degradation (ERAD) of misfolded proteins through induction of ERAD components such as ER degradation-enhancing  $\alpha$ -mannosidase-like protein (EDEP) or homocysteine-induced ER protein (HERP). Aside from these two signalling pathways specific to ER stress, another branch of the UPR is shared by the so-called integrated stress

response (ISR), which can be triggered by multiple stressors including viruses and alcohol [8]. This common pathway proceeds through phosphorylation of the eukaryotic initiation factor 2 $\alpha$  (eIF2 $\alpha$ ) which leads to the attenuation of global protein synthesis and induction of activating transcription factor (ATF) 4. The latter, in turn, up-regulates growth arrest and DNA damage-inducible protein 34 (GADD34), which can activate eIF2 $\alpha$  dephosphorylation and hence end the translational arrest; other up-regulated targets are involved in cell fate such as ATF3, cation transport regulator-like protein 1 (CHAC) and C/EBP homologous protein (CHOP), which contribute to cell cycle arrest and trigger apoptosis in cases of overwhelming stress. Thus, the UPR aims primarily at coping with ER stress and preserving cell survival, but when the severity and/or prolongation of the stress exceed a poorly defined threshold, the response can switch to a cell death commitment [5,9].

HCV is expected to cause ER stress because most steps of the virus infectious cycle take place in the ER or membranes derived thereof [10,11]. However, whether the UPR is induced by HCV has been the matter of conflicting publications, partly attributable to differences in the experimental systems used, the UPR branches and targets examined and the timeframe chosen before readout. Expression of the HCV genome in transgenic mice was either shown to activate [12] or not [13] the UPR. Cell-culture-based studies involving overexpression of viral proteins independently of an infection process suggested that while inducing ER stress, HCV proteins may modulate specific components of the UPR and hence alter the typical course of UPR signalling [10,11]. When a system enabling productive HCV infection became available using clones of the hepatocarcinoma-derived Huh-7 cell line, UPR activation was shown to occur in infected cells, with a strong induction of pro-apoptotic genes obviously coinciding with a phase of massive cell death [12,14–16]. However, the transformed Huh-7 cells diverge significantly from the normal, quiescent, highly differentiated hepatocytes that are the natural host cells of HCV. The only laboratory which has used the highly relevant cell-

culture model of primary human adult hepatocytes (PHH) reported that the UPR was induced upon inoculation with a modified virus replicating at low levels [17]. Finally, studies using liver biopsies from HCV-infected patients led to widely divergent results as to the occurrence of UPR activation [17–19].

An alcohol-induced UPR was first reported in the liver of mice subjected to intragastric ethanol feeding, which involved ER chaperones (BiP and GRP94) and transcriptional factors such as CHOP [20]. The occurrence of hepatocellular UPR was not systematically observed upon alcohol exposure but has been described in various animal models and in human alcoholics [21,22]. An in vitro model based on human hepatocytes is required to gain insight into the hepatocellular response induced by alcohol, but hepatocarcinoma-derived cell lines lack physiological relevance as they retain only a weak, if any, expression of ethanol-metabolizing enzymes.

We have developed a robust experimental system which enables productive HCV infection to be achieved in PHH maintained in a differentiated state for at least 2 weeks; this provides a long-awaited model which combines virological and physiological relevance [23]. Here, we have taken advantage of this unique model to reassess, systematically, the occurrence of activation of UPR target genes in human hepatocytes infected with HCV or exposed to ethanol during the long term, and to investigate for the first time the simultaneous effect of the two stressors.

## **2. Material and methods**

### *2.1. Statement of ethical approval*



Procedures for PHH isolation [23] were performed in accordance with the relevant French laws and regulations. All the liver tissue specimens were obtained in the course of routine work during surgical procedures carried out in Paris Public Hospitals (Assistance Publique-Hôpitaux de Paris (AP-HP), France). For all samples, informed consent was obtained from the patients, no-opposition statements were signed, and the specimens were anonymized. This procedure was approved by an Ethics Committee (Comité de Protection des Personnes (CPP) Ile-de-France III) and by the French Ministry of Health (References COL 2929 and COL 2930).

## *2.2. Isolation and primary culture of human hepatocytes*

PHH were isolated from normal-appearing liver tissue obtained from adult patients undergoing hepatectomy for the therapy of metastases or benign tumours and seronegative for HCV, hepatitis B virus and human immunodeficiency virus. Hepatocytes were isolated by a two-step perfusion technique and maintained in primary culture as described previously [23]. Briefly, the liver pieces were perfused with Liver Perfusion Medium (Life Technologies) followed by collagenase- and dispase-containing Liver Digest Medium (Life Technologies) at 37 °C until full digestion. After centrifugation at  $200 \times g$  for 1 min, hepatocytes were washed and seeded at a density of  $1.2-1.5 \times 10^5$  viable cells/cm<sup>2</sup> onto 6- or 12-well plates pre-coated with Bornstein and Traub type I collagen (Sigma-Aldrich). PHH were maintained at 37 °C in a humidified 5% CO<sub>2</sub> atmosphere, in complete hepatocyte medium consisting of Leibovitz's L-15 medium (Life Technologies) supplemented with 26 mM NaHCO<sub>3</sub>, 100 IU/L insulin (Novo Nordisk) and 10% heat-inactivated foetal calf serum (PAA laboratories). The medium was replaced 16-24 h after seeding with fresh complete hepatocyte medium complemented with 1 µM hydrocortisone hemisuccinate (SERB) until HCV inoculation and/or ethanol treatment 2 days later.

### 2.3. HCV inoculation and treatment of PHH

High-titre stocks of HCV derived from Huh-7.5.1 cell culture (HCVcc) were prepared as described previously [23,24]. Briefly, HCVcc preparations used in a previous study [23] were subjected to additional rounds of virus amplification by inoculation of naïve Huh-7.5.1 cells (at a multiplicity of infection (MOI) of 0.01 focus-forming units (ffu) per cell) which were then grown until infectious titres (see below for HCV titration method) reached at least  $10^7$  ffu/mL. Culture supernatants containing high titres of HCVcc were passed through cellulose acetate membrane filters of a 0.45- $\mu$ m pore size (ClearLine<sup>R</sup>, Dutscher), then concentrated approximately 10-fold in an Amicon Ultra-15 100,000 molecular weight cutoff ultrafiltration device (Millipore). The viral stocks obtained were aliquoted and stored at -80 °C until they were used to inoculate PHH 3 days after seeding (MOI = 0.1 ffu/cell), as previously described [23,24]. Briefly, the medium was replaced with the inoculum diluted in the smallest volume of fresh complete hepatocyte medium sufficient for covering the cells. For control experiments referred to as “mock”, UV-inactivated virus was prepared as described previously [25,26]: the same HCVcc stock diluted in fresh complete hepatocyte medium was exposed to continuous UVC light for 2 min beneath a UVC lamp (wavelength: 253.7 nm), and used as a non-infectious “inoculum” after the residual infectivity was assessed by HCV titration assay to verify that the infectious titre had indeed fallen below the threshold of detection of 10 ffu/mL. In all cases, the inoculum was removed after a 16-h incubation at 37 °C, and cell monolayers were washed 3 times with phosphate-buffered saline. PHH were maintained in complete hepatocyte medium, half of the medium being renewed every 3 days. Ethanol (100 mM) was added to the medium for the indicated periods of time; the loss of ethanol due to evaporation was assessed by the ECET-100 EnzyChrom<sup>TM</sup> Ethanol Assay Kit (BioAssay Systems) and compensated daily. Where indicated, thapsigargin (Sigma-Adrich) was added at 300 nM for the last 4 h of culture.

