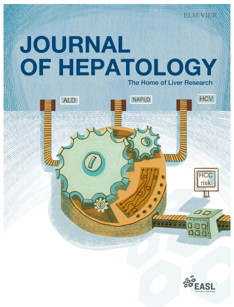

ADVANCED PRECLINICAL MODELS FOR EVALUATION OF DRUG INDUCED LIVER INJURY - CONSENSUS STATEMENT BY THE EUROPEAN DRUG-INDUCED LIVER INJURY NETWORK [PRO-EURO-DILI-NET]

Jose C. Fernandez-Checa, Pierre Bagnaninchi, Hui Ye, Pau Sancho-Bru, Juan M. Falcon-Perez, Felix Royo, Carmen Garcia-Ruiz, Ozlen Konu, Joana Miranda, Oleg Lunov, Alexandr Dejneka, Alistair Elfick, Alison McDonald, Gareth J. Sullivan, Guruprasad Aithal, M. Isabel Lucena, Raul J. Andrade, Bernard Fromenty, Michel Krannendonk, Francisco Javier Cubero, Leonard J. Nelson

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Paolo Angeli
Editor in Chief

Einar S. Björnsson
Associate Editor

June 2, 2021

Dear Drs. Angeli and Björnsson:

We would like to thank you for the acceptance of our *Seminar* manuscript: "**Advanced preclinical models for evaluation of drug-induced liver injury**" for publication in *Journal of Hepatology*, pending minor revision.

Following the suggestion from the reviewer and the editors, we have further reduced the length of the main text by about 900 words by presenting section 9.2 in the Supplementary Material. This approach ensures an additional decrease in the number of words without sacrificing the comprehensive nature of the review. As for the improvement in style and language, we can assure that the re-revised version was edited and approved by all co-authors, some of which are native English speaking, including last author Leonard J. Nelson.

We want to thank the editors and reviewers for the thorough evaluation of our manuscript and hope it may be found acceptable for publication in the *Journal.*

Sincerely yours,

Jose C Fernandez-Checa
On behalf of the authors

ADVANCED PRECLINICAL MODELS FOR EVALUATION OF DRUG INDUCED LIVER INJURY - CONSENSUS STATEMENT BY THE EUROPEAN DRUG-INDUCED LIVER INJURY NETWORK [PRO-EURO-DILI-NET]

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Author contributions:

Thematic structure and Introduction (LN, JCFC, FJC); DILI pathophysiology and emerging mechanisms in APAP (JCFC, FJC, CGR, HY); Emerging mechanisms and approaches (JMF-P, FR; LN); Towards capturing cellular complexity (LN, PSB, GS, JM, MK), Current hepatic-based microphysiological and advanced systems/ non-invasive technologies to screen DILI models (LN, POB, OL, AD, AM, AE); Emerging in vivo models for DILI (OK, JCFC, CGR); Implications of DILI in clinical contexts (BF, JCFC; GA, RA, ML).

Display Items: Figures 1-7; Key Points 1-7 and Table 1.

Key words: Acetaminophen, iPSCs, liver-on-a-chip, humanized models.

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ABSTRACT

Drug induced liver injury (DILI) is a major cause of acute liver failure (ALF) and one of the leading indications for liver transplantation in Western societies. Given the wide use of both prescribed and over the counter drugs, DILI has become a major health issue with a pressing need to find novel and effective therapies. Although significant progress has been made in understanding the molecular mechanisms underlying DILI, our incomplete knowledge of its pathogenesis and inability to predict DILI is largely due to both discordance between human and animal DILI in preclinical drug development and a lack of models that faithfully recapitulate complex pathophysiological features of human DILI. This is exemplified by the hepatotoxicity of acetaminophen (APAP) overdose, a major cause ALF due to its extensive worldwide use as an analgesic. Despite intensive efforts utilizing current animal and *in vitro* models, the mechanisms involved in the hepatotoxicity of APAP are still not fully understood. In this expert Consensus Statement, which is endorsed by the European Drug-Induced Liver Injury Network, we aim to facilitate and outline clinically impactful knowledge discovery by detailing the requirements for more realistic human-based systems to assess hepatotoxicity to guide future drug safety testing. We present novel insights and major players in APAP pathophysiology and describe emerging *in vitro* and *in vivo* pre-clinical models, as well as advanced imaging and *in silico* technologies, which may improve prediction of clinical outcomes of DILI including APAP hepatotoxicity.

1. Introduction

Drug-induced liver injury (DILI) is an infrequent, multifaceted and potentially life-threatening adverse reaction to medications and other chemical compounds that represents one of the most challenging liver disorders with regards to its prediction, diagnosis and management[1-4]. Idiosyncratic DILI (iDILI) leads to hospitalization of 23% of affected individuals[5], accounting for 11% of acute liver failure (ALF) cases in advanced economies, with acetaminophen (paracetamol, APAP) overdose, the prototypical example of intrinsic, predictable DILI, representing 50% of all attributable ALF cases[6]. In addition, 8% of acute DILI cases remain unresolved[7]. As a consequence DILI jeopardizes patient safety and represents a major concern for regulatory measures, drug attrition during clinical development and a leading reason for drug withdrawal from the market. The treatment of iDILI is not evidence-based so far[8] and relies often on *ad hoc* treatment with steroids or ursodeoxycholic acid, particularly in more severe cases[9]. Only for very specific instances, such as APAP intoxication, N-acetylcysteine has proven effective. Clinical aspects of DILI are covered in a recent review[1].

DILI pathogenesis is considered a multifactorial process involving several factors other than the generation of the toxic intermediate(s) from the parental drug metabolism, such as environmental, physiological and genetic factors as well as altered immunological responses. Thus, there is a need for the identification of mechanisms that contribute to DILI in order to develop protective/preventive therapeutic interventions[6, 10]. While some of these mechanisms are dose related, others derive from individual susceptibility to the toxic effects of a certain drug, leading to the classification of DILI as either intrinsic, which is considered predictable, reproducible and dose-dependent or idiosyncratic (iDILI), which is unpredictable and not necessarily dose-dependent. DILI covers a broad clinical and histological phenotypic spectrum, including hepatocellular damage, cholestasis, and acute steatosis, which are often detected late in Phase-III clinical trials or post-marketing.

APAP hepatotoxicity is the archetypal model of DILI and probably the most relevant to human DILI, with billions of analgesic doses consumed annually. APAP hepatotoxicity in humans can be modeled in rodents after administration of an acute or cumulative overdose, often after fasting. However, despite intensive efforts, the mechanisms involved in the hepatotoxicity of APAP are not fully understood, highlighting the imposed limitations of interspecies variability and differences in metabolism between humans and rodents, whilst existing in vitro hepatic cell systems based on human cell lines or rodent hepatocytes are sub-optimal. These factors have severely hampered pre-clinical efforts to accurately predict DILI and to unravel hidden mechanisms that occur *in vivo*.

In this review, we summarize the pathophysiology of DILI exemplified by the APAP paradigm, describing existing pre-clinical models for DILI. In addition, we assess emerging models, including the development of multi-parametric approaches and humanized models for better DILI prediction. Highlighting the utility of advanced technological integration and innovation to enhance phenotypic profiling that could lead to a better understanding of DILI. Future avenues are also explored including novel approaches in delineating mechanistic DILI and the utility of disruptive technologies, such as liver on-chip, to advance DILI prediction (**Key Point 1**).

