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Title page :

Non-oxidative ethanol metabolism in human hepatic cells *in vitro*: Involvement of uridine diphospho-glucuronosyltransferase 1A9 in ethylglucuronide production

Running title: Ethylglucuronide production by UGT1A9 in human hepatic cells

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Abstract

Ethanol is the most frequently psychoactive substance used in the world, leading to major public health problems with several millions of deaths attributed to alcohol consumption each year. Metabolism of ethanol occurs mainly in the liver via the predominant oxidative metabolism pathway involving phase I enzymes including alcohol dehydrogenases (ADH), cytochrome P450 (CYP) 2E1 and catalase. In a lesser extent, an alternative non-oxidative pathway also contributes to the metabolism of ethanol, which involves the uridine diphospho-glucuronosyltransferase (UGT) and sulfotransferase (SULT) phase II enzymes. Using liquid chromatography-high resolution mass spectrometry, ethylglucuronide (EtG) and ethylsulfate (EtS) produced respectively by UGT and SULT conjugation and detected in various biological samples are direct markers of alcohol consumption. We report herein the efficient non-oxidative metabolic pathway of ethanol in human differentiated HepaRG cells compared to primary human hepatocytes (HH). We showed dose- and time-dependent production of EtS and EtG after ethanol (25 or 50 mM) treatment in culture media of differentiated HepaRG cells and HH and a significant induction of CYP2E1 mRNA expression upon acute ethanol exposure in HepaRG cells. These differentiated hepatoma cells thus represent a suitable *in vitro* human liver cell model to explore ethanol metabolism and more particularly EtG and EtS production. In addition, using recombinant HepG2 cells expressing different UGT1A genes, we found that UGT1A9 was the major UGT involved in ethanol glucuronidation.

Keywords: Ethanol, Ethylglucuronide, Ethylsulfate, Hepatocytes, Uridine diphospho-glucuronosyltransferase, Sulfotransferase

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Abbreviations: BSA: bovine serum albumin, CYP: cytochrome P450, LC-HRMS: liquid chromatography coupled to high resolution mass spectrometry, UGT: UDP-glucuronosyltransferase, SULT: sulfotransferase, CHZ: chlorzoxazone, EtG: ethylglucuronide, EtS: ethylsulfate, CHZ-O-Glc: CHZ-O-glucuronide, OH-CHZ: 6-hydroxy-CHZ, HH: human hepatocytes

Highlights

- Ethylglucuronide (EtG) and Ethylsulfate (EtS) are two metabolites of ethanol used as direct markers of alcohol consumption.
- Using LC-HRMS, we report the dose- and time-dependent EtG and EtS production after ethanol treatment by human hepatocytes.
- Using recombinant HepG2 cells, we found that UGT1A9 was the major UGT involved in ethanol glucuronidation.

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1. Introduction

Ethanol is the most consumed legal drug worldwide and is considered to be the third leading cause of death in the United States (Nutt et al., 2007). Chronic excessive consumption of ethanol is associated with multi-organ toxicities, including hepatic, neurologic, cardiovascular or hematologic disorders (Bühler and Mann 2011; Smith et al. 2015; Osna et al. 2017; Obad et al. 2018; Le Daré et al. 2019). Alcohol is also involved in carcinogenesis of mouth, esophageal, hepatic, colic and breast cancer (Seitz and Stickel 2007). Thus, the mortality attributed to alcohol is mainly related to cancer, cirrhosis, road accidents, psychoses, and dependencies (Guérin et al., 2013).

In humans, ethanol is mainly metabolized through oxidative pathways in hepatocytes. Ethanol is initially oxidized into acetaldehyde by three enzymatic pathways located in different cellular compartments. This predominant phase I metabolism occurs in the cytosol via alcohol dehydrogenases (ADHs) but ethanol can also be metabolized by cytochrome P450 2E1 (CYP2E1) in the endoplasmic reticulum membrane (and to a lesser extent in mitochondria) and by catalase located in peroxisomes. Acetaldehyde is then oxidized by acetaldehyde dehydrogenase (ALDH), xanthine oxidase (XO) and aldehyde oxidase (AO) to produce acetate, which is released into the bloodstream and finally oxidized in carbon dioxide and water by tricarboxylic acid cycle (Zakhari 2006; Cederbaum 2012) (Supporting information 1A). Interestingly, ADH and ALDH show different characteristics depending on their genetic variants, which influence ethanol consumption and metabolism (Edenberg 2007). For example, ADH genotypes have been associated with differences in alcohol consumption and subjects with an ADH2*2 allele (highly active enzyme) have a decreased risk of alcoholism (Borràs et al. 2000). Also, in eastern Asia, 15-40% of the population have inactive ALDH2 isoenzymes and thus acetaldehyde levels are 5 to 20 times higher in these subjects than in individuals with active isoenzymes (Cederbaum 2012). Therefore, the resulting accumulation

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of acetaldehyde would be responsible for the lower tolerance to alcohol in these populations compared to the Caucasian population.

Besides this oxidative pathway, ethanol undergoes metabolism through the so-called non-oxidative pathway generating fatty acid ethyl esters (FAEE), phosphatidylethanol (PEth), ethylsulfate (EtS) and ethylglucuronide (EtG) (Kaphalia et al., 2004; Foti and Fisher 2005; Helander and Beck 2005). EtG and EtS are stable, non-volatile and water-soluble phase II metabolites produced by direct conjugation of ethanol to glucuronic acid *via* uridine diphospho-glucuronosyltransferases (UGTs) and to sulfate *via* cytosolic sulfotransferases (SULTs), respectively (Cabarcos et al. 2015) (Supporting information 1B). Notably, phase II conjugation enzymes exhibit a low affinity towards ethanol, which limits the contribution of this alternative pathway in the overall metabolism of this molecule (Cederbaum 2012). Although EtG and EtS are minor metabolites corresponding to less than 1% of ethanol excreted in urine (Dahl et al. 2002; Ingall 2012; Cabarcos et al. 2015), these stable compounds are reliable markers of ethanol consumption, particularly interesting given their sensitivity and specificity.

Indeed, their detection in blood or urine confirms recent ethanol consumption even at low doses (Foti and Fisher 2005; Helander and Beck 2005). Detection and quantification of these biomarkers are routinely used in clinical practice for evaluation, screening and diagnosis of patients with excessive ethanol consumption, as well as for monitoring abstinence (Ingall 2012) and forensic sciences (Andresen-Streichert et al., 2018).

While many reports described the detection of EtG and EtS in hair and biological fluids for clinical use and forensic medicine, intrinsic or exogenous factors that may modulate ethanol metabolism and production of EtG and EtS are not well characterized. Among these putative factors, genetic polymorphisms of metabolic enzymes, chronic or occasional consumption, nutritional factors, co-exposure to xenobiotics and liver diseases are thought to regulate the

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non-oxidative metabolic pathways of ethanol as observed for most other xenobiotics. Similarly, identity of the liver enzymes involved in the non-oxidative transformation of ethanol remains debated. For instance, contradictory data have been reported regarding UGT family members exhibiting the highest catalytic activities for EtG production. While several UGTs are obviously involved in the production of EtG, it was first described that UGT1A1 and 2B7 were the most prevalent isoforms generating EtG in cell free assay using recombinant UGTs (Foti and Fisher 2005). However, patients suffering from Gilbert's syndrome, a congenital metabolic disorder characterized with a decrease in UGT1A1 expression, show no differences in EtG formation following exposure to ethanol (Huppertz et al., 2015). Conversely, high rates of ethanol glucuronidation by UGT1A9 were reported using recombinant UGTs (Schwab and Skopp 2014). These discrepancies indicate that additional studies are required to better identify the UGT family members involved in EtG production.

Human hepatocytes in primary culture are considered as a suitable *in vitro* model to study hepatic metabolism of xenobiotics. However, hepatocytes isolated from patients present important differences in metabolic activities and lose their phenotype over time (including their capacity for drug metabolism and toxicity). Therefore, studies often combined different hepatic cell models including human hepatocytes in both 2D and 3D culture conditions and human hepatoma cell lines such as the differentiated HepaRG cells.

The HepaRG hepatoma cell line has emerged as a suitable alternative model to the use of human hepatocytes (Rogue et al., 2012). These cells differentiate into cholangiocyte- and hepatocyte-like cells in appropriate culture conditions (Cerec et al. 2007) and express most of the liver specific functions including the major CYPs (Aninat et al., 2006) and UGTs (Quesnot et al., 2018). In this context, the aim of this work was to evaluate the differentiated HepaRG cells for studying non-oxidative ethanol metabolism upon a single exposure that would mimic acute ethanol exposure in humans. For this purpose, we compared this

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metabolic pathway between HepaRG cells and human hepatocytes in primary culture through the quantification of EtG and EtS metabolites using Liquid Chromatography–High resolution–Mass spectrometry (LC-HRMS) following cell exposure to 25 and 50 mM ethanol corresponding to 1.15 and 2.30 g/L, respectively. Furthermore, expression of different SULT and UGT genes were investigated to study the capacity of ethanol to modulate their expression. Then, we brought further evidence that UGT1A1, UGT1A6 and more particularly UGT1A9, play a predominant role in EtG production.

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2. Material and methods

2.1. Reagents

Ethanol and propan-1-ol were purchased from VWR International (Fontenay-sous-bois, France). EtG, EtS and their deuterated analogs (EtG-D5 and EtS-D5) were obtained from Promochem (Molsheim, France). Chlorzoxazone (CHZ) and 6-hydroxychlorzoxazone (OH-CHZ) were purchased from Sigma-Aldrich (Saint Louis, MO). Chlorzoxazone β-d-Glucuronide (CHZ-O-Glc) was from Toronto Research Chemistry (Canada). Methanol was obtained from Fisher Scientific UK (Loughborough, UK). Phosphate-buffered saline (PBS), trypsin-EDTA and glutamine were purchased from Life Technologies (Paisley, UK). William's E medium (ref: 12551032) and William's E Medium without phenol red (ref: A12176-01) were purchased from Gibco (Thermo Fisher Scientific, Illkirch France). Penicillin and streptomycin were obtained from Life Technologies (Grand Island, NY). Fetal Bovine Serum (FBS) was purchased from Eurobio (Courtaboeuf, France) and from Hyclone™ GE Healthcare Life Sciences (Logan, UT). Hydrocortisone hemisuccinate was from Serb (Paris, France). Dimethyl sulfoxide (DMSO), formic acid, Minimum Essential Medium (MEM) culture medium and insulin were obtained from Sigma-Aldrich (Saint Louis, MO).