#### 2.4. Gene expression analysis

Gene expression was analysed by reverse-transcription quantitative PCR (RT-qPCR). RNA was extracted using RNeasy<sup>®</sup> Mini Kit (Qiagen) and reverse transcribed with the High-Capacity cDNA Archive Kit (Applied Biosystems). Real-time PCR was performed on an ABI Prism<sup>®</sup> 7900HT Sequence Detection System (Applied Biosystems). The relative amounts of mRNA were estimated by the delta-delta Ct method [27] using the gene encoding 60S ribosomal protein L13A (RPL13A) as the reference gene and mock-infected cells not exposed to any stressor and harvested the same day as control cells. The primer sequences are given in the Supplementary Table S1. Of note, the forward and reverse primers for RPL13A, which was used as the reference gene in all experiments, were designed to target two different exons (exon 7 and exon 8, respectively, which are separated on genomic DNA (gDNA) by a 181-bp intron); accordingly, the qPCR species generated from reverse-transcribed RNA (i.e., cDNA) and from gDNA are different (of 124 bp and 305 bp in size, respectively). The absence of the second species in the qPCR products permitted to ascertain that the RNA samples were devoid of gDNA contamination.

#### 2.5. Analysis of X-box binding protein 1 (XBP1) transcript splicing

PCR for XBP1 transcripts was performed using 5'-GGGAATGAAGTGAGGCCAGT-3' and 5'-TGAAGAGTCAATACCGCCAGA-3' as forward and reverse primers, respectively, and the following conditions: 40 cycles of denaturation (at 95 °C for 30 s) followed by annealing and elongation (at 60 °C for 1 min). PCR products were resolved by electrophoresis on a 2%-agarose gel, and visualized using ethidium bromide and UV trans-illumination. The expected size for the bands corresponding to the spliced (XBP1-S) and unspliced (XBP1-U) forms of XBP1 transcript was 111 and 137 bp, respectively. The additional 163-bp band

corresponded to a hybrid amplicon (XBP1-H). The density of each band was quantified using ImageJ software.

### *2.6. HCV replication assay*

To assess HCV replication, intracellular levels of negative-strand HCV RNA were quantified by a strand-specific RT-qPCR technique described previously (threshold of detection: 25 copies/reaction) [28].

### *2.7. Cytotoxicity assay*

Cytotoxicity was assessed by measuring the activity of lactate dehydrogenase (LDH) released into culture supernatants using the CytoTox 96<sup>®</sup> Non-Radioactive Cytotoxicity Assay (Promega) as per the manufacturer's instructions. Cytotoxicity was calculated as the ratio of LDH leakage from the cells under study versus control cells not exposed to any stressor at the same day normalized to cell number.

### *2.8. Cell line culture*

Huh-7.5.1 cells (a kind gift from F. V. Chisari, Scripps Research Institute, La Jolla, CA, USA) were grown in Dulbecco's modified Eagle's medium (Life Technologies), supplemented with 10 mM HEPES (pH 7.3), 1% non-essential amino-acids (Life Technologies), 2 mM L-glutamine (Life Technologies) and 10% foetal calf serum (PAA Laboratories) at 37 °C in a humidified 5% CO<sub>2</sub> atmosphere. The same HCVcc stocks used to inoculate PHH were used to inoculate Huh-7.5.1 cells at the same MOI.

### *2.9. HCV titration*

Infectious titres in viral stocks or in filtered culture supernatants of HCVcc-infected PHH or Huh-7.5.1 cells were determined by focus-formation assay (threshold of detection: 10 ffu/mL) as described elsewhere [29].

### *2.10. Western blotting*

Total cell lysates were prepared using RIPA buffer (50 mM Tris-HCl pH 7.9, 1% NP40, 150 mM NaCl, 0.1% sodium dodecyl sulphate, 0.5% sodium deoxycholate) supplemented with protease and phosphatase inhibitor cocktails (Sigma-Aldrich). Cell lysates were electrophoresed in sodium dodecyl sulphate-12% polyacrylamide gels under reducing conditions, in parallel with protein molecular mass standards (PageRuler™ Prestained Protein Ladder, ThermoFisher), and, subsequently, transferred onto nitrocellulose membranes (Bio-Rad). Membranes were blocked with Odyssey Blocking buffer (LI-COR) for 1 h at room temperature, and then probed overnight at 4 °C with primary antibodies against cytochrome P450 2E1 (CYP2E1) or alcohol dehydrogenases (ADHs) diluted in the same buffer. These were a rabbit antibody against CYP2E1 (a kind gift from I. de Wazier, Inserm UMRS-1147, Paris, France), a goat antibody against pan-ADH1 (Acris, catalog number #AP16311PU-N; diluted 1:1,000) or a rabbit antibody against ADH4 (Abgent, catalog number #AP10128b; diluted 1:1,000). Membranes were reprobbed with a mouse monoclonal antibody (Abcam, catalog number #ab8226; diluted 1:5,000) or a rabbit polyclonal antibody (Abcam, catalog number #ab8227; diluted 1:5,000) against  $\beta$ -actin. IRDye<sup>R</sup> 800CW goat anti-rabbit IgG (LI-COR, catalog number #926-32211), IRDye<sup>R</sup> 800CW donkey anti-goat IgG (LI-COR, catalog number #926-32214) and IRDye<sup>R</sup> 650 goat anti-mouse IgG (LI-COR, catalog number #926-65010) were used as secondary antibodies, all of them being diluted 1:10,000. After the final wash with phosphate-buffered saline containing 0.1% Tween-20, the signals were quantified using an Odyssey Infrared Imager (LI-COR).

### 2.11. Statistical analysis

The data are expressed as the means  $\pm$  SEM of the indicated number of independent experiments, i.e., experiments performed with PHH originating from different donors. Statistical analysis was carried out using R (*agricolae* package) software. The Kruskal-Wallis test (nonparametric comparison of k independent series) followed by the Fisher's least significant difference *post-hoc* method were performed to assess statistical significance (-, non-significant; \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ ).

## 3. Results

### 3.1. Effect of HCV infection on the hepatocellular UPR

To reassess whether HCV infection induces the hepatocellular UPR, PHH were inoculated with HCVcc at an MOI of 0.1, and transcript levels of key target genes of the different branches of the UPR were measured by RT-qPCR on different days over a period of 2 weeks after inoculation. Supplementary Fig. S1 (left and middle columns) presents the kinetics of UPR gene induction along with viral parameters (HCV genome replication and infectious titres) for two independent preparations of PHH (i.e., originating from two different donors), and Fig. 1a shows the changes in transcript levels of UPR markers as means  $\pm$  SEM of at least 6 independent experiments. While the kinetics of induction of different markers could vary slightly from one PHH preparation to another (Supplementary Fig. S1a and Fig. S1b, compare the left and middle columns), the statistical analysis of independent experiments with PHH isolated from at least 6 different donors showed a significant mean increase of approximately 1.5- to 2-fold at day 3 post-inoculation for all the markers examined, including the ER chaperones BiP and GRP94, the ERAD components HERP and EDEM (Fig. 1a, left panel)

and genes shared by the ISR (Fig. 1a, right panel). Similar levels of gene transcripts were maintained at day 9 post-inoculation, except for GRP94, ATF3 and CHAC the expressions of which returned to baseline. Thus, HCV infection in PHH induced a complete and moderate UPR.