2. DILI Pathophysiology

2.1. The APAP paradigm

APAP-induced liver damage is characterized by hemorrhagic centrilobular necrosis and high plasma transaminases levels in both humans and animals[2, 10]. Although APAP is normally metabolized to its glucuronidated and sulphated non-toxic metabolites in the liver, APAP overdose saturates these pathways and excess APAP is metabolized mainly by cytochrome P450 (CYP) CYP2E1 but also by CYPA2, CYP2A6, CYP2D6 and CYP3A4 into the highly reactive metabolite N-acetyl-*p*-benzoquinoneimine (NAPQI). This highly toxic byproduct is rapidly conjugated with glutathione (GSH) resulting in non-toxic mercapturic acid and cysteine conjugates that are excreted in the urine. In APAP overdose or in conditions of GSH limitation (e.g. fasting), free unconjugated NAPQI reacts with sulfhydryl groups on cysteine and lysine residues, generating adducts with proteins (APAP-protein adducts) in hepatocytes, and particularly in mitochondria, leading to mitochondrial dysfunction and cell death[11-13]. Despite being the most comprehensively studied and understood hepatotoxic drug, our understanding of the underlying mechanisms involved in APAP hepatotoxicity are still incomplete. Indeed, APAP can also elicit an idiosyncratic response in humans[2] and the use of APAP even at therapeutic doses can have deleterious effects[14, 15]. Although models of iDILI are lacking, it is postulated drug reactive metabolites may elicit an immune response in susceptible individuals[16].

The complex and multifactorial nature of APAP hepatotoxicity extends to DILI itself. Clearly, there is an imperative need to develop more realistic human models to foster a better understanding of the mechanistic basis of DILI. In turn, improved models that allow more accurate prediction of pre-clinical DILI may help uncover effective therapeutic interventions (**Key Point 2**).

2.2. Other DILI-causing drugs

Besides APAP, other classes of drug such as nonsteroidal anti-inflammatory drugs (NSAIDs) and statins are important causes of DILI, although with a relatively low overall incidence rate. Some of the molecular and cellular mechanisms underlying NSAID-DILI have been identified: (i) Mitochondrial injury, (ii) Induction of cholestasis, (iii) Protein adduct formation by reactive drug metabolites, and (iv) Possible direct consequences of COX inhibition[17]. In isolated rat liver mitochondria Diclofenac decreases hepatic ATP content and impaired ATP synthesis causing mitochondrial permeability transition (MPT), leading to generation of reactive oxygen species (ROS), mitochondrial swelling and oxidation of NADP and protein thiols[18]. Besides diclofenac, indomethacin, celecoxib and ibuprofen NSAIDs can induce ER stress response-related proteins, particularly CHOP, leading to apoptosis [19].

Statins are generally well tolerated and adverse effects are relatively rare [20]. Mitochondrial dysfunction due to significant increase in ROS, causing lipid peroxidation as well as the inhibition of the respiratory chain (complex I and III) triggering apoptosis, may explain the mechanisms of statin-induced hepatotoxicity[21].

3. Emerging Mechanisms and signaling cascades governing APAP hepatotoxicity

APAP overdose has high clinical relevance as the primary cause of ALF in advanced economies and a major reason of liver transplantation and is regarded as a model hepatotoxin. In the following section we briefly summarize the role of major players that contribute to APAP-induced liver damage. A key question in the pathophysiology of DILI is how a toxin or its (reactive) intermediate metabolites trigger cell damage, which has been intensively investigated in the case of APAP. In this regard, cell-specific (i.e. hepatocytes, immune cells) signaling cascades governing APAP hepatotoxicity have attracted much attention since activation/inhibition of these pathways could be of pivotal importance in patients who do not respond to standard treatment (**Key Point 3**).

3.1 MAPK family: c-Jun-N-terminal kinase (JNK)

The c-Jun-N-terminal kinase (JNK) is a member of serine/threonine kinases that belong to the mitogen-activated protein kinases (MAPK) family, which has been shown to play a causal role in APAP hepatotoxicity by mediating an amplification loop in APAP-induced mitochondrial targeting and oxidative stress[22]. In the liver, two JNK genes, *Jnk1* and *Jnk2*, are expressed[23]. Antagonizing JNK activation using the classical inhibitor SP600125 has protective effects against APAP-induced liver injury, by significantly reducing necrosis both *in vivo* and *in vitro*[24, 25]. Although SP600125 may have effects independent of JNK inhibition, combined *Jnk*1 and *Jnk*2 germ-line deletion or knockdown by antisense oligonucleotides in adult mice markedly protected against APAP hepatotoxicity[26]. In addition, simultaneous deletion of *Jnk1* and *Jnk*2 in adult hepatocytes in *Jnk*1+$2^{f/f}$ mice following injection with an associated adenovirus expressing Cre recombinase driven by the hepatocyte-specific promoter TBG (AAV-TBG-Cre) protected against APAP-mediated liver injury[27]. In contrast with these findings, a recent report in mice with hepatocyte-specific *Jnk1* and *Jnk2* deletion ($Jnk^{\Delta hepa}$) questioned the role of JNK in APAP-induced hepatotoxicity, as $Jnk^{\Delta\text{hepa}}$ mice developed greater liver injury than wild-type animals after APAP overdose, suggesting a beneficial role for combined JNK1 and JNK2 activation in hepatocytes [28]. Whilst the reasons underlying these opposing findings remains to be fully unraveled, in the latter study *Jnk2* was globally deleted in all cell types and *Jnk1* was specifically knocked down in hepatocytes but not in non-parenchymal cells, implying opposing roles for Jnk1 in different types of liver cells, as well as in infiltrating inflammatory cells..

The specific contribution of JNK1 and JNK2 in DILI remains controversial. No differences in APAP hepatotoxicity in *Jnk1* knockout mice were observed[26], despite a clear pro-apoptotic and profibrogenic function of *Jnk1* in TNF-induced cell death[29], and in liver fibrosis[30]. Regarding *Jnk2*, increased susceptibility towards APAP, TNF and LPS-induced liver injury was reported upon *Jnk2* deficiency[31], whilst *Jnk2* disruption protected against APAP-induced liver injury[26]. Recent findings have shown that both hepatocyte *Jnk*2 knockout, and knockdown ameliorated ibuprofen-mediated

DILI[32]. Recent investigations highlighted the critical role of immune cells in APAP-induced ALF, including activation of resident hepatic macrophages (Kupffer cells; KCs) following hepatocyte necrosis as well as massive CCR2-dependent recruitment of monocytes[33].

3.2 JNK activation factors

Preclinical findings in constitutive and conditional knockout mice have shown that JNK can be activated by many factors, ranging from various pathogens and cytokines, including TGFβ, IL-1β and TNF to oxidative stress and DNA damage in both hepatocytes and infiltrating cells[22, 34]. Phosphorylation of JNK is mediated by MAP2Ks[35], which, in turn, are phosphorylated and activated by MAP3K. The best characterized MAP3Ks are the apoptosis signal-regulating kinase-1 (ASK1) and mixed-linage kinase 3 (MLK3). ASK1 participates in APAP-induced JNK activation[36], which is achieved by dissociation from thioredoxin-1 (Trx-1) (**Figure 1**). MLK3, a member of the Ser/Thr protein kinases family, mediates the initial phase of JNK activation[37]. GSK-3β is also involved in the early-phase of JNK activation. Inhibition of GSK-3β in mice prevented JNK activation and ameliorated APAP-derived toxicity[25]. The MAP2Ks (MKK4 and MKK7) are capable of phosphorylating JNKs at Thr/Tyr residues[38]. Furthermore, MKK4 activates both JNK and p38 kinases, while MKK7 only activates JNK. An additional player that contributes to sustained JNK phosphorylation in APAP hepatotoxicity is through impaired MAPK phosphatases (Mkp). Mkp deficiency in mice has been shown to exacerbate APAP-induced liver injury along with sustained JNK activation, while Mkp activation prevents JNK activation and subsequent APAP hepatotoxicity[39, 40].