2.2. Cell cultures

2.2.1. HepaRG cells

Progenitor HepaRG cells were cultured as previously described (Aninat et al. 2006). Briefly, proliferating progenitor HepaRG cells were seeded at a density of 2.6×10^4 cells/cm^2 in 12-well plates and expanded in the following culture medium: William's E medium (A12176-01, Gibco) supplemented with 10 % FBS, 50 U/mL penicillin, 50 µg/mL streptomycin, 5 µg/mL insulin, 2 mM glutamine, 50 µM sodium hydrocortisone hemisuccinate. After 2 weeks, cells

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were quiescent and further cultured during two more weeks in the same medium supplemented with 2% DMSO in order to enhance differentiation and to obtain higher expression levels of liver specific functions in differentiated HepaRG hepatocyte-like cells. After 4 weeks, the cultures are differentiated in both cholangiocyte- and hepatocyte like cells (Cerec et al. 2007). The detection of ethylglucuronide and ethylsulfate was performed using this coculture model. To detect CHZ metabolites, cultures enriched in hepatocyte-like cells were used as previously described (Cerec et al. 2007; Corlu and Loyer 2015).

2.2.2. Primary human hepatocytes

Primary human hepatocytes (HH) were obtained from the processing of biological samples through the Centre de Ressources Biologiques (CRB) Santé of Rennes BB-0033-00,056 under French legal guidelines and fulfilled the requirements of the institutional ethics committee. Cells were isolated by collagenase-perfusion of liver biopsies from adult donors (Guguen-Guillouzo et al., 1982) and these cells were plated at a density of $1.5x10^5$ /cm^2 and cultured in the same William's E medium than HepaRG cells supplemented with 2% of DMSO. Cells were used 4 days after plating.

2.2.3. Parental and recombinant HepG2 cells.

HepG2 cells, obtained from American Tissue Culture Collection (ATCC, Los Altos, CA, USA) were seeded at a density of 6.6x104 cells/cm^2 in 12-well plates in MEM Eagle medium supplemented with 10% FBS, 50 UI/mL penicillin, 50 µg/mL streptomycin and 4 mM L-glutamine. The recombinant cells were derived from parental cells by transduction with lentiviral transgenes encoding the human CYP2E1, UGT1A1, UGT1A6 and UGT1A9 proteins, as previously described (Quesnot et al. 2018). All HepG2 cells were used 4 days after seeding.

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2.2.4. Ethanol treatment

Ethanol was added at different times in culture medium of cells located in 2 wells at the center of 12-well plates while the other wells were kept empty. One mL of medium containing ethanol (25 mM and 50 mM corresponding respectively to 1.15 g/L and 2.30 g/L) was incubated with cells in incubator under 95% air/5% CO_2 at 37°C. After treatment, supernatants were collected in order to be analyzed.

2.3. Ethanol quantification

Ethanol concentrations in culture medium of cells ~~supernants~~ were quantified by headspace gas chromatography – flame ionization detector (HS-GC-FID). Analyses were performed on a Thermo Scientific TRACE 2000 GC including a TriPlus Headspace autosampler (Thermo Scientific). Data acquisition, peak integration and calibration were performed using the ChromQuest 5.0 software (ThermoScientific, San Jose, CA). Briefly, 200 µL of sample and 200 µL of propan-1-ol (internal standard) were added in headspace crimp vials and incubated during 20 minutes at 80°C. GC separation was performed on a Innowax column (30 m x 0.25 mm; 0.25 µm) (Interchim, Montluçon, France) at 40°C with N_2 as mobile phase and detected at 250°C by FID. This method quantified simultaneously ethanol, methanol and acetaldehyde in samples. Quality controls were passed each day.

2.4. Quantification of EtG and EtS

Culture medium of cells (90 µL) were supplemented with internal standards (EtG-D5, EtS-D5) and treated with 1000 µL of methanol. Samples were then vortexed, centrifugated for 10 min at 3000 *g* and supernatants were evaporated to dryness at 50°C under a stream of nitrogen. Residues were dissolved in 100 µL of water and transferred for LC-HRMS analysis (Q Exactive®, Thermo Fisher Scientific). LC separation was performed using a C18 Accucore

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column (100 mm x 2.1, 2.6 µm) (ThermoScientific). The mobile phase used was H_2O LC-MS grade containing 0.1% (v/v) formic acid and was delivered at a flow rate of 500 µL/min during 3 minutes in a thermostated column at 30°C. Data were acquired in ~~positive~~ negative heated electrospray ionization mode coupled to Targeted SIM (Single Ion Monitoring) mode. The measured accurate *m/z* values of the ions ~~protonated species~~ were 124.9910, 130.0225, 221.0679 and 226.0994 for EtS, EtS-D5, EtG, and EtG-D5, respectively. Quantification was performed by extracting the exact mass of each of the protonated species using a 5 ppm extraction window. LC-HRMS method parameters are further detailed in the Supporting information 2.

2.5. *Quantification of CHZ and its metabolites*.

To quantify CHZ and its metabolites, William's E medium without red phenol was used. To quantify OH-CHZ, enzymatic hydrolysis of CHZ-O-Glc was performed for 2 h at 37°C and pH 5 with 200 IU of β-glucuronidase (Sigma Aldrich G0251).

Samples were analyzed by high pressure liquid chromatography (HPLC) with ultraviolet (UV) detection at λ = 284 nm by injecting samples (40 µL) on an Agilent Series 1100 (Waldbronn, Germany) equipment with a 75×3 mm Ace Excel 2 Super C18 column (ThermoFisher Scientific). Elution was performed with a constant flow rate of 0.7 mL/min using a linear gradient elution from 2 to 60% of acetonitrile for 10 min. The eluents were 0.1% acetic acid in water with 0.25% (m/v) of triethylamine hydrochloride.

2.5.1. Incubation medium used in experiments involving CHZ

In order to investigate UGT1A and CYP2E1 catalytic activity by competition between ethanol and CHZ, HepaRG cells were incubated with 300 µM CHZ in William's E culture medium without phenol red during 4 h prior to detection of the CHZ glucuronide (CHZ-O-Glc) with

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direct injection of culture media in HPLC column. HepaRG cells were incubated for 8 h with 300 µM CHZ and without or with 50 mM ethanol, and the CHZ-O-Glc production was analyzed by HPLC.

2.5.2. Pre-incubation medium of ethanol: Induction of CYP2E1 activity

In order to induce CYP2E1 expression prior to the evaluation of its activity in the presence of CHZ with or without ethanol, HepaRG cells were incubated during 48 h with William's E medium (1X) (A12176-01, Gibco) supplemented with 10 % FBS, 50 U/mL penicillin, 50 µg/mL streptomycin, 5 µg/mL insulin, 2 mM glutamine, 50 µM sodium hydrocortisone hemisuccinate and 1% DMSO. Then, cells were incubated with 300 µM CHZ in William's E culture medium without phenol red alone during 4 h prior to detection of CHZ-O-Glc with direct injection of culture media in HPLC column.

2.6. Real-time quantitative Reverse Transcription PCR (RT-qPCR) analysis

After cells lysis, total RNAs were isolated using a Macherey-Nagel NucleoSpin® RNAII kit, according to the manufacturer's protocol. Total RNAs (0.5 µg) were reverse-transcribed into first-strand cDNAs using a High-Capacity cDNA Achieve Kit (Applied Biosystems, Foster City, CA), according to the manufacturer's instructions. RNA quantity and purity were assessed with a Nanodrop ND-1000 spectrophotometer (Nyxor Biotech, Paris, France). Real-time qPCR was performed using the fluorescent dye SYBR Green method, with SYBR Green PCR Master Mix in 384-well plates and the StepOnePlus™ system (Applied Biosystems). Human GAPDH was used as the reference gene. Relative quantification values were expressed using the $2^{\Delta\Delta Ct}$ method as fold changes of the target gene, normalized to the reference gene, and related to the expression level in control experiments (arbitrarily set to 1).

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The sequences of the forward and reverse primers used in this study are available in the Supporting information 3.

2.7. Statistical analysis

The results were expressed as means ± standard error of the mean (SEM). Intergroup differences as a function of the treatment were analyzed in a one-way analysis of variance (ANOVA) with Tukey's post-hoc test. When two parameters were concomitantly studied, intergroup differences were tested in a two-way ANOVA with Bonferroni post-hoc correction. All analyses were performed using Prism 5.0 software (Graphpad Software, La Jolla, CA). For each analysis, two sided *p* value less than 0.05 were considered statistically significant.

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3. Results

3.1. HepaRG cells as a cellular model to study ethanol metabolism

In order to study the non-oxidative metabolism of ethanol, we have chosen a model of acute ethanol exposure of differentiated HepaRG cells and HH since these two cell models are metabolically competent. Two different concentrations of ethanol (25 and 50 mM) were used. HepaRG cells were exposed for 8, 24, 48 and 72 h while hepatocytes were treated for 24 and 48 h because of their limited availability. Cells exposed to ethanol were maintained in a CO_2 incubator dedicated to this treatment while control cells were cultured in a separate incubator to avoid ethanol dispersion and cross-contamination between conditions.

We first evaluated four models of ethanol evaporation using 12-well plates with different settings in absence of cells (Supporting information 4). Model 1: wells with medium containing ethanol surrounded by empty wells. Model 2: wells with medium containing ethanol surrounded by wells containing PBS. Model 3: wells with medium containing ethanol surrounded by PBS supplemented with ethanol at the same concentration. Model 4: same model as model 3 but with plates sealed with parafilm. Ethanol evaporation was very different depending on the models (Supporting Information 4), although models 1 and 2 exhibited the same profile with ~20% of the initial ethanol concentration at 24 h, ~10% at 48 h and <5% at 72 h. In model 3, ethanol evaporation was slowed down with ~75% of the initial ethanol levels at 24 h and ~40% at 72 h. In model 4, evaporation was even more slowed down in sealed wells. Indeed, ~30% of ethanol disappeared from the medium within 48 h and then the concentration remained stable.

We then performed experiments using the different models with HepaRG cells in the central wells. Indeed, considering the large variations between the different models regarding the remaining ethanol content in the culture media, we assessed LDH release and cellular ATP content in HepaRG cells cultured according to models 1, 3 and 4. We did not observe any

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LDH release from HepaRG cells at any time points or any concentrations (data not shown) suggesting no significant cytolysis. However, ethanol exposure induced a slight but significant decrease in cellular ATP content regardless of the different ethanol concentrations found in the culture media after evaporation in the 3 experimental settings (Supporting information 5).

In our study, *in vitro* ethanol concentrations of 25 mM (1.15 g/L) and 50 mM (2.3 g/L) were compatible with blood alcohol concentrations found in acute human consumption (Jones 2010). In addition, evidence-based survey showed that human ethanol elimination rate ranged from 10 to 35 mg/100mL/h (Jones 2010), which leads to estimate the entire elimination of 2.3 g/L blood alcohol within ~23 h. Using model 1, in which ethanol decay resembled to that observed *in vivo* (Jones 2010), we next studied ethanol elimination in absence and presence of HepaRG cells (Figure 1A, B) and HH (Figure 1C, D). In these conditions, our data showed that the decrease in ethanol content in the culture medium was mainly due to evaporation, although a significant reduction in ethanol concentrations was observed in culture wells containing cells compared to wells without cells. The decay curves were similar for both cell types and ethanol concentrations (25 and 50 mM) with a 4- to 5-fold decrease in ethanol contents at 24 h. At 48 h, ethanol concentration was < 3 mM and ethanol was barely detectable at 72 h. Taking into consideration our *in vitro* data and previous clinical investigations (Pizon et al. 2007; Jones 2010), further experiments were carried out using experimental model 1.