Because this pattern seemed widely different from the cellular response described in Huh-7 sublines [12,16], we compared PHH and Huh-7.5.1 cells upon inoculation with the same HCVcc stock at the same MOI. As expected in the actively dividing Huh-7.5.1 cells [23], the viral parameters, i.e., intracellular amounts of negative-strand HCV RNA (a hallmark of HCV genome replication) and extracellular infectious titres, increased during the first 6 days after inoculation, and reached levels that, compared to those attained in PHH, were higher by 2-3 orders of magnitude (Supplementary Fig. S1c, compare right column with left and middle columns). In Huh-7.5.1 cells no induction was observed for any of the ER chaperones at any day post-inoculation (Supplementary Fig. S1a, right column, and Supplementary Fig. S2a), in agreement with published data [16]. Expression of ERAD components was not induced at day 3 but increased significantly at day 9 post-inoculation and, most strikingly, a marked induction of ISR genes was observed at this time (Supplementary Fig. S1b, right column, and Supplementary Fig. S2a), consistent with previous observations [12]. Interestingly, this marked induction of pro-apoptotic genes without concomitant induction of ER chaperones coincided with a phase of extensive cell death visible by microscopic examination of the cultures from day 6 to day 12 post-inoculation (Supplementary Fig. S2b) and documented by a marked decline in cell count (Supplementary Fig. S1d, right column) and a massive leakage of LDH into the culture supernatant (Supplementary Fig. S2c). In contrast, daily examination of PHH monolayers showed no sign of cytotoxicity attributable to HCV infection at any time point of the 2-week kinetics post-inoculation (Fig. 2a), and no decrease in cell count (Supplementary Fig. S1d, left and middle columns) or significant increase in LDH release

(Fig. 2b) was observed in HCV-infected compared to mock-infected PHH. Collectively, the results show that the levels of HCV infection, the nature and magnitude of UPR activation and the resulting cell fate were completely different in normal, quiescent PHH compared to transformed, rapidly-dividing Huh-7.5.1 cells.

### *3.2. Effect of alcohol on the hepatocellular UPR*

Ethanol is metabolized in hepatocytes by ADHs 1 (class I) and 4 (class II) and CYP2E1. We previously showed that the expression of the CYP2E1 transcript was maintained in PHH throughout a 2-week culture period under our conditions, at levels that largely exceeded those found in Huh-7.5.1 cells [23]. Here, we further verified the expression of both ADHs and CYP2E1 at the protein level. Indeed, whereas these enzymes were expressed only weakly or not at all in Huh-7.5.1 cells at any time point of a 9-day time course, even upon ethanol exposure, they were readily detected in PHH at day 3 (Supplementary Fig. S3a) and their expression was maintained at similar levels at day 9 (Supplementary Fig. S3b). Moreover, ethanol further increased the levels of CYP2E1 in PHH at any time point (Supplementary Fig. S3b), which confirms the maintenance of the inducibility of this enzyme throughout the culture period. PHH thus represent a suitable model for studying the hepatocellular impact of alcohol.

To reassess whether alcohol activates the hepatocellular UPR, PHH were exposed to 100 mM ethanol during a prolonged period of 9 days. For all the UPR markers tested, an approximate 2- to 4-fold increase in gene expression was observed at day 3 and the transcript levels did not return to baseline at day 9 of the kinetics (Fig. 1b). This complete UPR elicited by ethanol in PHH was not accompanied by any significant cytotoxicity throughout the exposure period (Fig. 2). We concluded that prolonged exposure to ethanol induced a chronic UPR in physiologically relevant PHH.



### *3.3. Effect of HCV infection or ethanol on the hepatocellular UPR induced by a strong ER stressor*

To further characterize and compare the impact of HCV and ethanol on the hepatocellular UPR, we tested them in combination with thapsigargin, an established potent ER stress inducer which acts by depleting the ER calcium stores. Thapsigargin, or dimethylsulfoxide as the carrier control, was added for the last 4 h of culture to the medium of PHH at day 1 or day 3 after inoculation with HCVcc or continued exposure to ethanol. We verified that a 4-h treatment with thapsigargin induced a massive activation of all the UPR genes in PHH that were neither infected with HCV nor exposed to ethanol (Fig. 3a and Fig. 3b, white bars).

In PHH pre-exposed to ethanol for 1 day as compared to non-exposed PHH, the only significant change was a lesser induction of BiP upon thapsigargin treatment (Fig. 3a). However, pre-exposure to ethanol for 3 days further potentiated the massive induction of HERP, EDEM, ATF4, CHOP and CHAC which was triggered by thapsigargin alone. In particular, in PHH treated with ethanol plus thapsigargin versus thapsigargin alone, expression of the pro-apoptotic genes CHOP and CHAC was increased  $53.9 \pm 5.6$ -fold versus  $30.6 \pm 1.2$ -fold and  $36.7 \pm 5.6$ -fold versus  $13.9 \pm 0.6$ -fold, respectively.

Unlike ethanol exposure, HCV infection did not enhance the thapsigargin-triggered induction of UPR genes at any time point (Fig. 3b). On the contrary, treatment with thapsigargin 1 day post-inoculation produced a significantly lesser induction of BiP, ATF4 and CHOP in infected PHH versus mock-infected PHH:  $25.9 \pm 1.2$ -fold versus  $32.9 \pm 2.3$ -fold increase, respectively, in the expression of the pro-apoptotic gene CHOP, for example. Thus, HCV not only did not potentiate but, on the contrary, appeared to restrain the massive UPR triggered by thapsigargin. As such a massive UPR might be detrimental to viral replication [10,11], we assessed the consequence of thapsigargin treatment on HCV genome replication using quantitation of the intracellular amounts of negative-strand HCV RNA by a sensitive

and strand-specific technique [28]. These were reduced by about half upon thapsigargin treatment both at 1 day and 3 days post-inoculation (Fig. 4a). Thus, HCV replication was compromised under the unresolvable ER stress induced by thapsigargin.

The UPR can be initiated by three sensor proteins, which detect the occurrence of ER stress [7]. The most conserved and well-known of the three signalling pathways activated by these proximal ER stress sensors is the one which is mediated by inositol-requiring enzyme 1 (IRE1) and proceeds through an unconventional splicing of XBP1 transcript. As an approach to exploring a possible mechanism for the observed HCV-mediated attenuation of the thapsigargin-triggered UPR, we assessed XBP1 transcript splicing by semi-quantitative RT-PCR analysis (Fig. 3c). The ratio of spliced to unspliced XBP1 transcripts was not or only slightly increased by either HCV or ethanol alone at day 1, whereas a 4-h treatment with thapsigargin was sufficient to induce a massive increase in XBP1 transcript splicing, as expected for this strong ER stressor. Most interestingly, this massive induction of XBP1 transcript splicing triggered by thapsigargin was comparable in PHH pre-exposed to ethanol for 1 day versus non-exposed PHH, whereas it was greatly reduced in HCV-infected versus mock-infected PHH at 1 day post-inoculation. These results confirm that HCV, unlike ethanol, tended to curb the UPR induction triggered by thapsigargin, and further pointed to the IRE1-XBP1 signalling pathway as one possible target manipulated by HCV for this attenuation.