3.3 JNK amplification loop

Recent studies utilizing novel mouse liver models, a feedforward self-sustaining signaling pathway referred as the JNK amplification loop[41, 42] was reported to maintain sustained JNK activation, leading to liver damage and dysfunction in response to APAP. Activated JNK (p-JNK) translocates to mitochondria and binds to the Sab (SH3BP5) protein on the outer mitochondrial membrane[43, 44], impairing mitochondrial respiration and enhancing the release of ROS[45]. ROS release, in turn, activates ASK1 and MKK4, which sustains JNK activity and amplifies the toxic effect. The binding to the outer mitochondrial membrane of JNK via Sab further induces MPT, thus changing the permeabilization of the mitochondrial outer membrane and allowing the exit of molecules less than

1500 Da, including cytochrome *c*, apoptosis inducing factor (AIF) and endonuclease G[46]. Although release of cytochrome *c* and AIF is a hallmark of apoptosis by activating caspase 3/7 and leading to nuclear DNA cleavage, respectively, [47, 48](**Figure 1**), the major form of cell death in APAP toxicity is necrosis[49]. This may be due to the fact that the marked injury in the mitochondria and the pronounced reduction in ATP cannot sustain activation of the apoptosis cascade. It should be noted that currently, other less familiar modes of cell death including pyroptosis, necroptosis and ferroptosis with alternate mechanistic pathways are under active investigation as to their contribution to APAP hepatotoxicity[50]. Moreover, although autophagy is another form of cell death, it is considered a protective mechanism against APAP hepatotoxicity (see below).

3.4 ER stress and mitochondrial cholesterol accumulation

ER stress-mediated unfolded protein response (UPR) is an adaptive stress response resulting in accumulation of unfolded or misfolded proteins in the ER lumen[51]. ER stress can be detected late after APAP challenge (500 mg/kg) in murine models, and becomes highly significant 12 hrs following APAP administration[52].The ER stress response has three signaling arms: (i) Protein kinase RNA-like ER kinase (PERK), (ii) Activating transcription factor 6 (ATF6), and (iii) Inositol-requiring enzyme 1 α (IRE1 α). These pathways are maintained in an inactive state through binding to BIP in non-stressed cells. Upon APAP-mediated ER stress, IRE1α, PERK and ATF6 become activated, triggering an inflammatory response and cell death mediated via ASK1 and JNK [52](**Figure 1**). However, in genetically deleted XBP1 mice, constitutive IRE1α hyperactivation in hepatocytes resulted in reduced JNK activation and protection from APAP through suppression of CYP450 activity[53]. Recently, the steroidogenic acute regulatory protein (STARD1), a mitochondrial cholesterol transport protein, has been identified as a key player in ER-stress mediated DILI[27].

In this respect, STARD1 promotes cholesterol trafficking and accumulation in mitochondria, which in turn leads to mitochondrial GSH depletion and contributes to mitochondrial dysfunction, exacerbated ROS generation and necrotic cell death (**Figure 2**). An intriguing finding is the protection of mice with liver-specific STARD1 deletion despite preserved mitochondrial Sab/p-JNK activation, suggesting that the deleterious effect of p-JNK in mitochondrial dysfunction and hepatocyte cell death is dependent on STARD1[27]. In addition, hepatocyte-specific deletion of Sab or p-JNK1+2 was also protective against APAP hepatotoxicity, preventing APAP-induced ER stress and subsequent STARD1 upregulation. Further mechanistic studies utilizing human models and clinical samples (see below) will likely lead to confirmation of the molecular basis for the complimentary role of STARD1/mitochondrial cholesterol and the Sab/p-JNK axis in APAP hepatotoxicity - and the upstream events involved in p-JNK1/2 induced ER stress.

Furthermore, as mitochondrial dysfunction contributes to APAP hepatotoxicity, removal of damaged mitochondria through mitophagy has emerged as a critical mechanism in APAP-induced ALF[13]. Besides transcriptional regulation, autophagy can be also modulated by lysosomal lipid composition. Indeed, accumulation of lipids (e.g. cholesterol) in lysosomes has been shown to impair the fusion of autophagosomes (containing disrupted mitochondria) with lysosomes, contributing to perpetuation of damaged mitochondria, which sensitizes to APAP hepatotoxicity [54]. Thus, not only the generation of intermediates of APAP metabolism (e.g. NAPQI) acting directly in mitochondria can determine APAP hepatotoxicity, but secondary factors that delay mitochondria turnover via mitophagy can also contribute to APAP-induced liver failure. The latter can be of particular clinical relevance, in nonalcoholic steatohepatitis (NASH), which can potentiate DILI [55] (see Section 9). In fact, it has been previously reported that patients with NASH exhibit increased expression of STARD1 [56], suggesting that a subset of patients with advanced nonalcoholic fatty liver disease (NAFLD) and enhanced free cholesterol content with STARD1 expression, may develop liver injury on APAP ingestion.

In summary, it is clear that the JNK signaling pathway is a critical component in DILI, particularly in APAP pathogenesis. Since JNK differentially regulates important biologic targets, this cascade can be either beneficial or detrimental in different cells and tissues, and compensatory mechanisms need to be modulated or even, discarded. In addition, upstream or downstream pathways regulating the JNK-specific role in cell death during APAP hepatotoxicity are pivotal to developing new therapeutic interventions in patients with DILI.

3.5 Other signaling pathways and mechanisms

Apart from MAPK, other pathways have been reported to modulate APAP hepatotoxicity. Inhibition of protein Kinase C (PKC) prevents APAP hepatotoxicity via blocking ROS-mediated hepatic necrosis[57]. The receptor interacting protein kinases (RIPKs) that modulate necroptosis is an intensive area of study with a controversial role in DILI. RIPK3-deficient mice were protected from early phase APAP toxicity, which also resulted in the prevention of ROS-JNK associated signaling[58]. In contrast, other studies found no evidence that RIPK3 or the pseudokinase MLKL participate in APAP-mediated injury[59]. Indeed, RIPK1 inhibition reversed APAP-induced JNK activation and liver damage, a possible mechanism associated with ASK1 and endoplasmic reticulum (ER) stress[36, 60]. As these studies used global RIPK1/3 deletion, conditional deletion in adult mice would be needed in order to unequivocally demonstrate the role of RIP1/3 in APAP hepatotoxicity.

Liver sinusoidal endothelial cells (LSECs) form the wall of hepatic sinusoids, regulate hepatic vascular tone and contribute to the maintenance of a low portal pressure. LSECs help maintain hepatic stellate cell quiescence, and thus essentially inhibit intrahepatic vasoconstriction and fibrosis development. In line with their key role in hepatic homeostasis, LSECs play a key role in the initiation and progression of chronic liver disease and DILI [61]. Pioneering studies identified LSEC as a target for APAP toxicity[62], with further investigations revealing the ability of APAP to cause LSEC apoptosis via Trail[63], leading to hepatic congestion and hemorrhagic lesions. Quite intriguingly, recent findings revealed that the accumulation of free cholesterol in the endolysosomes of LSEC exacerbates APAP hepatotoxicity via TLR9/inflammasome pathway[64]. These findings highlight that hepatic steatosis, and in particular increased liver cholesterol, emerge as a risk factor for APAP hepatotoxicity (see section 9.1).