It has been established many years ago that CYP2E1, mainly expressed in hepatocytes, is an ethanol-inducible CYP oxidizing ethanol *in vivo* (Koop 1992; Ingelman-Sundberg et al., 1993). Similarly, ethanol increases CYP2E1 expression and activity in HH in primary culture (Mahli et al., 2019) and in HepaRG cells (Do et al., 2013). Hence, we next determined whether acute ethanol exposure induced CYP2E1 expression and activity. We observed that

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CYP2E1 mRNA expression was induced in a dose-dependent manner after ethanol treatment (25 or 50 mM) at 8 and 24 h in HepaRG cells (Figure 1E). We also verified whether the increase in *CYP2E1* mRNA levels correlated with enhanced catalytic activity by detecting the 2 main CHZ metabolites produced via CYP2E1-dependent oxidation (Quesnot et al. 2018). We found an increase in the amounts of OH-CHZ and its down-stream metabolite CHZ-O-Glc in the culture media of HepaRG cells following a 24 h ethanol treatment (Figure 1F).

3.2. Production of EtG and EtS by HepaRG cells and HH

In order to demonstrate that differentiated HepaRG cells and HH *in vitro* can produce phase II metabolites of ethanol (EtS and EtG) upon an acute exposure, we developed and validated an original LC-HRMS method to detect and quantify these metabolites in cell culture media (Supporting information 2). Using this approach, we showed that both EtS and EtG were detected as early as 8 h in media of HepaRG cells exposed to ethanol and accumulated in a time- and dose-dependent manner in both cell types (Figure 2). In particular, concentrations of these metabolites were almost twice as high upon 50 mM ethanol treatment as compared to 25 mM ethanol. The profiles of metabolite's accumulation appeared quite similar between HepaRG (Figure 2A, C) cells and HH (Figure 2B, D). For 25 mM ethanol, we observed an equal production of metabolites in both cell types with 1 µM of EtG and 0.5 µM of EtS at 24 h. For exposure to 50 mM ethanol, both cell models produced ~1 µM of EtS while EtG concentration reached 1.7 and 3 µM in HH and HepaRG cells, respectively. The rapid accumulation of metabolites found within the first 24 h of ethanol exposure followed by the relative steady amounts of EtG and EtS after 24 h was correlated to the strong decrease in ethanol concentration in the culture media due to the evaporation of ethanol at 37°C (Figure 1).

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To further determine whether the amounts of EtG and EtS correlated with the remaining concentrations of ethanol in the culture media, we measured EtG and EtS levels in media of HepaRG cells treated for 72h with 25 and 50 mM ethanol using models 1, 3 and 4. Indeed, ethanol concentrations in these models remained much higher over the 3-day treatment because of less evaporation (Supporting Information 4). As expected, EtG and EtS concentrations were higher in these models than in model 1 (Figure 2E, F). Finally, we also demonstrated the production of EtG and EtS by precision cut liver slices (PCLS) prepared from a human liver biopsy and treated with ethanol for 48 h and confirmed that the amounts of both metabolites were higher when PCLS were cultured in sealed flasks that maintained elevated levels of ethanol during the incubation time (Supporting information 7).

3.3. SULT and UGT expression

It has been previously reported that ethanol induces different SULT and UGT gene family members in rat liver *in vivo* and in primary rat hepatocytes (Farinati et al., 1989; Kardon et al., 2000; Li et al., 2000). To confirm that HepaRG cells express most of the hepatocyte specific functions (Rogue et al., 2012) in our culture conditions, we first compared the relative mRNA levels of several ADHs, ALDHs, SULTs, and UGTs as well as CYP2E1 between HH and differentiated HepaRG cells (Supporting information 6). As previously reported (Rogue et al., 2012), all the genes expressed in HH were also detected in HepaRG cells confirming their high levels of differentiation.

We next studied the expression of different SULTs and UGTs at the mRNA levels between untreated and ethanol-treated differentiated HepaRG cells (Figures 3 and 4). At 8 h, we did not observe any elevation of mRNA SULT expression (*SULT1A1, SULT1B1, SULT1E1, SULT2A1*) in ethanol-treated HepaRG cells compared to untreated cells while at 24 h, mRNA SULT expression of these different SULTs was slightly increased after ethanol treatment

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compared to the control cells (Figure 3). Similarly, the mRNA levels of *UGT1A1*, *UGT1A6* and *UGT2B15* were slightly but significantly more elevated in ethanol-treated cells than in control cultures while no significant differences were found for *UGT1A9*, *UGT2A3*, *UGT2B4* and *UGT2B7* (Figure 4). The overall SULT and UGT induction remained rather weak in our acute condition of ethanol treatment of HepaRG cells.

3.4. Ethanol and CHZ are competitors for CYP2E1 and UGT enzymatic activities.

Previous studies have reported that UGT1A1, UGT1A9 and UGT2B7 catalyzed the formation of EtG using recombinant UGTs (Foti and Fisher 2005; Schwab and Skopp 2014) while patients suffering from Gilbert's syndrome who have much lower expression in UGT1A1 show no differences in EtG formation following exposure to ethanol (Huppertz et al., 2015). We have recently demonstrated that CHZ [5-chloro-2(3*H*)-benzoxazolone], a centrally acting myorelaxant indicated for musculoskeletal pain (Chou et al., 2004), produced CHZ-O-Glc following the CYP2E1-mediated formation of OH-CHZ (Quesnot et al., 2018). Moreover, CHZ-O-Glc synthesis is catalyzed by UGT1A1, UGT1A6 and UGT1A9 but not by UGT2B7 (Quesnot et al., 2018). Thus, we investigated whether ethanol could modulate the UGT-mediated metabolism of CHZ, postulating that a decrease in CHZ-O-Glc production in presence of ethanol would further indicate that UGT1A enzymes generate EtG.

HepaRG cells were incubated for 8 h with 300 µM CHZ and without or with 50 mM ethanol, and CHZ-O-Glc production (reflecting UGT1A activity) was analyzed by HPLC (Figure 5). The HPLC chromatograms (Figure 5A) showed that the peak of CHZ-O-Glc was higher in culture media of cells treated with CHZ only compared to cells co-incubated with both ethanol and CHZ. Peak area quantification indicated that co-incubation with 50 mM ethanol reduced by 50% the production of CHZ-O-Glc (Figure 5B). Conversely, CHZ concentration was higher in culture media of cells co-incubated with ethanol and CHZ compared to cells

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incubated with CHZ only (Figure 5B). Together, these data demonstrated that ethanol was a competitor of CHZ for glucuronide conjugation and strongly suggested that UGT1A enzymes are involved in EtG synthesis.

3.5. Glucuronidation of ethanol by UGT1 subfamily.

In order to determine the UGT1A family members involved in EtG production, we took advantage of the different recombinant HepG2 cell lines recently established in our laboratory respectively expressing *UGT1A1, UGT1A6* and *UGT1A9* (Quesnot et al., 2018) in order to compare the production of EtG in each of these cell lines. The parental HepG2 cells are hepatic cells expressing very low levels of these UGTs (Quesnot et al., 2018) and presenting reduced xenobiotic metabolism (Donato et al., 2015). The enforced expression of one UGT gene thus allows to determine the involvement of the corresponding enzyme in EtG production.

We first confirmed that the recombinant HepG2 cell lines expressed high levels of the three *UGT1A1, UGT1A6* and *UGT1A9* genes at the mRNA level (Figure 6A-C). As expected, each individual recombinant cell line expressed very high mRNA levels of one UGT gene. We also set up a RT-qPCR condition using "panUGT1A" primers that totally match with conserved sequences of UGT1A1, UGT1A6 and UGT1A9 in order to compare whether the overall expression of these UGTs was similar in the different recombinant cell lines (Figure 6D). We found that *UGT1A1, UGT1A6* and *UGT1A9* mRNA levels were not significantly different using the "panUGT1A" primers indicating that the overall expression of these different UGTs in the three cell lines was relatively similar. As negative control, we used parental HepG2 cells (control) and the recombinant CYP2E1 HepG2 cell line (Quesnot et al., 2018) that expresses very high levels of CYP2E1 mRNAs and low amounts of the *UGT1A1, UGT1A6* and *UGT1A9* genes (Figure 6E). Using these cell lines, we did not detect by LC-HRMS any

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EtG in the culture media of the parental and CYP2E1 HepG2 cells after 24 h of 50 mM ethanol treatment (Figure 6F), while the glucuronide was found in the supernatants of UGT expressing cells with the highest amounts detected for the *UGT1A9* recombinant cells.

4. Discussion

In this study, we have further characterized the *in vitro* human HepaRG hepatoma cells with the goal to investigate ethanol metabolism, and more particularly the production of EtG and EtS metabolites.

Our first effort was to develop a model of ethanol acute exposure that would resemble the physiological ethanol elimination in humans by controlling ethanol concentration in the culture wells. To this end, we choose 25 and 50 mM in culture media corresponding to blood concentrations of 1.15 and 2.3 g/L during an acute ethanol intoxication in human. Ethanol is a highly volatile molecule that disappears from the culture medium at 37°C through evaporation in air, thus contaminating adjacent wells of the same culture plate (Blein et al., 1991; Borgs et al., 1993). Hence, in order to avoid contamination by ethanol between wells, we have chosen 12-well culture plates where only the two central wells were filled, while peripheral wells were left empty (model 1, Supporting Information 4). In addition, other models were tested concerning the peripheral wells such as (i) wells containing PBS, (ii) wells containing PBS with ethanol and (iii) wells containing PBS with ethanol in culture plates sealed with parafilm (Models 2 to 4, Supporting information 4). However, Model 1 seemed the most appropriate since the decay profile of ethanol concentration was fast and comparable to the *in vivo* ethanolemia decay profile following acute ethanol exposure in humans (Pizon et al. 2007; Jones 2010). Indeed, at the chosen concentrations, we observed a complete disappearance of ethanol at 48 h using the GC-FID analytic method, as observed in humans (Jones 2010). In addition, no cytotoxicity was observed at any time points or for any concentrations in both

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HepaRG cells and HH. However, while the LDH release assay showed no cytolysis, cellular ATP content was moderately decreased. This suggests that ethanol could affect energy metabolism and oxidative phosphorylation without inducing major cell death, even when ethanol concentrations remain high over 72 h. Reduced cellular ATP most probably reflects the various deleterious effects of ethanol on mitochondrial function, including fatty acid oxidation and oxidative phosphorylation (Fromenty and Pessayre 1995).

In the selected model of ethanol exposure, alcohol decay was largely due to the evaporation under our conditions of culture since less than 10% difference in ethanol concentrations was observed between wells with and without cells (HH or HepaRG). Next, we set up a reliable analytic method to detect ethanol metabolites in culture media of hepatic cells to demonstrate that HepaRG cells are competent for ethanol metabolism. After 48 h with 50 mM ethanol, the production of phase II metabolites represented only few µM of EtG and EtS, *i.e* less than 1% of the initial ethanol concentration, in agreement with previous data reporting phase II metabolism *in vivo* (Dahl et al., 2002; Cabarcos et al., 2015).