#### *3.4. Effect of HCV infection and ethanol in combination on the hepatocellular UPR*

To investigate the hepatocellular impact of HCV infection and ethanol in combination, PHH inoculated with HCVcc at an MOI of 0.1 were exposed continuously to 100 mM ethanol over a prolonged period of 9 days and the resulting UPR was compared to that elicited by each stressor separately. In PHH subjected to both stressors versus neither of them, a significant activation of about 2- to 4-fold was observed for all UPR genes both at day 3 and

day 9 (Fig. 5, black bars). In HCV-infected PHH, ethanol exposure further increased the levels of all ER-stress-specific markers both at day 3 and day 9 and also significantly increased the expression of a number of ISR genes (ATF4 and ATF3 at both time points and CHAC at day 3) (Fig. 5, black versus white bars). Conversely, the gene activation levels were similar in PHH exposed to ethanol irrespective of whether they were infected or not, except for three UPR genes, namely BiP, EDEM and GADD34, the expressions of which were significantly enhanced by HCV at day 9 post-inoculation (Fig. 5, black versus grey bars). These three key components of the UPR are known to assist protein folding in the ER, to target misfolded proteins to the ERAD pathway and to mediate a negative feedback loop to end the translational arrest induced as part of the ISR, respectively [7]. This suggests that their selective induction by HCV in PHH exposed to ethanol may contribute to coping with this increased stress. Importantly, ethanol exposure of HCV-infected PHH neither caused cytotoxicity (Fig. 2), nor affected the levels of negative-strand HCV RNA (Fig. 4b). This indicates that both cell survival and HCV replication were, indeed, maintained in spite of the additional stress caused by ethanol.

#### **4. Discussion**

Even though HCV and alcohol are generally considered to provoke an ER stress in hepatocytes, the existence of conflicting publications has left unclear whether the downstream response itself, i.e., the UPR, is actually activated and, if so, which of its branches are involved. In this work we first wanted to reassess the occurrence of UPR induction in PHH, the closest in vitro model of normal human adult hepatocytes, over a 9-day period of productive infection with HCV or continuous exposure to ethanol. We focused on the genes of the response itself, comprising genes that are specific to ER stress and those shared by the

ISR, because it is the pattern of activated target genes which eventually determines the outcome ranging along a spectrum that goes from homeostasis restoration to cell death commitment and includes cell dysfunction [5]. Each of the two stressors was found, indeed, to activate the UPR in PHH. However, the response differed both quantitatively and qualitatively. Indeed, the degree of gene induction appeared only modest upon HCV infection as compared to ethanol exposure. Moreover, when combined with thapsigargin used as a classical ER stress inducer, ethanol amplified the UPR, with an exacerbated induction of pro-apoptotic genes (CHOP, CHAC), whereas HCV not only did not intensify but instead appeared to restrain the activation of key UPR genes. The second goal of our study was to investigate the impact of the combination of HCV and alcohol in PHH. Interestingly, we found that the resulting UPR did not simply represent the sum of the responses induced by each stressor separately; instead, HCV appeared to enhance selectively the expressions of three pro-survival genes (BiP, EDEM and GADD34) and HCV replication was spared in spite of the added stress due to ethanol. Collectively, the results suggest that whereas alcohol activates indiscriminately all the branches of the hepatocellular UPR, HCV apparently manipulates UPR signalling towards the maintenance of its host cell survival. This seems to be a viral strategy to permit maintenance of its own replication.

HCV generally is believed to activate the hepatocellular UPR as a response to ER stress, yet most publications supporting this view have used ectopic expression of part or all of the viral genome in cells that significantly differ from normal human hepatocytes [10,11]. In fact, the evidence is even less clear when considering only those studies involving a bona fide infection process in physiologically relevant human hepatocytes. Indeed, the few publications that used chimeric mice with humanized livers reported increased expression of either BiP and CHOP [30] or BiP but not CHOP [31] in HCV-infected hepatocytes. Moreover, the comparison of liver biopsies from HCV-infected patients versus normal liver tissues showed

no evidence of UPR [19], or only activation of the UPR sensors with no induction of downstream target genes [18], or else activation of UPR genes including BiP [17]. Our *in vitro* model in PHH enabled us to study the impact of HCV infection in normal human hepatocytes while avoiding potential confounding factors inherent to *in vivo* studies. Despite the inter-individual variability of PHH originating from different donors, we found a significant induction of almost all the UPR target genes. These results confirm previous data obtained in a system where PHH inoculated with a modified virus supported only low levels of HCV replication [17], and extend them to a situation of robust, productive HCV infection [23]. While, at first glance, the magnitude of UPR activation appears, nevertheless, to be modest, it should be emphasized that any biochemical change induced by HCV in infected cells is diluted among the majority of cells that remain uninfected whereas other stressors such as ethanol are expected to affect the whole cell population. Such a mechanism was put forward as a possible explanation for the discrepancy between the absence of detectable UPR gene induction in the liver tissue from HCV-infected patients and the detection of signs of ER stress *in situ* in small clusters of hepatocytes scattered in the liver parenchyma [18]. However, direct evidence supporting this hypothesis would rely on single-cell analysis of viral replication and host gene expression with the use of sophisticated high-resolution techniques that are not yet widely available.

It generally is admitted that HCV is a non-cytotoxic virus, *in vivo*, and that the death of hepatocytes during HCV-associated hepatitis is attributable mainly to their clearance by cytotoxic T cells. With this in mind, the detection of a transient phase of massive cell death upon HCV infection of Huh-7 sublines was, in fact, unexpected [14,15]. Here, we found that upon inoculation with the same HCV stock at the same MOI, Huh-7.5.1 cells underwent massive cell death between day 6 and day 12 post-inoculation whereas PHH showed no evidence of cytotoxicity throughout the 2-week time course. At least two facts can be put

forward to explain this difference between the two sources of human hepatocytes. First, the phase of massive cell death seen in the Huh-7 system occurs when virtually all the cells in the culture are infected [14], i.e., when the levels of infection are far higher than those supported by PHH or which occur in the liver of patients during natural infection [32–35]. Second, cell death coincided with a massive induction of pro-apoptotic genes of the ISR but not of chaperones characteristic of the ER-stress-specific branch of the UPR. Whereas it is established that HCV infection in Huh-7 sublines triggers activation of the apoptotic gene CHOP, discrepancies exist as to whether BiP also is induced [12,15,16]. In fact, such transformed cells were reported to express high levels of BiP constitutively [36], which make them an unsuitable model for studying the occurrence of an ER-stress-specific response. In contrast to Huh-7.5.1 cells, PHH inoculated in parallel displayed only a slight, if any, induction of ISR-associated genes accompanied by an activation of ER-stress-specific genes including its master regulator BiP. Thus, HCV is not cytotoxic for PHH, consistent with these normal hepatocytes developing a complete UPR response that does not reach the threshold leading to cell death.