3.6 Adaptive and cellular protective mechanisms: Autophagy | Keap1/Nrf2

Macroautophagy (autophagy) is a nonselective bulk degradation process aimed at recycling cellular components and damaged organelles in response to a variety of stimuli, such as nutrient deprivation and toxic stress, including APAP hepatotoxicity. Using primary mouse hepatocytes and GFP/light chain 3 transgenic mice, Ni and colleagues described that APAP-induced autophagy correlated with recycling of damaged mitochondria [65]. APAP suppressed mTOR complex 1 and APAP-induced autophagy was blocked by NAC, suggesting APAP mitochondrial protein binding and the subsequent production of ROS elicited APAP-mediated autophagy. Importantly, pharmacological inhibition of autophagy further exacerbated APAP-induced hepatocytoxicity; while induction of autophagy by rapamycin inhibited APAP-induced liver injury. The hepatoprotective role of autophagy in APAP hepatotoxicity was due to the elimination of damaged mitochondria by a more selective process called mitophagy [65] which impacted removal of APAP-protein adducts[66]. Interestingly, the APAP-induced mitophagy appears to be predominant in zone 3 of the liver compared to zone 1 (coinciding with the site of APAP metabolism), suggesting mitophagy as an adaptive mechanism to promote cell survival and restrict the expansion of necrotic areas [67]. In line with these findings, adiponectin has emerged as an adaptive mechanism to ameliorate APAP hepatotoxicity by promoting mitophagy through stimulation of autophagosome formation by AMPK-dependent activation of Unc-51-like kinase 1[68].

Besides mitophagy, ROS generation can be offset by an antioxidant stress response controlled by nuclear erythroid-2-related factor 2 (Nrf2)[69]. The Kelch-like ECH-associated protein 1(Keap1)/Nrf2 system is recognized as an important cytoprotective pathway combating cellular oxidative injury[70]. Liver conditional Keap1 knockout or activators of Nrf2 provide protection against APAP-induced liver injury, while deletion of Nrf2 results in hypersensitivity to APAP hepatotoxicity[71]. Indeed, farrerol, a 2,3-dihydro-flavonoid isolated from rhododendron, has been shown to confer rapid (within 1 hour) protection against APAP hepatotoxicity by activation of Nrf2 and autophagy [72]. Thus, whether targeting autophagy and Nrf2 in combination with NAC may be a relevant approach to reduce APAP-mediated ALF remains to be investigated.

4. Emerging mechanisms and approaches in DILI

4.1 Extracellular Vesicles

Like other cell types hepatocytes secrete extracellular vesicles (EVs), both under physiological and pathological conditions, including in chronic injury, such as liver fibrosis, and in DILI[73]. EVs are membrane-bound vesicles released to the extracellular milieu, protected by a lipid bilayer, which also include protein receptors and signal triggering molecules. The EVs carry diverse cargo that include proteins, active enzymes, coding and non-coding RNA, DNA, and metabolites[74-76]. Three different types of EV can be released from cells (exosomes, microvesicles (MVs), and apoptotic bodies) and are closely related to the mechanism of biogenesis. Exosomes are the smallest EVs (30-150nm)[77], which are formed in multivesicular bodies (MVBs) of the endocytic and secretory pathway[78, 79]. Microvesicles (50-3000nm) are formed directly by outward budding of the plasma membrane [80]. Whilst apoptotic bodies are EVs (>500 nm) that originate from cells undergoing apoptosis[81]. In liver, the first descriptions of hepatocyte-derived vesicles were obtained from primary culture of rat hepatocytes [82], and isolated hepatic stem cell cultures[83]. Many researchers have since contributed to the characterization of liver-derived EVs in different contexts of liver disease[84-86].

Of relevance to DILI, hepatocyte-derived EVs can be influenced by drug metabolism, affecting the protein cargo composition, morphology and number[87, 88]. As characterized in in vitro systems APAP and diclofenac among others, the released EVs have been shown to contain liver-specific mRNA (*e.g.* ALB gene)[89], liver-specific miRNA (such as miR-122) [84], and liver specific proteins such CPS1, MAT1 and COMT [87, 89-93]. Several studies have unraveled the diverse cargo of EVs in DILI (**Figure 3**), including the presence of CYPs, such as CYP2A1, 2B3, UDP-glucuronosultransferases (UGT), and 2B2 isoforms, and sulfotransferase 1A1 in rats[82]. Notably, CYPs 1A2, 2B6, 2E1, 3A4 and UGT 1A1, and other isoforms have been detected in circulating EVs isolated from plasma of DILI patients[93, 94].

Apart from CYPs, EVs may also harbor other active enzymes, such as Arginase 1 (Arg1) [95] or the carboxylesterase 3 (CES3)[90]. Due to this capability of transporting active liver enzymes, it is likely that circulating EVs are also involved in the pathogenesis of DILI, since they can reach different tissues, such as lung or brain[96], and modify acceptor cell response, such as the contractile capability of blood endothelium[95] - potentially playing a role in the pathogenesis of lung hypertension related to liver damage. Moreover, the presence of active CYP2E1 in EVs suggest they may exacerbate APAP-induced toxicity in hepatocytes and monocytes[97]. The presence of drug-induced protein modification within the EV cargo can potentially have negative effects through covalent binding to certain drugs, such as amoxicillin or flucloxacillin. These protein-adducts can also induce the activation of dendritic cells when exposed to EVs released by hepatocytes, indicating that EVs could play a role in drug-induced autoimmune hepatitis[98].

4.2 AOP Framework

As DILI is a highly heterogeneous process, the mechanistic characterization underlying this wide spectrum of hepatotoxic manifestations is a requisite to improve prediction. Effective experimental approaches for DILI evaluation require novel preclinical test systems that faithfully mimic these heterogeneous pathways. Context-specific *in vitro* models for assessing hepatotoxicity have been characterized recently[99] - and several new technological approaches are being developed in the search for more predictive systems (see Sections 5 and 6) (**Key Point 4**).

Although a substantial amount of mechanistic data on DILI is currently known (e.g. obtained through GWAS [100] or transcriptomics approaches [101]), significant gaps remain on hepatotoxic outcome following chemical exposure. The lack of detailed understanding of the mechanistic pathways underlying the multifactorial nature of DILI has impeded the development of improved treatment and cell systems to test advent therapies. The development of the AOP concept [102] has gained momentum together with parallel improvements in test systems to bridge this gap in knowledge.

AOP is a mechanistic representation of critical toxicological effects that propagate over different layers of biological organization, from the initial interaction of a chemical compound with a molecular target, to an adverse outcome at the individual or population level (**Figure 4**). An AOP describes a sequence of events starting with a molecular initiation event (MIE; the molecular target), with progression through a series of key events (KE), linked by key event relations (KERs), which may occur at the sub-cellular/ cellular-/or tissue-level, up to the whole organism. It describes only toxicodynamic interactions and pathways, and as such, is compound agnostic, i.e. independent of any specific chemical or its dose level. AOP is a valuable approach to incorporate mechanistic knowledge as demonstrated for APAP, chlorpromazine and other DILI-causing drugs [102, 103] - a multi-scale data integration tool in which newly obtained mechanistic data can be used to feed the linear AOP structure (see below, and **Figure 4**). Moreover, AOP can enhance our mechanistic knowledge [102], as it can identify deficits in existing tests and models intended to predict DILI. The AOP framework could in particular be helpful in delineating underlying causes and mechanisms in iDILI. As such, piecing together currently fragmented data sets of studies on iDILI in an AOP scheme will fill knowledge gaps, allowing the design of effective experimental approaches to unveil pathway(s) and help reduce the unpredictability of iDILI. Additionally, AOP is geared towards the indication of potential DILI biomarkers as early indicators of DILI, as practical read-outs in experimental approaches, as well in pharmacovigilance. Initial AOPs for several forms of DILI have been recently established[104] and contain substantial mechanistic information on liver fibrosis, steatosis, and cholestasis.

The linear schematization of current mechanistic information in AOP generates relevant data on metabolic alterations, such as bile acid homeostasis [105], mitochondrial dysfunction [106], or the role of innate immune responses [107]. In addition, this workflow points to AOP as a practical tool for the design, development, and validation of improved experimental models for DILI prediction. Indeed, a recent AOP approach that integrates mechanistic knowledge of multiple data sources enabled selection of a number of *in vitro* assays as effective predictors of DILI risk[108].