Regarding the oxidative metabolism, we first looked at the CYP2E1 expression in HepaRG cells following exposure to ethanol. CYP2E1 is considered as the main CYP responsible for microsomal and mitochondrial ethanol metabolism into acetaldehyde, which has been associated with alcohol-induced liver damage (Lieber 2005; Knockaert et al. 2011). In addition, CYP2E1 expression is known to be induced by ethanol consumption (Gonzalez et al., 1991; Takahashi et al., 1993; Zhukov and Ingelman-Sundberg 1999; Balusikova and Kovar 2013). Herein, we report a moderate but significant induction of *CYP2E1* mRNA expression after an acute ethanol treatment in a dose-dependent manner demonstrating that differentiated HepaRG cells have kept an ethanol-inducible CYP2E1 gene expression. Moreover, we showed for the first time an ethanol-mediated induction of CYP2E1 catalytic activity in human hepatoma cells. Since induction of CYP2E1 mRNA is predominantly

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described during chronic exposure to ethanol, it may be postulated that the early increase in activity of this enzyme is more likely attributed to the stabilization of CYP2E1 mRNA and/or protein by ethanol (Zhukov and Ingelman-Sundberg 1999).

The oxidative metabolism of ethanol was difficult to study under our culture conditions. Indeed, ethanol is oxidized to acetaldehyde by ADH and CYP2E1, then further metabolized to acetate by ALDH (Cederbaum 2012). Although both HepaRG cells and HH express these enzymes (Quesnot et al., 2018), we did not detect acetaldehyde nor acetate by our methods most likely because of too low concentrations in the culture medium. Acetaldehyde has a boiling point at only 20.2 °C and the capacity of ALDH to remove acetaldehyde notably exceeds the capacity of its generation by the different pathways of alcohol oxidation. The absence of acetate detection can also be explained by its rapid metabolism to CO_2, fatty acid, cholesterol or ketone bodies (Cederbaum 2012).

Because our knowledge on the enzymes involved in the conjugation of ethanol is incomplete and partly controversial (Stachel and Skopp 2015), we investigated the non-oxidative metabolism of ethanol. EtG and EtS are non-volatile and water-soluble non-oxidative metabolites formed in the liver and excreted in urine. These markers have also been shown to be stable *in vitro* (Walsham and Sherwood 2014). Following a single exposure of both HepaRG cells and HH to ethanol, we detected ethanol-related phase II-metabolites using an innovative and fully validated LC-HRMS method. To our knowledge, this is the first quantification by LC-MS of EtG and EtS in culture media that revealed the non-oxidative production of ethanol metabolites in a dose and time dependent manner in metabolically active hepatic cells *in vitro*. These results also suggested that UGTs and SULTs were not saturated for 25 and 50 mM ethanol since the amounts of both metabolites were much higher in culture settings that maintained elevated amounts of ethanol over 3 days of treatment. The amount of EtG produced was greater than EtS, similar to what has been found *in vivo* (Halter

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et al., 2008; Kummer et al., 2013). In addition, the EtS/EtG ratio (molar concentration) was lower for HepaRG cells (0.36) than for human hepatocytes (0.59). I*n vivo*, in urine, this ratio has previously been found to be less than 1 (Halter et al. 2008; Winkler et al. 2013). Altogether, these results would suggest that UGTs have higher metabolic activity towards ethanol than SULTs both *in vivo* and in the *in vitro* models of hepatocytes used in this study.

SULT1A1 and SULT2A1 are the main SULT isoforms expressed in liver (Riches et al., 2009; Kurogi et al., 2012; Stachel and Skopp 2016). Here, we confirmed that *SULT1A1* and *SULT2A1* mRNA are expressed at comparable levels between HepaRG and HH. Furthermore, *SULT1A1, SULT1B1, SULT1E1* and *SUL2A1* mRNA expression was slightly induced after 24 h-ethanol treatment in HepaRG cells. The phase II-UGT enzymes are also expressed at high levels in liver, especially UGT2B4, UGT2B15, UGT1A9 and UGT2B7 (Ohno and Nakajin 2009). Our results also confirmed similar UGT mRNA expression levels between HepaRG cells and HH. UGT mRNA expression was barely induced by ethanol excepted *UGT2B15* at 25 mM after 24 h, *UGT1A1* at 50 mM after 8 and 24 h and *UGT1A6* at 50 mM after 24 h. To our knowledge, there are no data available on the effect of ethanol on UGT expression in human. Only one study showed induction of UGT1A1 expression after chronic ethanol exposure in rats (Kardon et al., 2000).

Several UGTs are most likely involved in the production of EtG but contradictory data have been published regarding the most prevalent isoforms generating EtG. In cell free assays using recombinant UGTs, UGT1A1, 1A9 and 2B7 were identified as the most implicated enzymes (Foti and Fisher 2005; Ohno and Nakajin 2009; Court et al., 2012; Al Saabi et al., 2013; Schwab and Skopp 2014; Stachel and Skopp 2016). To further identify which UGTs can catalyze ethanol glucuronidation, we used recombinant HepG2 cells expressing CYP2E1, UGT1A1, UGT1A6 and UGT1A9. Our results demonstrated that recombinant HepG2 cells expressing UGT1A1, UGT1A6 and UGT1A9 were able to metabolize ethanol in EtG while

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native HepG2 cells and HepG2 cells expressing CYP2E1 did not produce this glucuronide. Furthermore, the UGT1A9 isoform seems to be the most active in ethanol glucuronidation in agreement with a previous study showing that UGT1A9 and UGT2B7 appeared to be the main isoforms producing EtG in recombinant microsomes (Stachel and Skopp 2015). Further experiments would be required to establish additional recombinant HepG2 cells expressing other UGTs and SULTs and to study their involvement in EtG and EtS production.

Our results also demonstrated that HepaRG cells represent a suitable hepatic cell model to study ethanol metabolism. Indeed, we showed that expression of phase I and phase II genes and ethanol metabolism are very similar between these cells and HH. After characterizing this experimental model, we would like to extend this study to the metabolism of ethanol in pathological conditions observed in humans. Indeed, our laboratory has developed culture conditions of HepaRG cells to induce steatosis (Michaut et al., 2016), inflammation (Al-Attrache et al., 2016) and cholestasis (Bachour-El Azzi et al., 2014; Sharanek et al., 2016). These diseases are frequently related to, or associated with, alcohol consumption. Thus, our models will allow a better understanding of the metabolism, and therefore the toxicity of ethanol in different pathological conditions in human hepatocytes.

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Authorship contributions

Participated in research design: Hugbart Chloé, Le Daré Brendan, Vène Elise, Lagente Vincent, Fromenty Bernard, Bouvet Renaud, Morel Isabelle, Loyer Pascal, Gicquel Thomas.

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Wrote or contributed to the writing of the manuscript: Hugbart Chloé, Loyer Pascal, Le Daré Brendan, Gicquel Thomas.

Conflicts of Interest

Authors declare that they have no conflicts of interest that might be relevant to the content of the manuscript.

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FIGURE LEGENDS

Figure 1. Evaporation of ethanol (EtOH) in culture multiwell plates in absence and presence of cells and effect of ethanol (EtOH) on CYP2E1 expression and activity in differentiated HepaRG cells and Human Hepatocytes in primary culture (HH). Evaluation of ethanol elimination from culture media in 12 culture well-plates in absence (dashed line) or presence (plain line) of differentiated HepaRG cells (A, B) and human hepatocytes (HH: C, D) at 25 and 50 mM of ethanol in open multiwell plates with only 2 central wells containing ethanol (Model 1, Supporting information 4). Statistics: ***p<0.001, *p<0.05 for ethanol (EtOH) concentrations in cultures of HepaRG cells and HH compared to control cultures without cells at each time.
Differentiated HepaRG cells and HH were incubated during different times (8, 24, 48 or 72 h) with EtOH (25 or 50 mM). The effects of EtOH treatment on CYP2E1 expression was analyzed by RT-qPCR at 8 and 24h of treatment in differentiated HepaRG cells (E). The effect of EtOH (50 mM) on CYP2E1 activity was measured in differentiated HepaRG cells using the chlorzoxazone (CHZ) as a substrate and by detecting its primary metabolite, the 6-hydroxychlorzoxazone (OH-CHZ), and the secondary metabolite, the chlorzoxazone O-Glucuronide (CHZ-O-Glc) by HPLC-UV in culture media at 4h of 50 mM EtOH incubation (control amount corresponds to 100%) (F). The data are expressed as the mean ± SEM for three independent experiments performed in triplicate. Statistics: **p<0.01 and *p<0.05 for cultures exposed to ethanol treatment compared with the control cultures (E) or CHZ (F).

Figure 2. Ethylglucuronide (EtG) and ethylsulfate (EtS) production by differentiated HepaRG cells and Human Hepatocytes in primary cultures (HH) incubated with ethanol (EtOH). Differentiated HepaRG cells and HH were incubated during different times (8, 24, 48 or 72 h) with EtOH (25 or 50 mM) in open multiwell plates with only 2 central wells containing ethanol (Model 1, Supporting information 4). Concentrations of metabolites EtG (A, B) and EtS (C, D) were measured by LC-HRMS in the culture media of these cells. Differentiated HepaRG cells were also incubated during 72 h with EtOH at 25 mM (E) and 50 mM (F) in other multiwell plates settings (model 1, 3 and 4, Supporting Information 4) to obtain various remaining concentrations of ethanol within the wells containing cells and concentrations of metabolites EtG and EtS were measured. Results are expressed in µM and the data are expressed as the mean ± SEM for three independent experiments performed in triplicate. Statistics: ***p<0.001 **p<0.01 and *p<0.05 for cultures exposed to 25 mM EtOH treatment compared with 50 mM EtOH treatment at each time. ###p<0.001 and ##p<0.01 for cultures exposed to 50 mM EtOH treatment compared with 8 h at 50 mM EtOH treatment. $$$p<0.001 $$p<0.01 and $p<0.05 for cultures exposed to 25 mM EtOH treatment compared with 8 h at 25 mM EtOH treatment. αp<0.05 for cultures exposed to 25 mM EtOH treatment compared with 24 h at 25 mM EtOH treatment. Statistics (E, F): Results are expressed in µM and the data are expressed as the mean ± SD of one experiment performed in quadruplicate.