An increased expression of most UPR markers was found in liver biopsies from patients with chronic alcohol-related liver disease [21]. However, only some of the UPR target genes and pathways were reported to be induced by short-term ethanol exposure of human hepatocarcinoma-derived cell lines engineered to express ethanol-metabolizing enzymes [8,37], possibly due to their intrinsic inability to activate the ER-stress-specific response [36]. More unexpectedly, PHH also showed no significant activation of ER chaperones when exposed to ethanol over the short term (24 or 30 h) [8,38]. Importantly, the UPR should be viewed as a dynamic process. Therefore, an undetected marker at early time points does not necessarily rule out its activation upon prolonged exposure to the stressor and time course studies are needed to reveal how the pattern of activated markers varies with the duration of

the stress. Here, we subjected PHH to prolonged ethanol exposure and we observed a significant activation of all the UPR genes at day 3 that did not return to baseline at day 9, which is indicative of a chronic UPR. Of note, we observed that none of the UPR genes was induced in Huh-7.5.1 cells after 3 days of ethanol exposure. This suggests that the metabolism of ethanol probably contributes to the UPR activation seen in PHH, which, unlike Huh-7.5.1 cells, were demonstrated to have sustained expression of the alcohol-metabolizing enzymes. Indeed, alcohol-induced ER stress may be related to the production of acetaldehyde and reactive oxygen species generated from ethanol-induced CYP2E1 and to perturbations in calcium homeostasis and in homocysteine metabolism [8,22].

Ethanol was reported to potentiate the response to ER stress caused by other factors such as human immunodeficiency virus protease inhibitors in PHH [38], primary mouse hepatocytes or hepatic stellate cells [39], palmitic acid in primary rat hepatocytes [40] and tunicamycin or thapsigargin in cells of neuronal origin [41]. In our study, PHH pre-exposure to ethanol also potentiated the UPR triggered by thapsigargin, with a massive induction of the pro-apoptotic genes CHOP and CHAC. In sharp contrast, the thapsigargin-triggered induction of UPR target genes was not enhanced in HCV-infected PHH and a slight, yet significant attenuation in expression of the ISR genes ATF4 and CHOP was observed at 1 day post-inoculation. Our comparison of HCV and ethanol thus highlights the striking capacity of HCV to manipulate the UPR towards an adaptive response, a property likely to be essential for a virus prone to persist in its host [10,11]. Interestingly, the massive induction of XBP1 transcript splicing triggered by thapsigargin was reduced in HCV-infected PHH. This is reminiscent of the impact of murine cytomegalovirus on the UPR [42]. Indeed, this virus was reported to induce only a slight and transient increase in XBP1 transcript splicing and to suppress its induction by tunicamycin via IRE1 downregulation. Although additional studies would be required to unravel the exact mechanisms by which HCV tends to curb the UPR,

these data suggest that manipulation of the IRE1-XBP1 signalling pathway may be a strategy shared by different viruses.

An increased serum and intra-hepatic viral load was reported in HCV-infected patients with excessive alcohol consumption, suggesting that ethanol might promote HCV replication [43,44]. However, studies using Huh-7 sublines as cellular models gave conflicting data as to whether ethanol decreases or increases HCV genome replication and a variety of mechanisms have been incriminated to explain the observed effects [45–51]. Theoretically, a decrease in HCV replication would be expected to occur as a result of the cellular stress induced by ethanol, as illustrated here under conditions of overwhelming ER stress caused by thapsigargin. In our model of HCV infection in physiological PHH, the addition of ethanol did not affect significantly the level of HCV genome replication as evaluated, directly and rigorously, by the specific quantitation of negative-strand HCV RNA. Interestingly, the UPR resulting from the combination of ethanol and HCV did not simply represent the sum of the effects due to each stressor separately; instead, HCV appeared to increase specifically the expression of BiP, EDEM and GADD34 all of which can contribute to coping with the ER stress added by ethanol. Future studies are needed to unravel the mechanism of the alcohol-induced increase in viral load in the absence of any effect on the step of HCV genome replication. One hypothesis is that alcohol might affect the step of viral particle morphogenesis, which is expected to be promoted under conditions that increase the capacity of folding of the viral envelope glycoproteins in the ER.

In conclusion, the hepatocellular UPR has been incriminated in the pathogenesis of a variety of liver injuries and diseases, most notably steatosis for which a causal relationship has been supported by an increasing body of experimental evidence [4–6]. However, the UPR certainly should be viewed as a multitude of responses which differ in the patterns of activated markers and subsequent outcomes, not all of them being pathologic [5,6]. Here, we



found different patterns of UPR activation depending upon the duration of the stress and the nature of the stressor, HCV alone, alcohol alone, or HCV and alcohol in combination. The latter pattern could not be predicted simply by the addition of the effects of the two stressors taken separately, owing to their complex interplay and the peculiar ability of HCV to subvert UPR signalling. It is all the more important to characterize in detail the different types of UPR which result from different situations as new therapeutic avenues targeting specific UPR signalling pathways are being developed with the aim of switching the UPR from a disease-causing response to an adaptive, beneficial response [6,52].

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## **Author contributions**

PP, MG, HR and ARR conceived and designed the study. CH, EBB, VP, BL, MV, LA and AC performed the experiments. CH, EBB, VP, BL, MV and AC analysed and interpreted the data. FC and YC provided resources. CH, EBB, VP, HR and ARR drafted or wrote the manuscript. All authors approved the final version for submission.

## Conflict of interest declaration

The authors declare no conflict of interests.

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## Figure legends

**Fig. 1.** Effect of HCV infection and ethanol on the UPR in PHH. PHH were (a) inoculated with HCVcc or (b) continuously exposed to ethanol, and transcript levels of ER-stress-specific (left panels) and ISR (right panels) genes were quantified at day 3 (*D3*) or 9 (*D9*). Shown are the changes (*n*-fold), relative to the levels in mock-infected PHH not exposed to any stressor (normalized to 1), expressed as the means  $\pm$  SEM of  $\geq 6$  independent experiments (-, non-significant; \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ ).

**Fig. 2.** Absence of cytotoxicity of HCV infection and ethanol, alone and combined, in PHH. PHH were inoculated with HCVcc (*HCV*), continuously exposed to ethanol (*Eth*) or both (*HCV + Eth*), and compared with mock-infected PHH not exposed to any stressor (*Mock*). (a) Phase-contrast micrographs of the cultures at day 3 (*D3*) or 9 (*D9*) (scale bars, 100  $\mu\text{m}$ ). (b) LDH leakage in culture supernatants. Shown are the means  $\pm$  SEM of  $\geq 3$  independent experiments performed in triplicate. No significant difference was observed.