Toxicogenomic approaches can further reveal fundamental molecular mechanisms and improve prediction of toxicity through integration of cross-omics technology including epigenomics, transcriptomics, proteomics and metabolomics measurements, along with PBPK-based experiments This has been used in 3D human liver and heart microtissues with advanced *in silico* bioinformatics to predict DILI[109].

4.3 Toxicogenomics approaches

Next generation genomic technologies are now being used as powerful tools in the armament to investigate DILI. Toxicogenomics allows detailed analysis of altered gene and protein expression profiles and across biological scales (which are also relevant to AOP: molecular-single cell – population levels) in response to xenobiotic exposure[110]. Its potential for application is enhanced by the availability of accessible databases that can facilitate and harness generated Omics and imaging data [e.g. Open TG-GATEs[111]]. Genome Wide Association Studies (GWAS) have resulted in highly revealing findings, such as HLA polymorphisms related to DILI[112]; whilst transcriptomics-based Big Data-driven analysis has identified adverse outcomes at cellular and organism levels[101]. Recently, a GWAS-based polygenic risk score prediction strategy has allowed potential of DILI susceptibility and genetic variation at the level of the hepatocyte using a combined genomic, cellular and organoid 'polygenicity-in-a-dish' approach to delineate a spectrum of DILI causing agents including Fasiglifam (TAK-875)[113].

Recent application of single-cell transcriptomics in DILI has for the first time, enabled the identification of unique subsets of MYC-dependent, activated liver-resident cellular types or 'states' (Kupffer, stellate, and liver sinusoidal endothelial cells) in APAP-induced ALF in mice, which correlated with human ALF[114]. This approach may allow pathway-specific ALF therapeutic intervention strategies. Metabolomics can also be highly informative for DILI prediction; whereby detection of endogenous metabolites/ reactive metabolites can be complemented with the development of AOP for design of more effective approaches for DILI prediction [115].

Thus, whilst current methods to study DILI pathology has involved mostly well-defined, end-point assays such as immunostaining, multi-parametric image analysis (cell viability), ultrastructural imaging, qRT-PCR, Western blot or flow cytometry techniques; there are now increasingly available powerful complementary assays that, when coupled with emerging human-based multi-cellular models, can shed light on human-specific toxicity mechanisms.

5. Towards capturing hepatocellular complexity

5.1 Choice of cells - not all cells are equal

Conventional hepatic culture models for drug discovery assays mostly use rodent primary hepatocytes or human immortalized cell lines. However, these rapidly lose polarity and differentiated phenotype[116, 117] and are not representative of normal liver tissue. Such models often lack the functional repertoire of primary human hepatocytes (PHHs), including the ability to metabolize drugs (CYP activity). However, PHHs have a short culture life-span, exhibit phenotypic variability and instability in culture with intermittent supply and high unit costs[118].The multi-billion dollar drug development process is often hampered by the fact that candidate drugs, which show promise in preclinical animal models, subsequently do not show efficacy in humans, due to interspecies metabolic differences[116]. DILI is a leading cause of drug withdrawal from the market, highlighting the fact that current preclinical models of toxicity are not universally predictive of drug effects in humans[119]. DILI accounts for a 30% attrition rate of pharmaceutical compounds overall[120], therefore a robust and scalable human hepatic *in vitro* cell culture platform would enable physiologically-relevant preclinical data for drug screening for DILI.

5.2 HepaRG Cell line

The human liver derived HepaRG cell line is now considered the closest surrogate to PHHs for DILI applications. The HepaRG cell line is a unique and sustainable intrinsic human co-culture model system for reproducible measurements of drug uptake, metabolism and toxicity. The hepatic HepaRG bipotential progenitor cell line is able to differentiate to mature hepatocyte-like cells (HLCs) and biliary epithelial cells (BECs). Various liver-specific phenotypic functions[15, 121] are stably expressed in HepaRGs, including the major CYPs - at levels comparable to those found in PHHs, with high functional stability for several weeks. This cell line has been used as a scaffold-free spheroid to test a number of compounds to screen toxicity profiles and thresholds of a number of compounds [122]. Altogether, HepaRGs provide a high-fidelity, sustainable organotypic model system for exploring mechanisms of APAP toxicity and other forms of DILI such as chlorpromazine[122-124]. Coupling organotypic human HepaRG cells with various combinations of non-parenchymal cell (NPCs) types: hepatic stellate cells (HSCs), KCs, and LSECs provides a rational approach to providing context-specific models to investigate DILI, *viz*: i) Immunomodulation (HepaRG:KCs); ii) Vascular signals (HepaRG:LSECs), and Fibrogenic (HepaRG:HSCs) models. Stepwise integration of these cell types within a microphysiological system as well as novel 2D-3D platforms (see below, and Section 6), could be an important step in enhancing our understanding of DILI pathophysiology to solve the prediction dilemma in drug development. In principle, this approach may begin to discern what factors are lacking from current models to improve model relevance for DILI and thus unravel novel toxicity mechanisms leading to DILI.

5.3 Alternative *In vitro* Hepatic Models: *iPSC-derived hepatic tissue*

Alternative strategies to provide liver cell surrogates are found in two forms of human pluripotent stem cells (hPSCs): human embryonic stem cells (hESCs) and induced pluripotent stem cells (iPSCs). Both have the potential to serve as a source of hepatocytes-like-cells (HLCs) and other key cellular players for drug discovery and DILI research. However, ethical considerations, with moratoria or outright ban of hESC use in many countries, have prevented their widespread adoption. Therefore, hiPSCs, which are derived through the reprogramming of somatic cells, such as fibroblasts, are the mainstay of HLC-based, and multicellular human liver models (**Figure 5**) [125-127].

iPSCs have the potential to expand indefinitely and differentiate to any cell type (**Figure 5**). These characteristics make iPSCs an ideal source to obtain patient-specific cell types or to generate cells with specific genomic features resembling those of a particular human population using genome editing technologies[128]. Moreover, the pluripotent state of iPSC means they can act as a single source for the generation of the different hepatic cell types facilitating multicellular *in vitro* systems with the same genetic background or pre-existing disease[129, 130]. This technology allows production of highly novel cellular models for studying unique and unexplored aspects of DILI such as specific cell responses and multiorgan interaction.

[131-134]. Early protocols for HLC generation were based on addition of stage-specific morphogenic cues mimicking hepatic embryonic development. More recently, growth factor-free approaches have been reported using small molecules that activate or mimic the effect of growth factors with a significant reduction in costs[135-138]. The iPSC-derived HLCs exhibit many hepatic functions, including serum protein production, urea synthesis and xenobiotic metabolism. Human iPSC-derived HLCs have similar attributes to the hepatoma cell lines, HepG2)[139] and HepaRG cells (at least in 3D culture)[140], with lower metabolic activity compared with PHHs, and exhibit a mixed adult/foetal phenotype[141]. To improve HLCs functionality, strategies include generation of HLCs in 3D using collagen matrices to achieve cellular polarity, induction of mature hepatocyte genes by small molecules, or mimicking liver maturation (postpartum) by exposing HLCs to bile acid synthesis components, drug metabolism, amino acid transport or microbiome composition[142-146]. Remarkably, supplementation of the growth medium with high concentrations of defined amino acids drove metabolic maturity (PHHs levels of CYP activity) of both HLCs and HepG2 cells [140].

As all liver cell types are involved in disease process and DILI, different approaches have evolved to generate HSCs, LSECs, and KCs. iPSC-derived human cholangiocytes with functional characteristics of primary cholangiocytes[147] have been developed and used to model disease (Alagille syndrome, chronic cholestasis due to reduced intrahepatic bile ducts) and for drug validation - highlighting hiPSCs utility[148, 149].