Figure 3. Expression of sulfotransferases (SULTs) in differentiated HepaRG cells. Cells were cultured in medium alone (Control) or medium supplemented with ethanol (EtOH) at 25 mM and 50 mM for 8 or 24 h. The mRNA expression levels of *SULT1A1* (A), *SULT1B1* (B),

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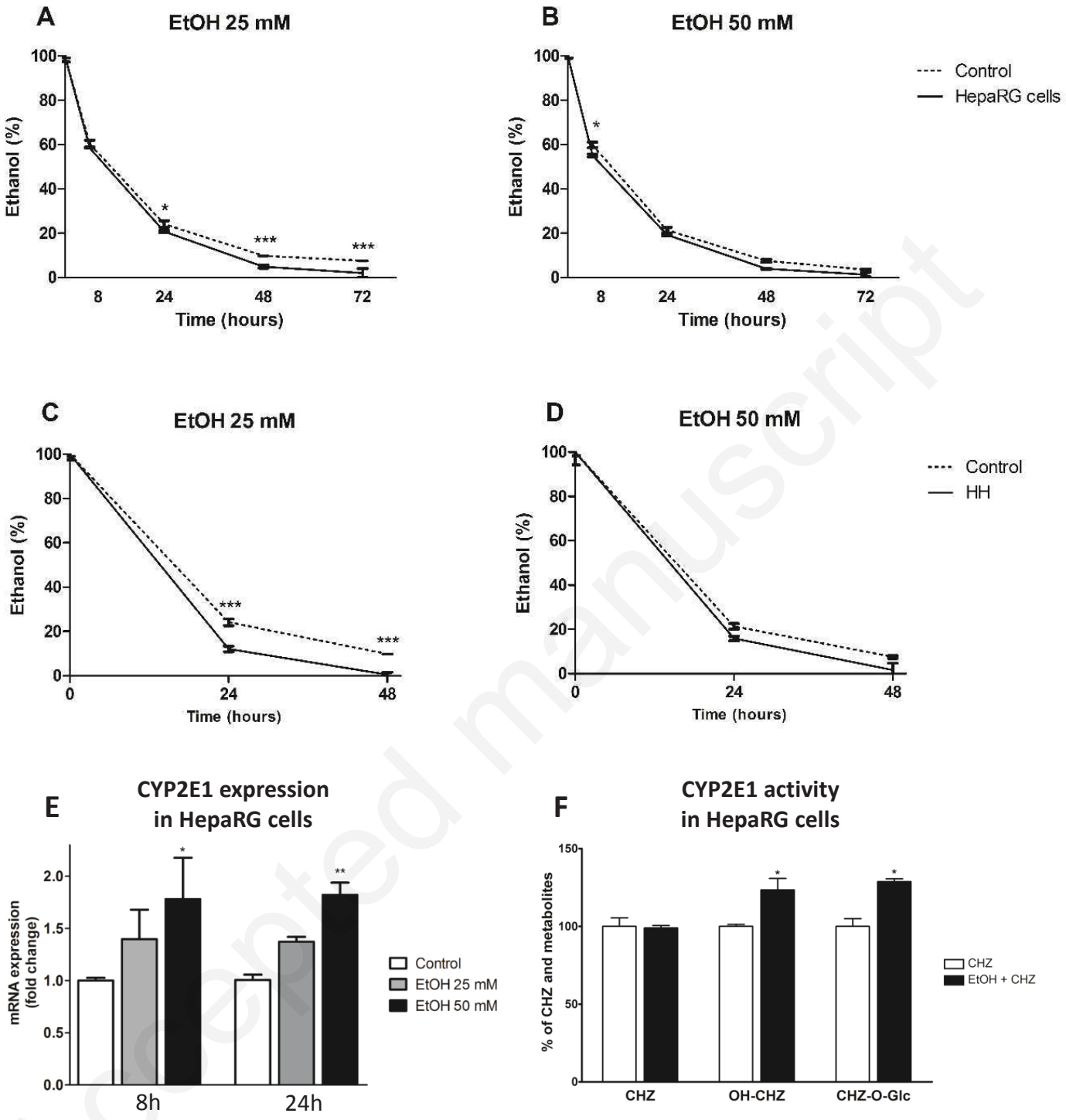
SULT1E1 (C) and *SULT2A1* (D) were determined using RT-qPCR. Results are normalized to GAPDH gene expression. The data are expressed as the mean ± SEM for three independent experiments performed in triplicate. Statistics: **p<0.01 and *p<0.05 for cultures exposed to ethanol treatment compared with the control cultures.

Figure 4. Expression of uridine diphosphate-glucuronosyltransferases (UGT) in differentiated HepaRG cells. Cells were cultured in medium alone (control) or medium supplemented with 25 mM or 50 mM ethanol (EtOH) at for 8 or 24 h. The mRNA expression levels of *UGT1A1* (A), *UGT1A6* (B), *UGT1A9* (C), *UGT2A3* (D), *UGT2B4* (E), *UGT2B7* (F) and *UGT2B15* (G) were determined using RT-qPCR. Results are normalized to GAPDH gene expression. The data are expressed as the mean ± SEM for three independent experiments performed in triplicate. Statistics: **p<0.01, *p<0.05 for cultures exposed to ethanol treatment compared with the control cultures.

Figure 5. UGT enzymatic activity in differentiated HepaRG cells treated by ethanol. Cells were incubated with incubation medium (William's E medium without red phenol) containing 300 µM of chlorzoxazone (CHZ) or incubation medium (William's E medium without red phenol) supplemented with both CHZ (300 µM) and ethanol (EtOH) at 50 mM for 4 h. The production of the CHZ-derived glucuronide (CHZ-O-Glc), reflecting UGT1A catalytic activity was analyzed by HPLC. Typical HPLC chromatograms (Fig. 5A) showing the peaks of CHZ-O-Glc and CHZ in culture media of cells incubated with CHZ and CHZ+EtOH. Peak area quantification of CHZ and CHZ-O-Glc (B) as the mean ± SEM for three independent experiments performed in triplicate. Results are expressed as percent of control cultures (with CHZ) set at 100%. Statistics: ***p<0.001 for cultures exposed to CHZ treatment compared with the cultures co-incubated with CHZ and EtOH.

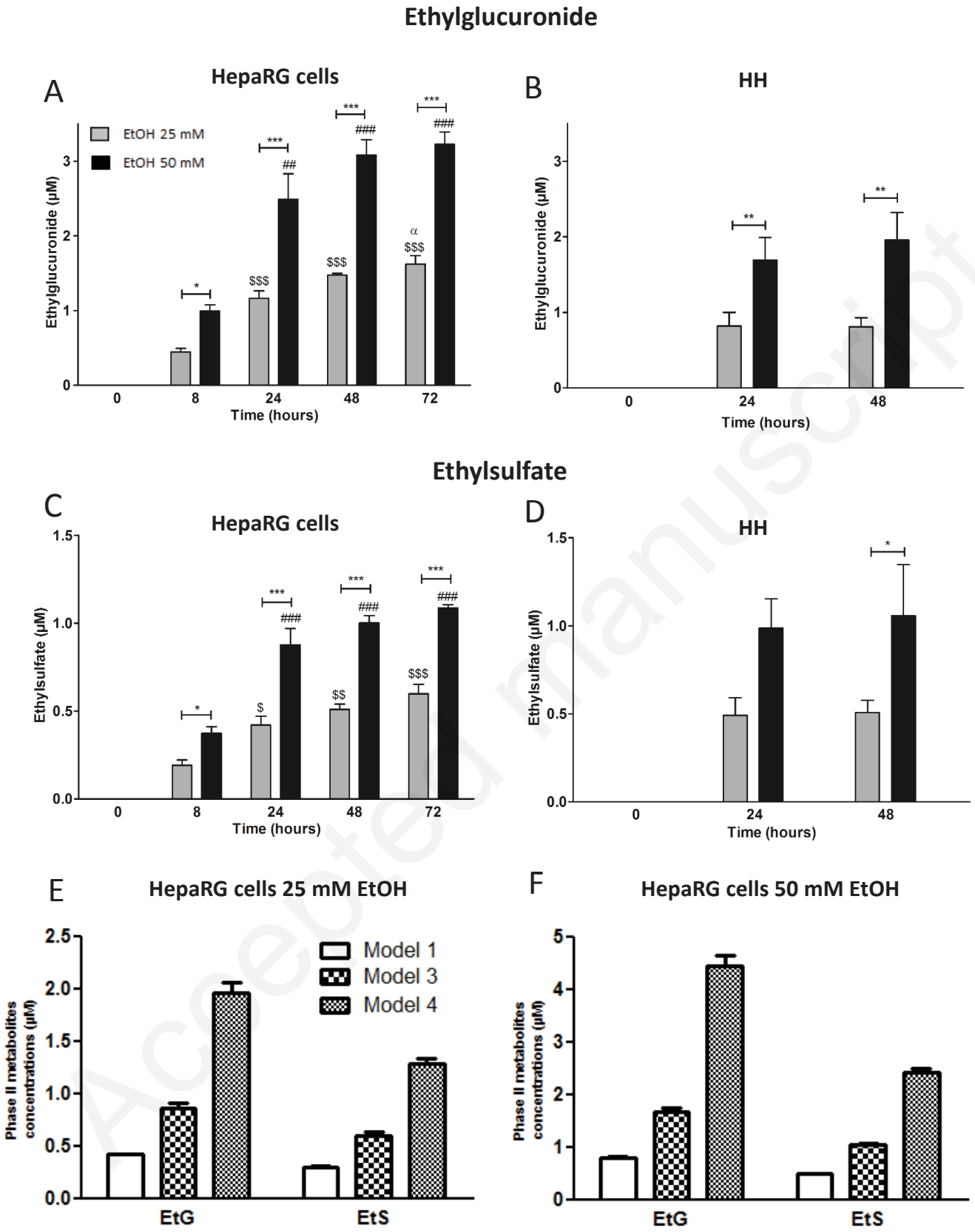
Figure 6. Glucuroconjugation of ethanol in recombinant HepG2 cells expressing UGT1A genes. The mRNA expression levels of *UGT1A1* (A), *UGT1A6* (B), *UGT1A9* (C), *panUGT1A1* (D) and *CYP2E1* (E) were determined using RT-qPCR in parental and recombinant HepG2 cells expression *UGT1A1*, *UGT1A6* or *UGT1A9* proteins. Results were normalized to GAPDH gene expression. Cells were incubated with ethanol at 50 mM for 24 h and EtG concentrations were measured by LC-HRMS in the culture media of these cells (F). The data are expressed as the mean ± SEM for three independent experiments performed in triplicate. Statistics: ***p<0.001 for cultures compared to control cultures. ###p<0.001 for cultures compared to other cultures.