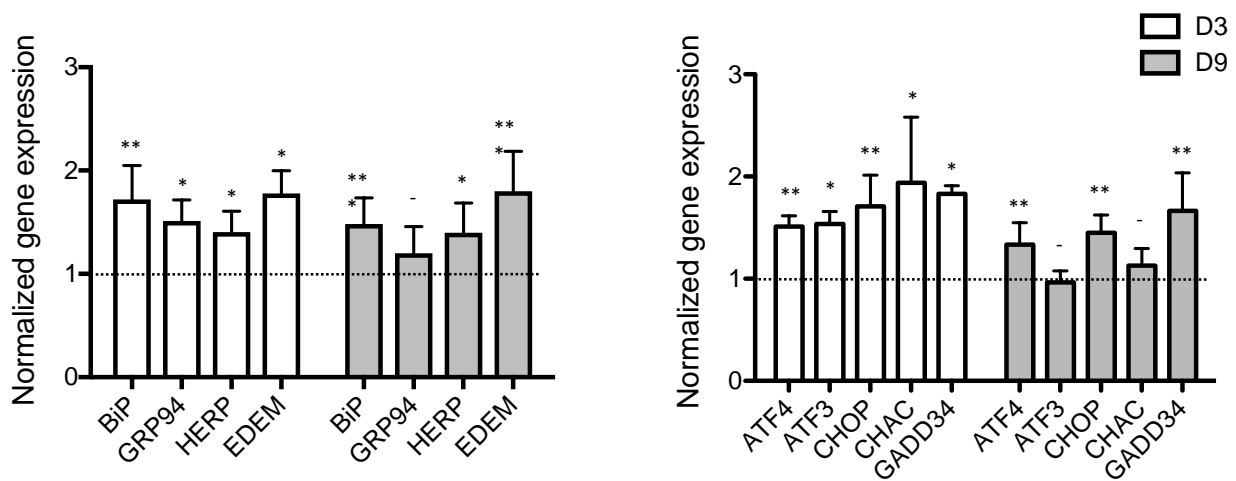
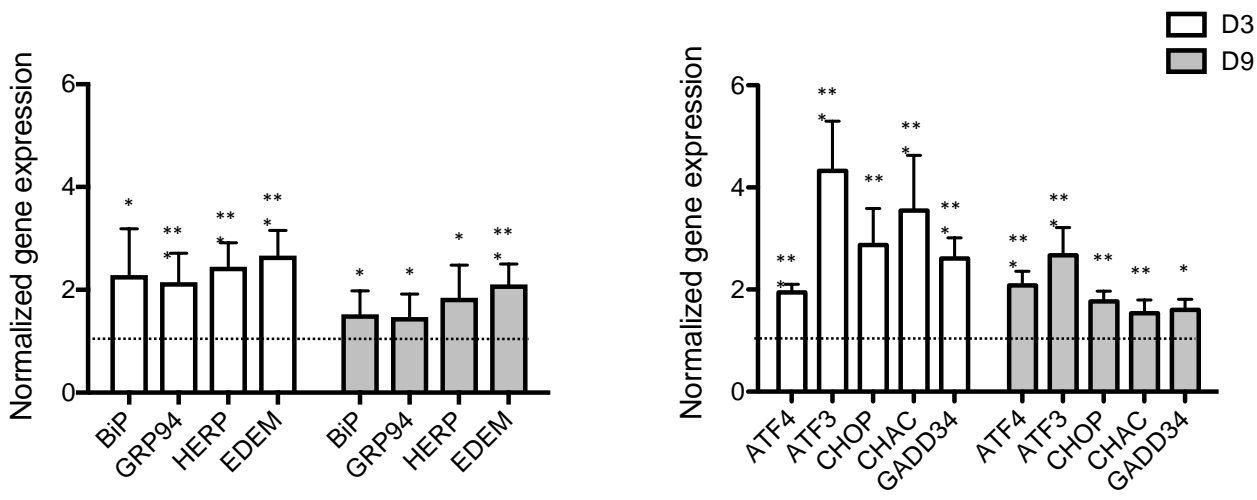
**Fig. 3.** Effect of HCV infection and ethanol on the UPR induced by thapsigargin in PHH. Thapsigargin (*Tg*) was added for 4 h to mock-infected PHH (*Tg + Mock*) or to PHH that had been inoculated with HCVcc (*Tg + HCV*) or continuously exposed to ethanol (*Tg + Eth*) (**a-c**) 1 day (*D1*) or (**a and b**) 3 days (*D3*) before. (**a and b**) Transcript levels of ER-stress-specific (left panels) and ISR (right panels) genes were quantified. Shown are the changes (*n*-fold), relative to the levels in mock-infected PHH not exposed to any stressor (normalized to 1), expressed as the means  $\pm$  SEM of  $\geq 4$  independent experiments (-, non-significant; \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ ). (**c**) Semi-quantitative RT-PCR analysis of XBP1 transcript splicing. (Left) Photograph of a representative ethidium-bromide stained agarose gel. (Right) Semi-quantitative analysis of the gel. Shown are the changes (*n*-fold) in the ratio of spliced XBP1 transcript (*XBPI-S*) to unspliced XBP1 transcript (*XBPI-U*), relative to that in mock-infected PHH not exposed to any stressor (normalized to 1).

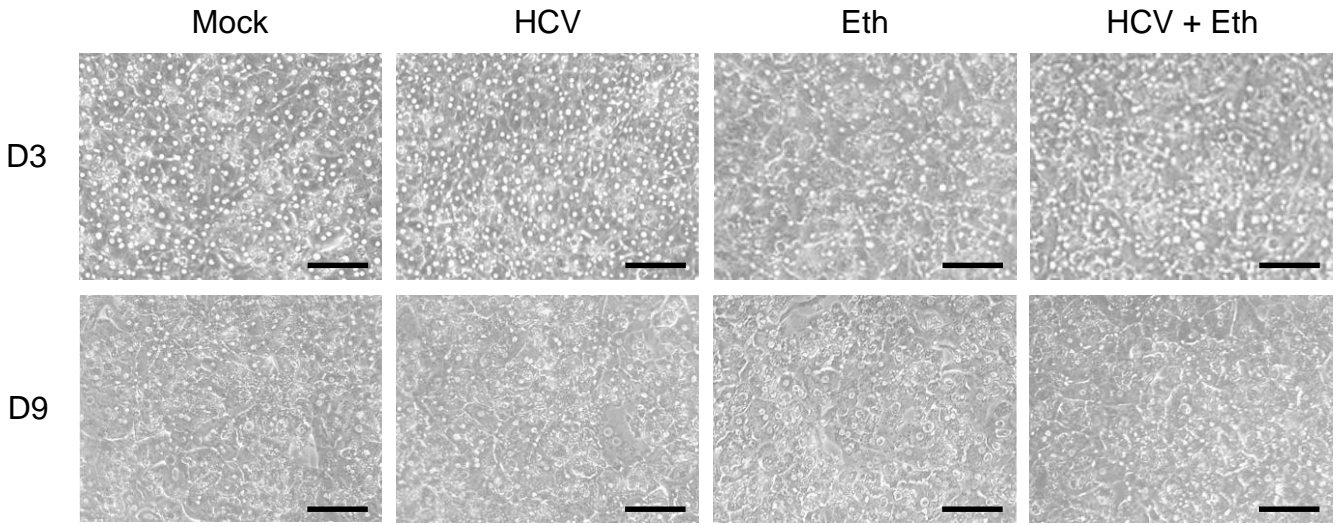
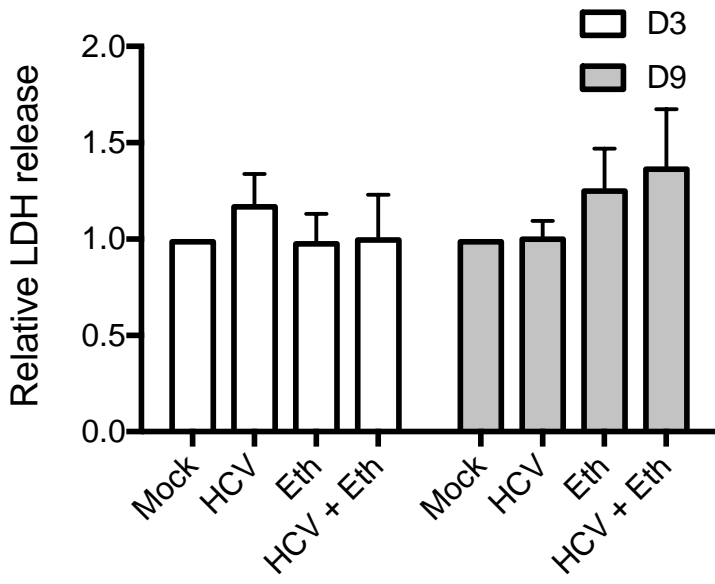
**Fig. 4.** Effect of thapsigargin and ethanol on HCV replication in PHH. PHH were inoculated with HCVcc and (**a**) treated with thapsigargin for 4 h (*HCV + Tg*) 1 day (*D1*) or 3 days (*D3*) later or (**b**) continuously exposed to ethanol (*HCV + Eth*) for 3 days (*D3*) or 9 days (*D9*). Levels of negative-strand HCV RNA were quantified to assess HCV replication. Shown are the changes (*n*-fold), relative to the levels in infected PHH not exposed to any additional stressor (*HCV*), expressed as the means  $\pm$  SEM of  $\geq 3$  independent experiments performed in triplicate (\*\*,  $p < 0.01$ ; otherwise, non-significant).