NPCs are essential for liver homeostasis and immunological function, and play a key role in DILI. Therefore, generation of NPCs has been an intense area of research for development of complex *in vitro* systems. Tissue-resident human macrophages with KC characteristics have recently been generated, which exhibit low mismatch-background inflammatory response when co-cultured with hepatocytes [150]. HSCs also play a crucial role in response to injury/ wound healing in the liver and are the main cell type responsible for not only extracellular matrix (ECM) production and degradation, but also ECM deposition and remodelling in fibrosis. In this regard, Coll and collaborators [151] generated HSC-like cells displaying features of quiescent HSCs that could be activated by inflammatory and pro-fibrogenic stimuli, such as LPS or TGF-ß[145].

A feature of pluripotent stem cells is their ability to recapitulate aspects of liver organogenesis/ development in the dish. In a landmark study, Takebe et al., combined vascular endothelial (HUVEC) cells and mesenchymal stem cells with specified iPSC-derived hepatic endoderm. This approach resulted in the formation of a 3D structure resembling a liver bud, which upon transplantation in mice protected against DILI[152]. The use of iPSCs for *in vitro* organogenic recapitulation of the liver has been used to generate liver organoids containing HLCs and other non-parenchymal cells, which could be useful for liver disease modelling, toxicity testing and drug screening[153-155].

As an alternative to primary hepatocytes, iPSC-derived cultures of HLCs have several applications for early preclinical hepatotoxicity assessment and drug screening in 2D and 3D culture systems[156]. iPSC-derived 3D organoids demonstrated a toxic response to clinically-relevant concentrations of drugs withdrawn from the market due to hepatotoxicity [157].

DILI is frequently characterized by common pathogenic mechanisms observed in chronic liver disease, such as inflammation, fibrosis and cholestasis. In order to link these responses to toxicity, more complex *in vitro* systems that capture aspects of *vivo* architecture, and containing different liver cell populations such as HSC, cholangiocytes and inflammatory cells are required.

5.4 3D Liver Cell Models

New *in vitro* cell and tissue engineering technologies are being developed to improve hepatocyte performance and are expected to generate more robust data on the potential risks of environmental agents and pharmaceuticals to humans. To achieve more efficient DILI prediction models, it will be necessary to develop new test systems that expand capabilities of target molecules more efficiently, reduce animal testing, increase drug development efficiency and are able to predict adverse effects[158-166].

The major shortcomings of the currently available 2D *in vitro* liver systems are insufficient hepatocyte-like function and metabolic competence. A valid alternative for *in vitro* toxicology testing comprises more predictive cell models closer to the *in vivo* environment. These are summarized in Table 1, whilst bioengineering aspects of 3D liver systems, including the use of dynamic or static bioreactor devices are discussed further in **Supplementary Information** (Sections 1.1 and 1.2).

6. ADVANCED TECHNOLOGIES

6.1 Current hepatic-based microphysiological systems

Development of reliable medium-high throughput screening (HTS)-compatible human hepatic organotypic culture systems would have a significant impact on streamlining the drug development pipeline. Commercially available bioengineered liver models, including Emulate Inc., HμREL® Biochip, RegeneMed, Hepregen and LiverChip systems (**Table 1**), are based on hepatocyte-stromal cell interactions providing biomimetic cues to enhance hepatic phenotype/ functionality. These systems, however, utilize either heterologous hepatic co-cultures (rodent, primate or PHHs), combined with complex multi-step microfabrication manufacturing processes (e.g. soft-lithography, microfluidics), significantly increasing unit costs. Furthermore, the Hepregen system contains 3T3-J2 mouse fibroblasts, seeded on rat collagen-I, which can stabilize the function of the co-cultured PHHs. Such systems, however, are bio-incompatible, as they may introduce confounding variables in drug metabolism assays, given the presence of xeno-derived proteins and the fact that fibroblasts are not abundant in the functional liver acinus. Distinct challenges therefore remain with regard to realization of a standardized, cost-effective, fully customized and widely available, organotypic *in vitro* human model (**Key Point 5**).

6.2 'Liver-on-a-chip' Models

Organ-on-a-chip models (OoCs) are being developed as potentially improved experimental devices to overcome the limitations of current *in vitro* models of DILI. OoCs are multicellular models connected by microfluidic flow that mimic features and functions of the organ represented. OoCs are rapidly emerging as an alternative to animal models to study human disease, while academic research and industrial drug discovery have implemented this approach for drug target identification, validation, as well as efficacy and safety testing. Compared with 2D micro-engineered or bioprinted co-culture models, OoCs are generally less amenable to HTS due to their inherent complexity, and the need to incorporate biosensors for longitudinal real-time monitoring of biological events. Instead, they aim presently to address more complex physiological outcomes, including the pre-clinical phase of drug development.

Complex events of liver drug metabolism, as described for APAP, are a predominant feature of adverse drug reaction events leading to DILI. Therefore, to emulate organ physio-/pathophysiology in OoCs, through integration of increasingly sophisticated and more realistic hepatic models, in combination with microfluidics and miniaturization, are central. This goal requires the convergence of tissue engineering processes and technologies to attain physiologically-relevant systems. 3D bioprinting technology is a relatively new and rapidly evolving technology that is strategically placed to significantly enhance development and utility of biomimetic OoCs for preclinical applications. In addition, 3D bioprinting can be implemented as a stand-alone system to fabricate multicellular human hepatic models for HTS-amenable screening formats. Recent work has addressed major issues including limited structural complexity and resolution of many 3D-bioprinter systems, enabling assembly of complex vascular networks within 3D-printed hydrogels[167]. Many novel OoC integrative bioengineering approaches have been adopted, with new designs and innovations continuing to evolve at a rapid pace. For example, Bhise et al. [168] developed an integrated Liver-on-a-chip platform for drug toxicity assessment, based on a bioreactor interfaced with a 3D-bioprinter. Hepatocyte (HepG2/C3A cells) spheroid-laden hydrogel constructs were bioprinted directly into a ‘bioreactor’ chip. This system exhibited a functional hepatic phenotype with an *in vivo*-like response to APAP toxicity. Ever more sophisticated, multi-cellular OoCs are now emerging. Digital light processing-based 3D-bioprinting systems can rapidly print tri-cultures of hiPSC-derived HLCs, endothelial and mesenchymal cells on hexagonal 3D-hydrogel scaffolds. The biomimetic liver lobule patterns demonstrated a robust functional metabolic profile (CYP expression) and suitability for hepatotoxicity screening and DILI prediction, as well as downstream personalized drug-screening applications [169]. Ingber's group recently developed a species-specific Liver-Chip that recapitulates complex liver cyto-architecture, phenotypic profile and species-specific drug toxicities using rat, dog, and human cells[170]. Crucially, this system could identify both species-specific toxicity of drugs, such as APAP, and identify toxic events in hepatocyte and vascular channels.

Various levels of *in vivo*-like complexity have been achieved with improved PHHs stability and functionality based on urea, albumin production, and CYP450 activity[171]. This has also been demonstrated using hepatocytes co-cultured with stromal cells present *in vivo* (e.g. liver KCs; sinusoidal epithelial cells, SECs)[172-174].

Whilst an organotypic human liver C3A cell line/HUVEC co-culture system demonstrated profound susceptibility to APAP-induced toxicity in endothelial cells (reflecting the situation found *in vivo*) as compared with the monocultures; it is speculated that the vascular signals were likely hepatoprotective in the (APAP-resistant) co-cultures[175]. Proteomic analysis of LSECs may provide mechanistic insights allowing the identification of sensitive and specific biomarkers through comparison and validation of omics data from preclinical animal, in vitro human models and clinical biospecimens (see also sections 4.2 and 4.3).