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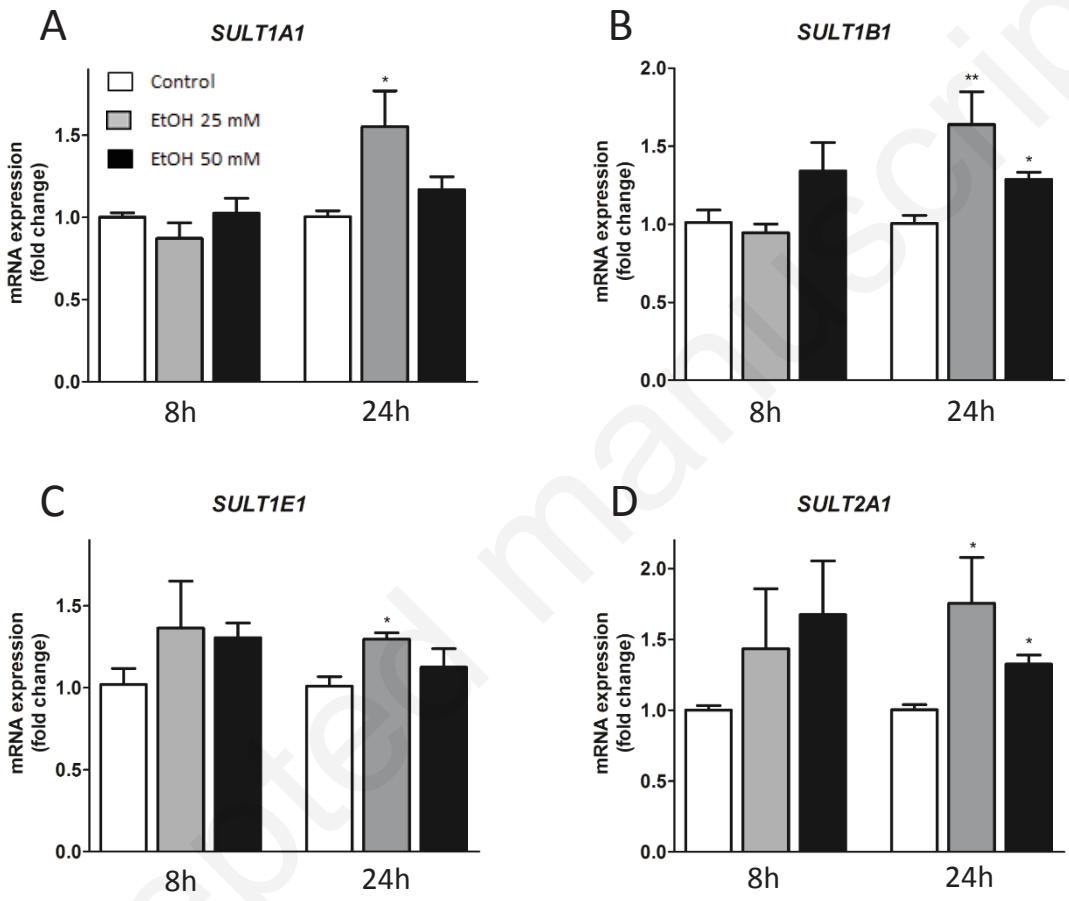
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Figure 1



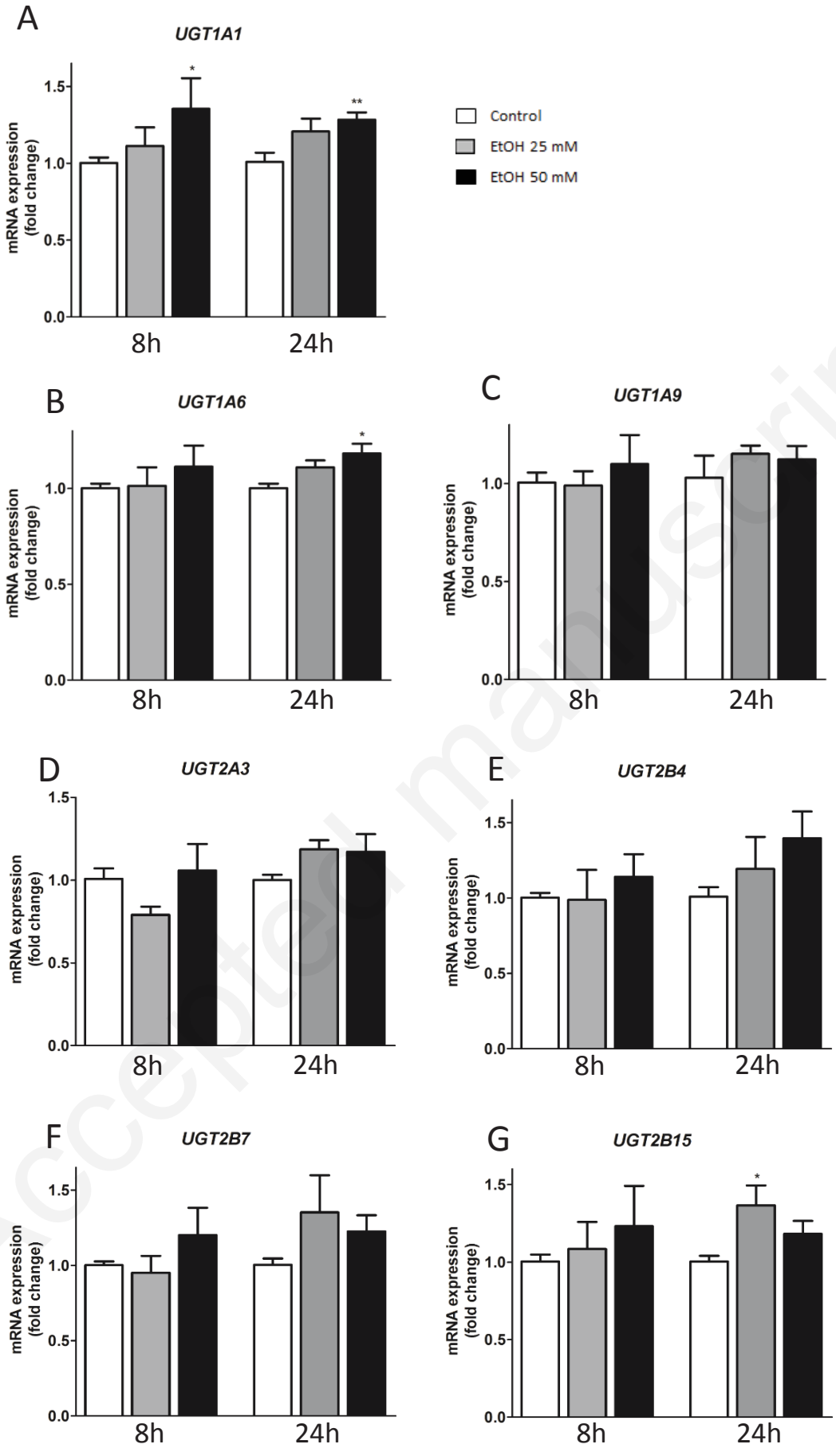
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Figure 2



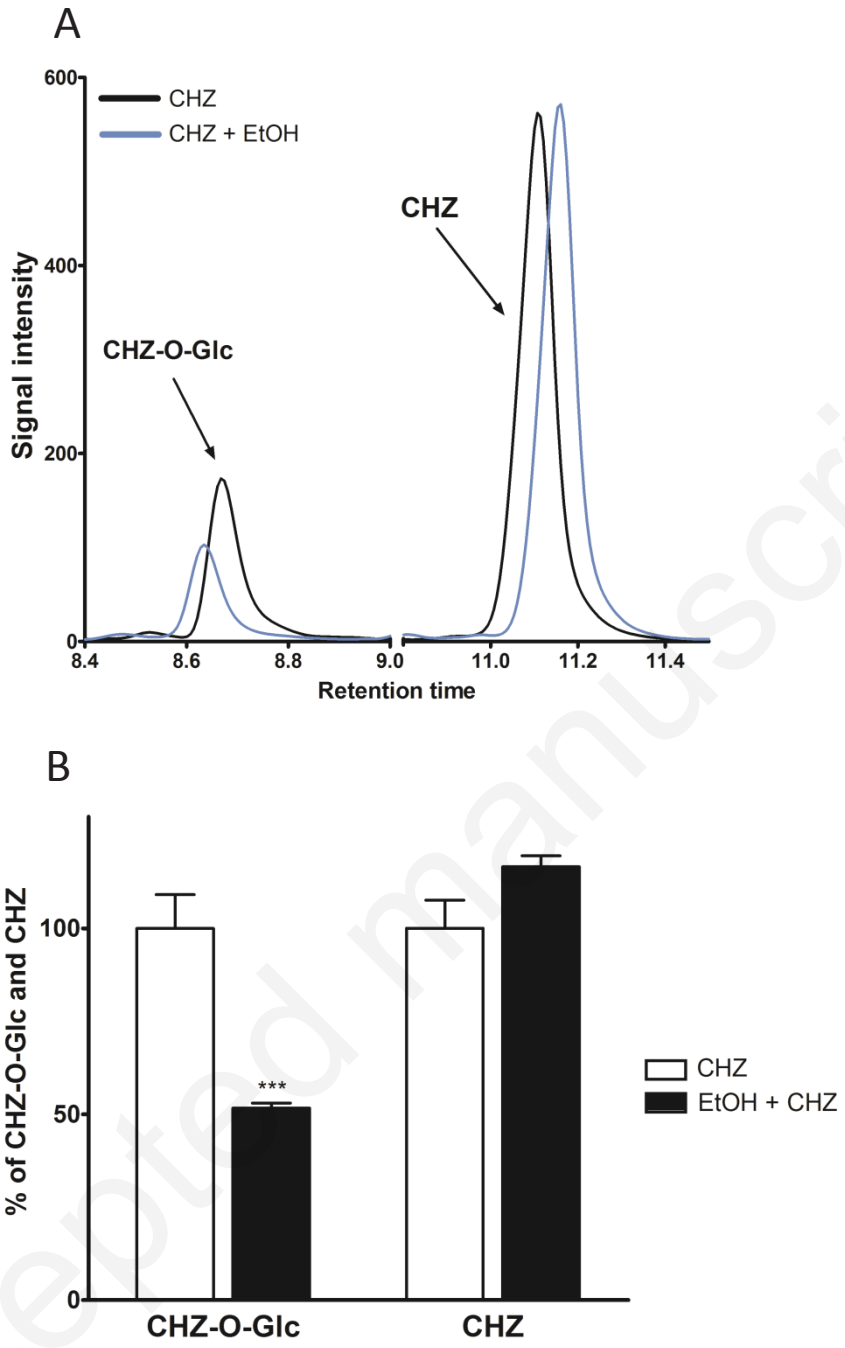
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Figure 3