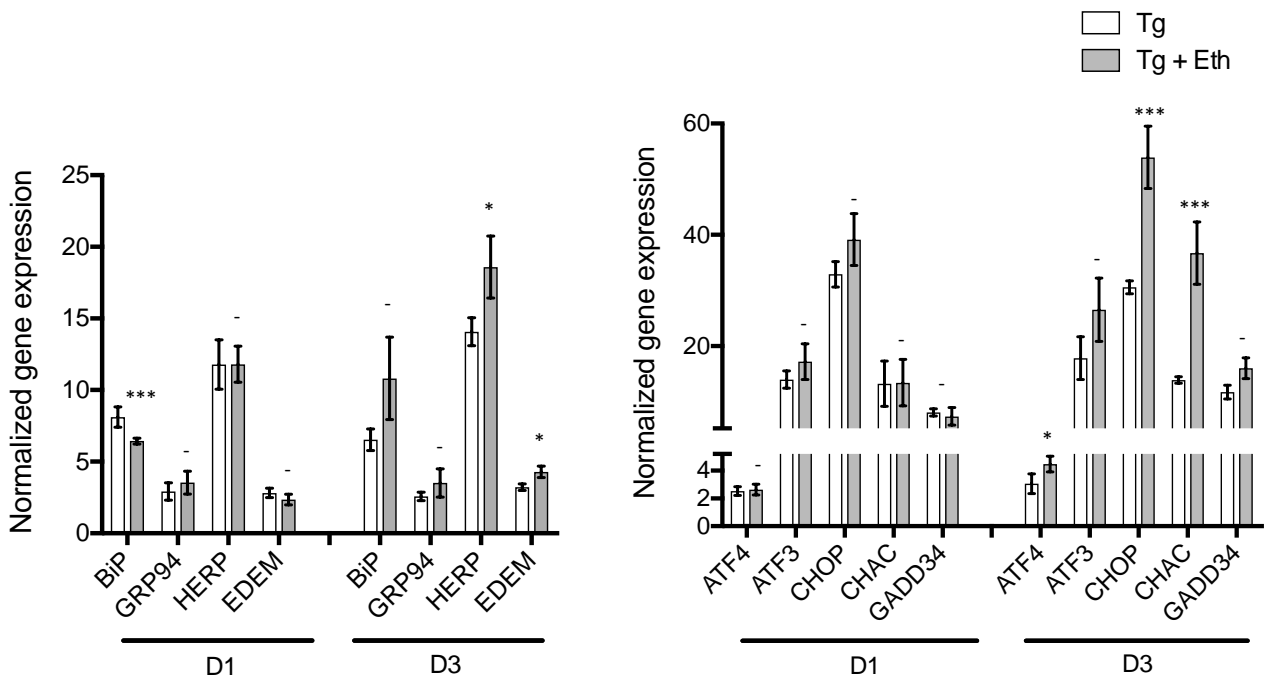
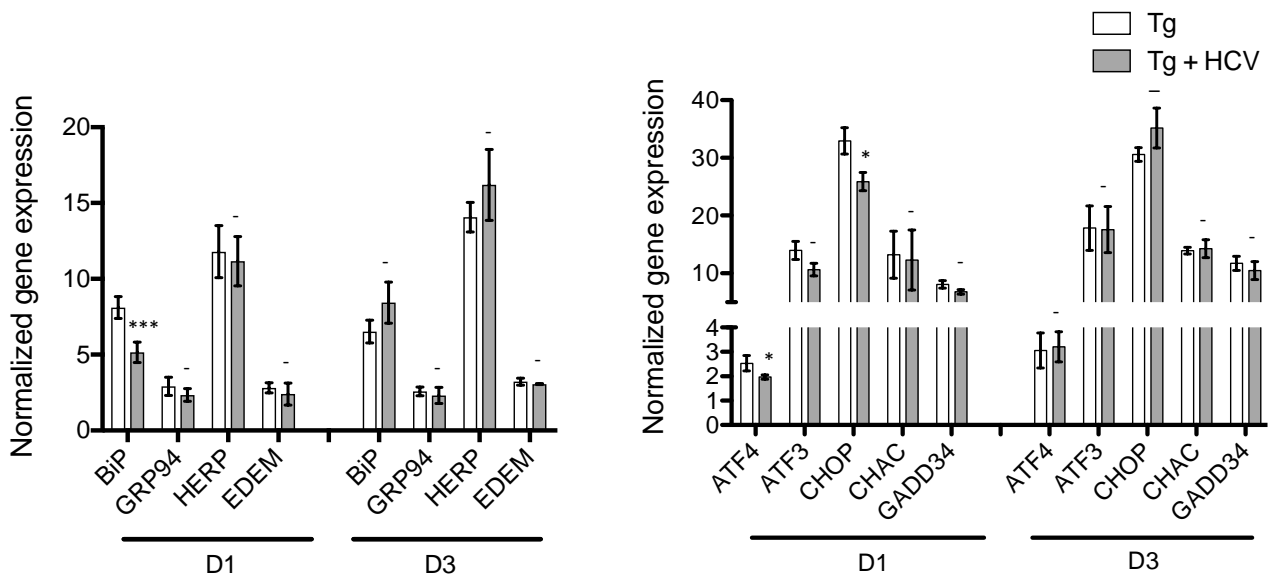
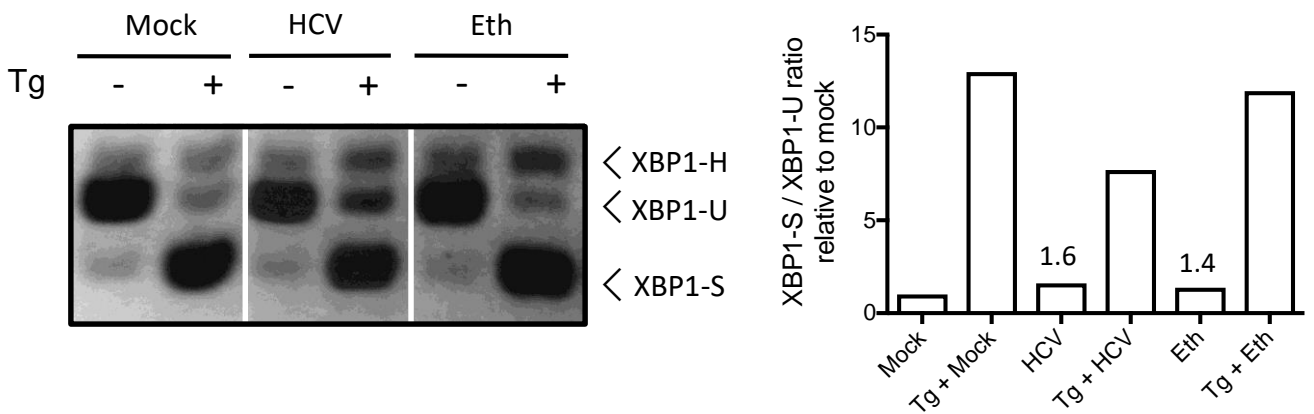
**Fig. 5.** Combined effect of HCV infection and ethanol on the UPR in PHH. PHH were inoculated with HCVcc (*HCV*), continuously exposed to ethanol (*Eth*) or both (*HCV+Eth*), and transcript levels of (**a**) ER-stress-specific and (**b**) ISR genes were quantified at day 3 (*D3*)