OoCs may also find a particularly relevant niche in the investigation of multi-organ systems, allowing the examination of how bidirectional signals (eg metabolic, pro-inflammatory) in drug metabolism can affect other organs, and to study drug pharmacokinetics and ADME. In particular, OoCs have attracted the interest of the pharmaceutical industry by demonstrating the ability to predict metabolic drug clearance rates in accordance with clinical data [176]. The circulation of drugs and metabolites between liver and intestine has been explored using "liver-gut" models that replicate the intestinal barrier function. The parent compound Phenacetin passed through the gut barrier and was metabolized to APAP by hepatic cells[177]*,* while a model including KCs mimicked inflammatory gut-liver interactions[178].

Examples of dual-organ OoCs include liver/kidney interaction recapitulated the nephrotoxicity of ifosfamide when metabolized by liver cells[179], whilst skin and tumor compartments proved efficacious for substance testing[180]. More complex multi-organ models are under development, including: i) Liver/cardiac/muscle/neuronal system to investigate drug toxicity (doxorubicin, atorvastatin, valproic acid, APAP and N-acetyl-m-aminophenol) [181], and ii) A gut/skin/liver/kidney system in which organ-level functions were maintained for 28 days[182]. Recently, improved drug and toxicological readout was demonstrated with liver/lung/cardiac organoids derived from primary and IPS cells linked with microfluidics[183].

Increasing OoC complexity has recently been explored with up to 10 interconnected organs to explore drug and metabolite bio-distribution together with a pharmacokinetic model[184]. At present, OoC technology is still in its infancy, and while it is demonstrating important tissue engineering principles and proof of concept, complexity and interactions resulting from multi-organ models is presently very challenging to decipher whilst incompatibility with HTS is inherent. This makes OoC models (and eventually multi-organ OoCs) more suited to mechanistic studies and to predict safety and efficiency of compounds as well as their pharmacokinetics, later in the drug discovery pipeline.

7. Non-invasive technologies to screen DILI models

High-content live cell confocal microscopy is particularly suited to screen DILI models. Real-time stress response pathways such as oxidative stress, UPR and DNA damage can be evaluated quantitatively at the single cell level[185], and in individual 3D spheroids to screen for DILI[186]. In addition, novel fluorescence dyes (e.g. Thioflavin T) that react with aggregated proteins can be utilized for the determination of unfolded proteins aggregations and thus monitor ER stress induced by hepatotoxic drugs in live cell imaging settings[187, 188].

Mitochondrial stress and lysosomal dysfunction are also important mechanistic targets of DILI (see Section 3). Endosomes, cellular vesicles' motion and mitochondrial fission-fusion are highly dynamic events (>3 μm/s) that require both super-resolution and fast acquisition (<<30 ms for 100-nm-resolution). While PALM, STORM or STED super-resolution microscopy cannot currently provide such fast live imaging, a novel implementation of SIM using spinning disk confocal microscope optics has been developed [189, 190] to achieve a spatial and temporal resolution of respectively, 120 nm[189, 190], and 6 μm/s [191] (**Key Point 6**).

7.1 Optical Screening of 3D Organotypic Models for DILI

3D tissue-like DILI models can be very challenging for optical microscopy. Novel high-resolution and super-resolution optical imaging methods have very recently been developed achieving 3D optical sectioning in real-time [192-197], allowing insight into 3D DILI *in vitro* model systems[185, 186, 192-197]. Finally, optical coherence tomography is particularly suited to imaging dense tissue-like structures at mm depth. Indeed, we have recently demonstrated label-free and non-destructive measurement of APAP hepatotoxic response in 3D human liver spheroids which correlated well with cellular metabolic activity assays[198].

7.2 Super-resolution fluorescence nanoscopy

A new technology revolution of microscopy imaging called super-resolution fluorescence nanoscopy has been developed allowing molecular scale resolution, localization (<2 nm) and tracking of molecules, using a light microscope[199]. This affordable and flexible system (MINFLUX nanoscopy) will open up enormous possibilities in DILI, including 3D phenotypic profiling, imaging of protein complexes (drug-protein adducts) in pharmacological, as well as ADME and toxicology studies, with simultaneous two-colour (fluorophore) staining and recording. The Adaptive Optics system permits sharp deep tissue images down to 250μm; while live-cell imaging of 3D-organoids to a depth of 37μm into the sample can be attained using the easy3D STED imaging system. Fluorescence nanoscopy has already shown its applicability as a discovery tool in key areas transferable to mechanistic DILI studies. Indeed, nanoscopy studies of mitochondrial apoptotic mechanisms - demonstrating assembly of Bax/Bak proteins in the mitochondrial outer membrane – have revealed the structural mechanism of membrane rupture, intracellular tracking of cancer-derived exosomes, and in *in vivo* mouse models[196, 200].

7.3 Impedance biosensing

As a non-invasive alternative to optical imaging, impedance-based cellular assays (IBCA)[201] have the advantages of label-free and real-time monitoring *in vitro* liver models, and provide unique dynamics and quantitative insights into the impact of hepatotoxic drugs on cell-cell junctions[15, 202]. Recent advances in IBCA also allow the measurement of 3D models[203]. Advances in non-invasive imaging technologies in parallel with improved cell systems are powerful tools for improving DILI prediction and to reveal critical DILI events such as cellular reactive metabolite/ oxidative stress at the molecular level and in real-time.

8. Emerging *In vivo* Models of DILI

8.1 Mouse models with humanized livers

The biology and metabolism between mice and humans differ and hence the pharmacokinetics and toxicity profile of drugs can be substantially different between humans and mice. Although some limitations have been partially addressed using alternative approaches such as human hepatic cell lines, liver microsomes, PHHs or engineered human micro-livers[204], these models are of limited predictive value regarding the pharmacokinetics and toxicity of drug metabolism *in vivo* and hence of relative relevance for human safety[205]. The development of chimeric models with bioartificial livers repopulated with human adult hepatocytes could be an important advance for predicting human pharmacokinetics, drug interactions and *in vivo* safety (**Figure 6**). This will be briefly described in the following sections (**Key Point 7**).

8.2 Generation of chimeric mice with humanized livers

Several models of chimeric mice suitable for repopulation with human adult hepatocytes have been developed over the years. The first chimeric mice with a partially humanized liver was described almost 20 years ago using urokinase-type plasminogen activator-transgenic SCID (uPA$^{+/+}$/SCID) mouse[124, 206]. The degree of repopulation of human hepatocytes in these initial studies was modest (about 15%), which was sufficient in the context of hepatitis viral infection, but inadequate to investigate human phamacokinetics (ADME) in mice *in vivo*. Tateno et al generated chimeric uPA$^{+/+}$/SCID mice, replacing 70% of the liver with PHHs following anti-human complement factor treatment (estimated by serum levels of human albumin and cytokeratin 8/18 immunostaining[207]. Another model used TK-NOG mice expressing a herpes simplex virus type 1 thymidine kinase transgene in the liver of highly immunodeficient NOG mice[208]. Mouse hepatocytes deletion was performed by exposure to ganciclovir followed by xenotransplantation of human hepatocytes. Both models exhibited substantial repopulation of human hepatocytes in the liver of chimeric mice were useful in investigating the expression and activities of enzymes involved in drug metabolism.

Azuma et al[209] also generated robust expansion of human hepatocytes in *Fah*$^{-/-}$/*Rag2*$^{-/-}$/*Il2rg*$^{-/-}$ (FRG) mice (humanized liver FRG mice). Fumaryl acetoacetate hydrolase (Fah) is involved in the tyrosine catabolic pathway, and genetic deletion of Fah acts as a molecular switch to control the demise of Fah$^{-/-}$ murine hepatocytes as its ablation causes massive damage to the endogenous mouse hepatocytes, driven by the accumulation of fumaroyl acetoacetate. The injection of human Fah$^{+/+}$ hepatocytes through the spleen, lead to the gradually repopulation of the liver of FRG mice over time.