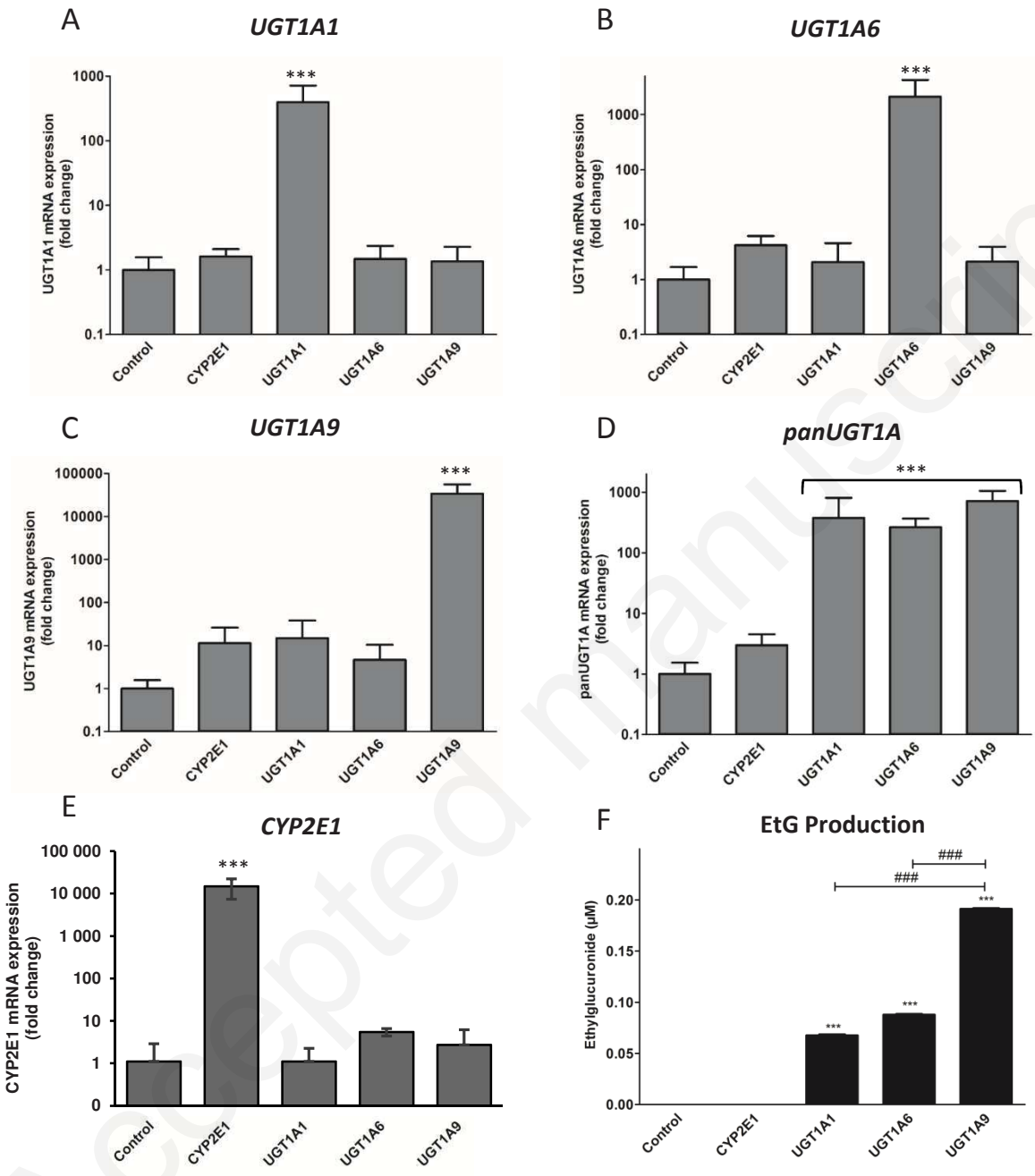
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Figure 4



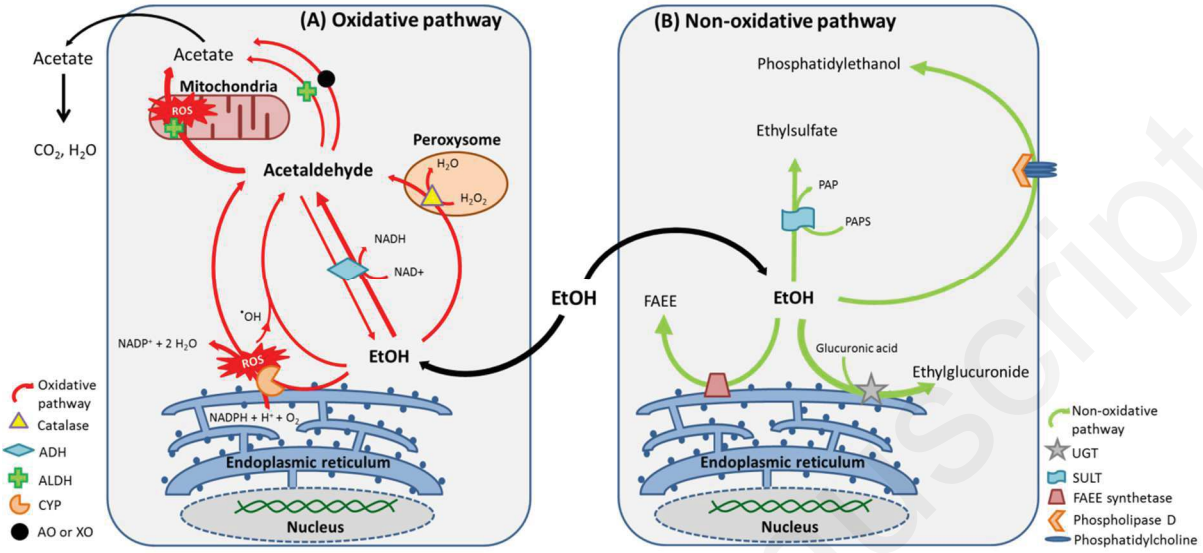
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Figure 5



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Figure 6



Supporting information 1 Oxidative (A) and non-oxidative (B) ethanol metabolic pathways in the hepatocyte. ADH: alcohol dehydrogenase; ALDH: aldehyde dehydrogenase; AO: aldehyde oxidase; CYP: cytochrome P450; EtOH: ethanol; FAEE: fatty acid ethyl ester; PAPS: 3'-phosphoadénosine 5'-phosphosulfate; ROS: reactive oxygen species; SULT: sulfotransferase; UGT: uridine diphosphate glucuronyltransferase; XO: xanthine oxidase

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Liquid chromatography	Mass spectrometry
• Column: C18 Accucore (10 cm) • Temperature: 20°C • Mobile phase: 100 % H_2O with 0.1% (v/v) formic acid • Mode: Isocratic • Run time: 3 min • Flow rate: 0.5 mL/min • Tray temperature: 15°C • Injected volume: 5 µL	• Source: HESI-II • Probe: 300°C • Mode: negative • Spray voltage: 3 kV • Sheath gas and auxiliary gas: N_2 • Capillary temperature: 300 °C • Source lens: 60 V • Acquisition data: t-SIM • Resolution: 70,000 FWHM • C-trap capacity: 10^6 charges • Maximum injection time: 200 ms

Compounds	RT (min)	ion [M^-] (*m/z*)
EtG	0.66	221.0679
EtG-D5	0.65	226.0994
EtS	1.26	124.9910
EtS-D5	1.23	130.0225

Supporting information 2 Method parameters for the detection and quantitation of EtG and EtS in culture media by Liquid Chromatography – High resolution – Mass spectrometry (LC-HRMS)

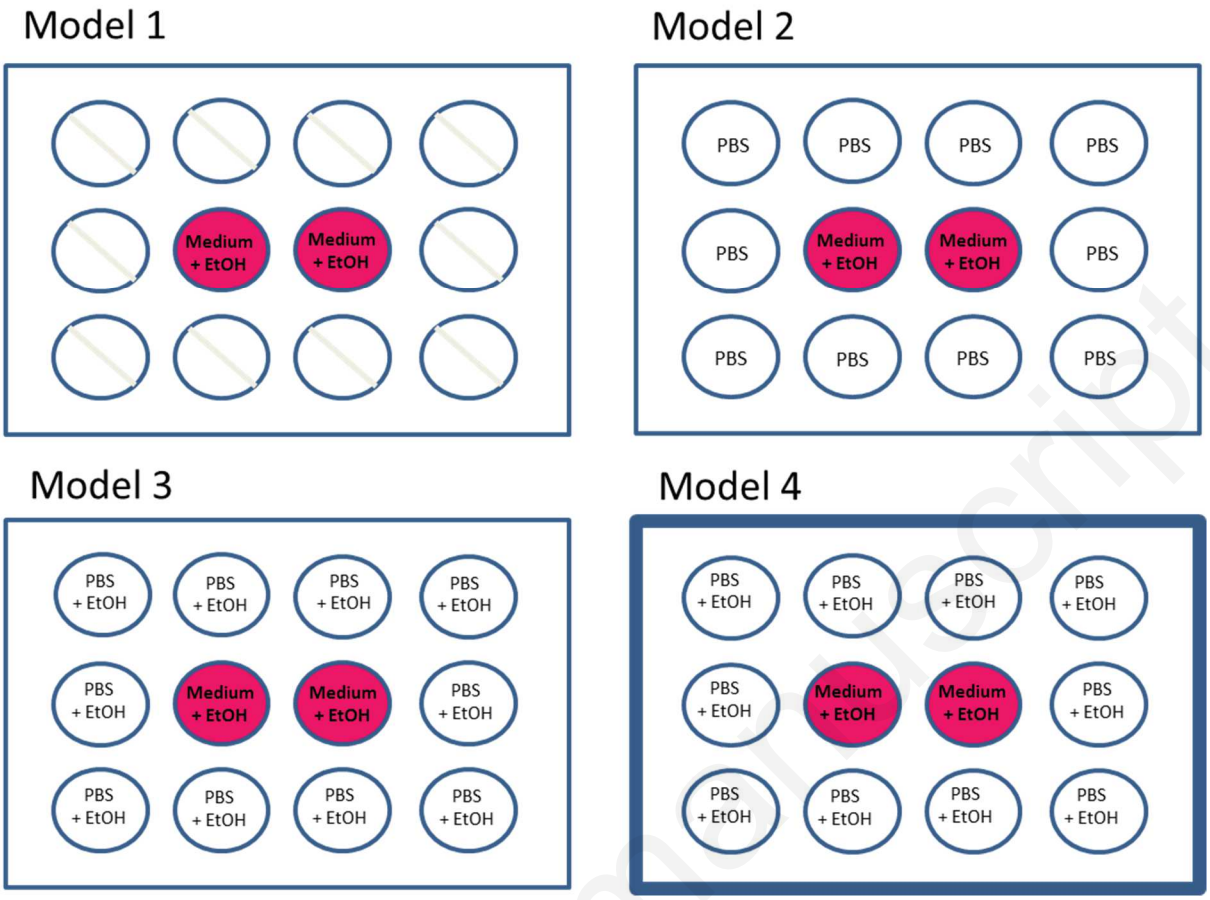
Method validation: This method was validated for linearity, precision and accuracy. Limits of quantification (LOQ) were respectively 0.01 mg/L and 0.05 mg/L for EtS and EtG and limits of detection (LOD) were respectively 0.005 mg/L and 0.01 mg/L for EtS and EtG. No matrix effect was observed in supernatants samples.