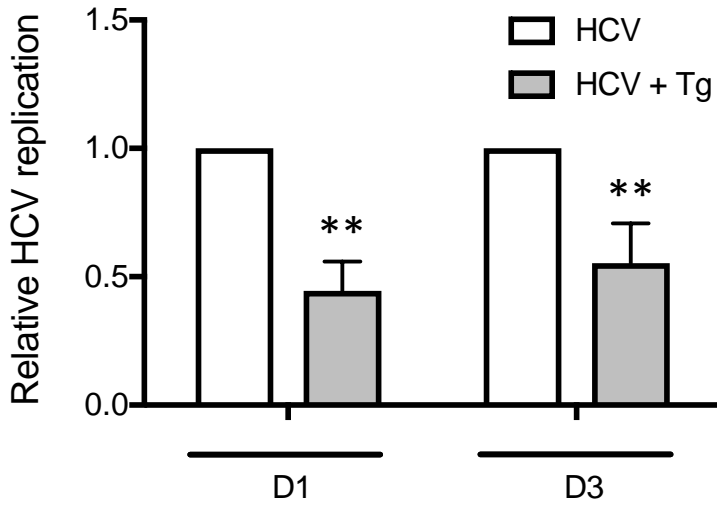
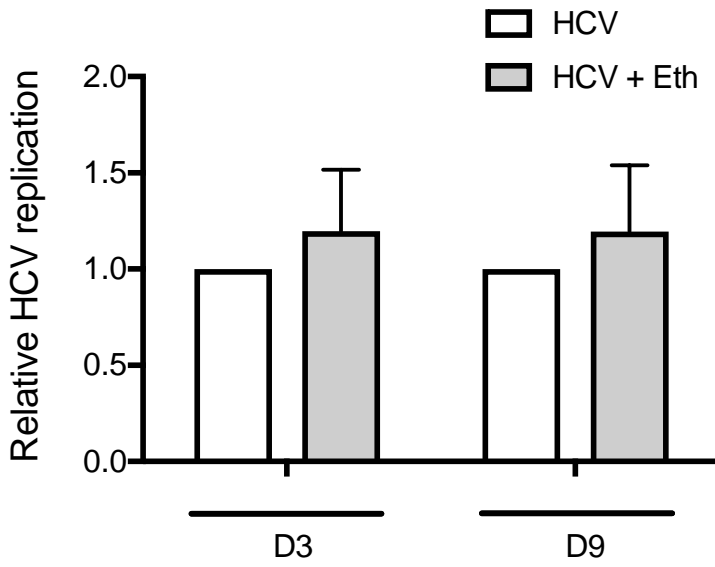
or 9 (*D9*). Shown are the changes (*n*-fold), relative to the levels in mock-infected PHH not exposed to any stressor (normalized to 1), expressed as the means  $\pm$  SEM of  $\geq 6$  independent experiments (-, non-significant; \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ ).

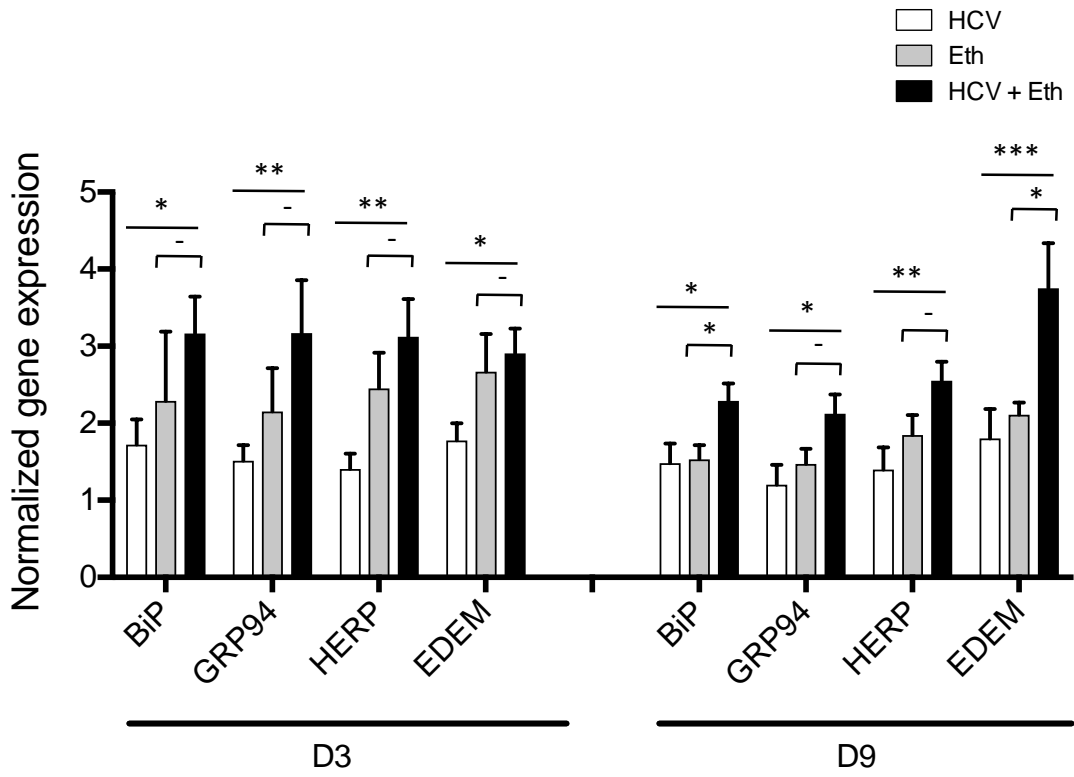


**a****b**

**a****b**

**a****b****c**

**a****b**

**a****b**