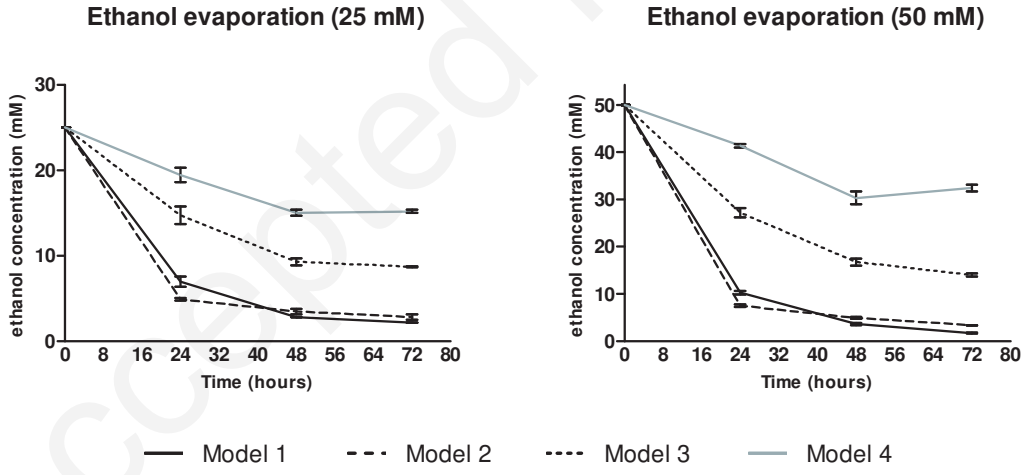
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Gene	Sense primer 5'→ 3'	Antisense primers 5'→ 3'
CYP 2E1	TTGAAGCCTCTCGTTGACCC	CGTGGTGGGATACAGCCA
UGT 1A1	TGACGCCTCGTTGTACATCAG	CCTCCCTTTGGAATGGCAC
UGT 1A6	CAGTGCCGTATGACCAAGAA	GTCTGAGGAGCAGTTAGGAATG
UGT 1A9	TGTCTTTAAACAAACTCCTGCAA	TGGAAAGCACAAGTACGAAGTATATA
UGT 1A (Pan UGT1A)	ACATAATGAGGCCCTGATCAG	AGGAATCGACAGGTACTTAGC
UGT 2A3	TTCCTCAACCATACCAACCTAAC	TTGAAACAGTGACCCCAGAG
UGT 2B4	CCTCACCCACTCTTACCAAATG	ATCGACCCCAGAGAAAACAC
UGT 2B7	CAACGTAATTGCATCAGCCC	AAGCTCTGGTCTTTGGATGAC
UGT 2B15	GTGTTGGGAATATTATGACTACAGTAAC	GGGTATGTTAAATAGTTCAGCCAGT
SULT1A1	GGATGTGGCAGTTTCCTACTA	TTTCGGGTTCTCCTTCATGTC
SULT1B1	GCCTATGGTTCCTGGTTTACTC	CACCAGCCGTCCCTTTAC
SULT1E1	GGTTCCTTATGGTTCCTGGTATAA	CTGATATCCTCTTTCAGGTCTTCG
SULT2A1	CCAGTTATTCCCCAAGTCTTTCT	AAACATCTCTGGGATTTCTCA
GAPDH	ATGACATCAAGAAGGTGGTG	CATACCAGGAAATGAGCTTG

Supporting information 3 Sequences of the primers (sense and antisense) used for quantitative PCR.

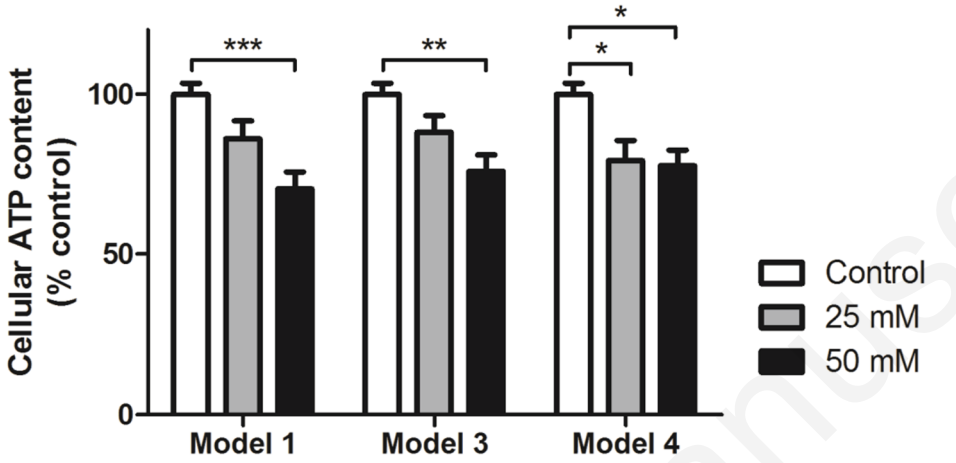
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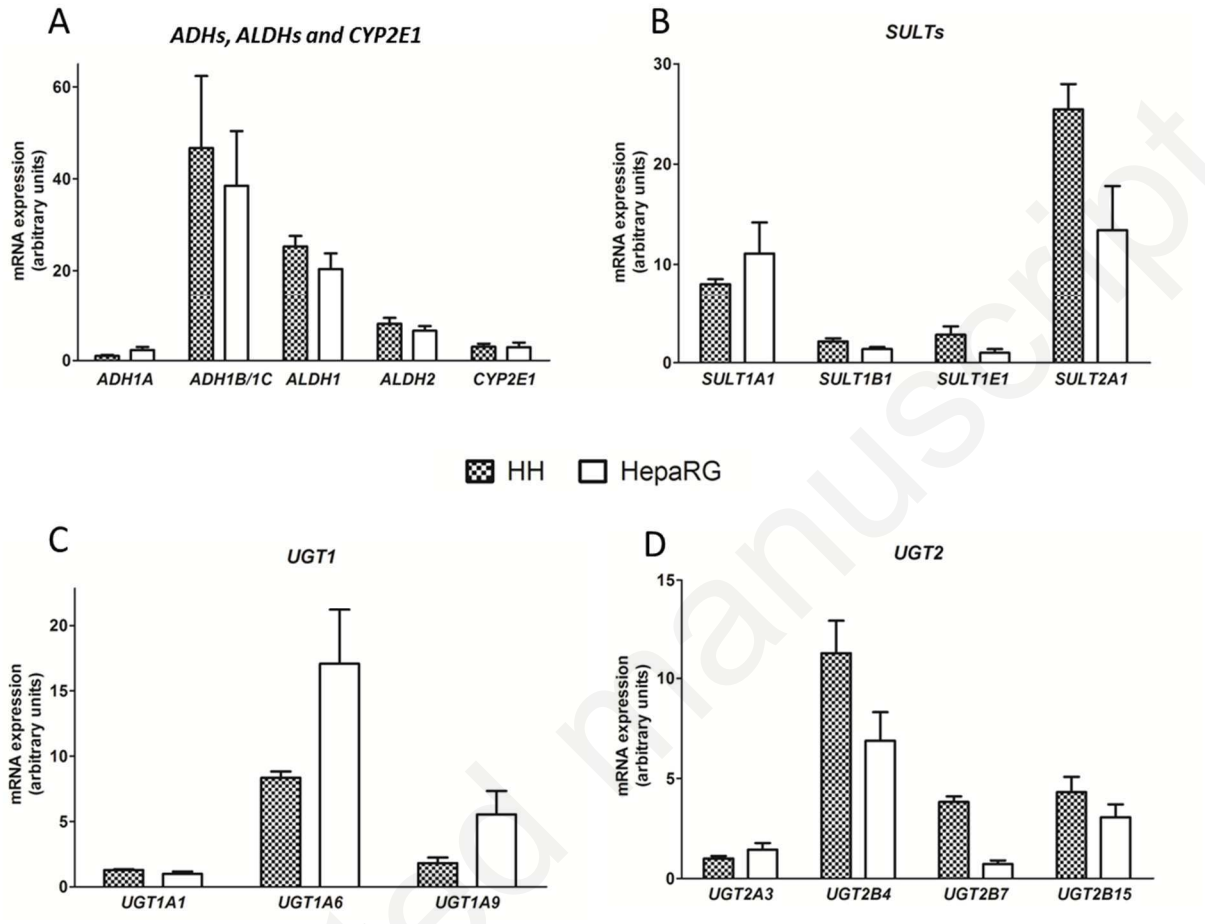
Supporting information 4 In order to set up a model of ethanol elimination mimicking the situation in humans, we tested four models of ethanol evaporation using 12-well plates. Model 1: wells with medium containing ethanol surrounded by empty wells. Model 2: wells with medium containing ethanol surrounded by wells containing PBS. Model 3: wells with medium containing ethanol surrounded by PBS supplemented with ethanol at the same concentration. Model 4: same model as model 3 but with plates sealed with parafilm.

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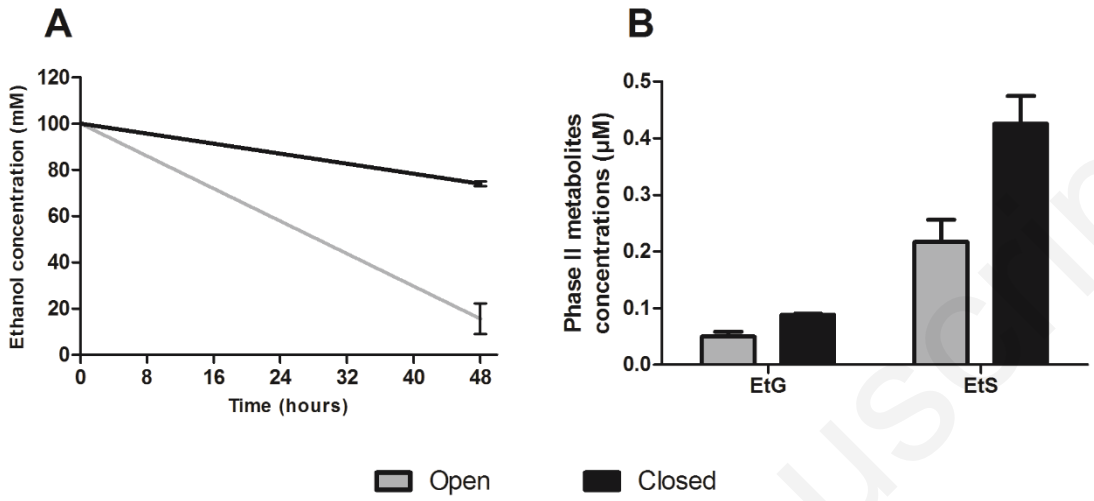
Supporting information 5 Evaluation of cell energy status by measuring ATP content in HepaRG cells in models 1, 3 and 4 of ethanol exposure at 25 and 50 mM of ethanol compared to untreated control cultures. Statistics: ***p<0.001, *p<0.05 in ethanol treated versus control HepaRG cells.

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Supporting information 6 Comparison of *ADH1A, ADH1B/1C, ALDH1, ALDH2* and *CYP2E1* (A), *SULT 1A1, SULT1B1, SULT1E1* and *SULT2A1* (B), *UGT1A1, UGT1A6* and *UGT1A9* (C), *UGT2A3, UGT2B4, UGT2B7* and *UGT2B15* (D) relative mRNA levels between human hepatocytes (HH) and differentiated HepaRG cells.

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Supporting information 7 (A) Ethanol concentration in culture medium of precision cut liver slice (PCLS) with open (grey chart) or closed (dark chart) culture flasks after 48 h exposure. (B) Ethylglucuronide (EtG) and ethylsulfate (EtS) concentrations after 48 h of ethanol exposure with open or closed flask.

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