Initial studies reported the use of these chimeric mice models to investigate the expression, levels and activities of human drug metabolizing enzymes and transporters. For instance, the expression and enzyme activities of several CYPs in the liver of humanized uPA/SCID mice were similar to those in the donor liver or even greater than those found in cryopreserved human hepatocytes[205, 207, 210]. Furthermore, protein and enzyme activity levels of human UGT, sulfotransferase, *N*-acetyltransferase and glutathione-*s*-transferase in the liver of humanized liver uPA/SCID mice were reported to be similar to those in the donor liver[211]. Similar findings in terms of expression and enzymatic activities of CYPs with respect to the donor livers were reported in the liver of TK-NOG mice repopulated with human hepatocytes[212]. Therefore, these data validate the functional retention of human drug metabolizing enzymes and transporters in the humanized liver of chimeric mice, further highlighting the utility in predicting relevant drug-drug interactions in humans[213, 214].

8.3 Drug metabolism and DILI in chimeric mice with humanized CYP and human liver chimeric mouse models

In addition to mouse models with humanized livers, several human CYP-transgenic mouse models have been generated. Most human CYP family members that are involved in xenobiotic metabolism, including members of the CYP1-CYP4 gene families, have been introduced into the mouse genome as a transgene and have been summarized recently[215]. Although these models are potentially useful, metabolism of drugs in these transgenic humanized models reflects the action of a single human CYP transgene. Hence the relevance to human drug metabolism and safety may be of limited significance for human drug metabolism as this process may involve the function of multiple CYPs.

Moreover, the advantage of chimeric mouse models with humanized liver over that of CYP transgenic humanized models is that the former has been shown to generate human-specific metabolites and hence are of potential relevance for clinical drug development [215]. For instance, chimeric mice with humanized livers were recently used to study the metabolism of fenclozic acid, a drug that was developed as an alternative to high-dose therapy with aspirin in the mid 70s, and while it showed a good safety profile in experimental animals, it had to be withdrawn from late-clinical development because of hepatotoxicity. Interestingly, although fenclozic is off the market, these studies are useful to illustrate the ability of the chimeric mice to generate human-specific metabolites, such as the presence of fenclozic acid with side-chain extension in the plasma, which is not detected in conventional mice[216]. However, a drawback of the chimeric mice with humanized liver is that the remaining murine hepatocytes contain an expanded set of CYPs that form the major class of drug-metabolizing enzymes. To exploit the potential of the human hepatocytes repopulating the livers of chimeric mice, and to provide xenobiotic metabolism, Barzi et al generated a chimeric model in which the NADPH-cytochrome P450 oxidoreductase gene (POR) was knock out in a liver restricted manner in *Il2rg*$^{-/-}$ */Rag2*$^{-/-}$ */Fah*$^{-/-}$ (PIRF) mice. This provided a model with the advantage that drug metabolism in this engineered liver reflected the predominant activities of human CYPs[217]. Indeed, in response to anticancer drug gefitinib or the retroviral drug atazanavir, the *POR*-deleted humanized PIRF mice developed higher levels of the major human metabolites and were consequently able to better predict human drug metabolism[237].

Despite the relevance of APAP in human DILI, few studies have investigated APAP hepatotoxicity in chimeric mice with humanized livers. In this regard, Sato et al, examined the susceptibility of uPA^{++}/SCID mice whose liver was repopulated with human adult hepatocytes to APAP hepatotoxicity compared with control mice[218]. APAP administration resulted in vacuolation of hepatocytes and hepatocellular degeneration, leading to the detection of some areas of TUNEL-positive cells in the human hepatocyte zones. The hepatotoxic effects of APAP in the chimeric livers were milder compared to the severe liver injury observed in the control mice[218]. Further analysis indicated that APAP-related changes correlated with human CYP2E1 expression. In addition to these findings, a recent study reported the APAP hepatotoxicity of chimeric FRG mice in a NOD background (FRGN) These mice underwent xenotransplantation with human adult hepatocytes that had been pre-sensitized with valproic acid (VPA) pretreatment[27]. Comparable with wild-type mice, VPA pretreatment sensitized humanized, fed FRGN mice to APAP hepatotoxicity, although the degree of injury was somewhat lower than that seen in wild type mice, in line with findings in uPA^{++}/SCID chimeric mice. A caveat from these studies is that the degree of APAP-induced hepatotoxicity was milder with respect to wild-type mice. Given immature hepatocytes such as oval cells are reported to be resistant to APAP toxicity[219], it is conceivable that the reduced toxicity of APAP in the chimeric mice (uPA$^{+/+}$-SCID or FRGN) may be due to functional immaturity of the repopulating human hepatocytes. In addition, whether reduced presence of inflammatory cells in the liver of chimeric mice (e.g. macrophages or neutrophils) contribute to the milder hepatotoxicity remains to be investigated. In this regard, FRGN but not FRG mice is an amenable model for double humanization following reconstitution with hepatocytes and hematopoietic cells. This is achieved by treatment with human CD34^{+} stem cells[220] and may be a useful approach to faithfully reproduce the observed hepatotoxicity of APAP in humans and to pinpoint the interactions between human hepatocytes and inflammatory cells. The outcome of FRGN mice humanized simultaneously with both human hepatocytes and hematopoietic cells in DILI still remains to be established.

8.4 Zebrafish, as a DILI model

Zebrafish is a vertebrate model organism widely used in development and genetics, and potentially provides a powerful tool for modelling DILI[221-223] (**Figure 7**). Advantages of the zebrafish model include a significant level of genomic, histological and functional similarity with humans, transparency of embryos and larvae allowing thorough imaging of the liver *in vivo*, and the availability of large numbers of offspring increasing the feasibility and statistical power of drug screening experiments. Multiple types of assays have been described to characterize DILI in the zebrafish, including the detection of accumulated lipids in zebrafish larvae/liver as well as quantification of changes in liver size and numbers of liver cells by using transgenic zebrafish lines expressing hepatic-specific fluorescent proteins[224-226]. Further applications of zebrafish for DILI modelling are discussed below and in **Supplementary Information** (section 1.3).

9. Implications of DILI in clinical contexts

9.1 NAFLD and ageing as a susceptibility state for DILI

NAFLD, also now referred to as metabolic associated fatty liver disease (MAFLD), is currently the most prevalent chronic liver disease worldwide due to its association with the obesity epidemic. NAFLD is a spectrum of liver disorders beginning with steatosis, which can progress to nonalcoholic steatohepatitis (NASH), cirrhosis and ultimately hepatocellular carcinoma. Although many drugs can induce steatosis as an early sign of potential hepatotoxicity, in this section we focus on growing evidence indicating that NAFLD can sensitize to DILI.

There are increasing clinical reports suggesting that patients suffering from obesity and NAFLD may be more susceptible to DILI [55, 227-231]. This paradigm implies two possible scenarios. First, drugs such as APAP (in the context of overdose), halothane and isoflurane may cause more severe and/or more frequent ALF in individuals with NAFLD[228, 232]. Second, pharmaceuticals such as irinotecan, methotrexate and tamoxifen seem to be more hepatotoxic in obese patients than in lean individuals by triggering the transition from steatosis to NASH, and/or worsening pre-existing steatosis, necroinflammation and fibrosis[55, 233, 234]. In spite of these emerging and distinct clinical situations, well-designed prospective clinical studies are urgently needed in order to identify the full repertoire of drugs, which pose a particular risk in NAFLD patients[55, 233]. However, detection of DILI using the standard clinico-biological parameters could be difficult in patients with NASH[235]; while not all drugs necessarily pose a specific risk in NAFLD. For instance, hepatotoxicity induced by amiodarone and statins do not seem to be more frequent in NAFLD patients[233]. Interestingly, the cytotoxicity induced by amiodarone, atorvastatin and lovastatin was not greater in a cellular model of NAFLD using the HepaRG cell line [236]. In contrast, the antiretroviral ritonavir was found to be less cytotoxic in this model, although clinical investigations are warranted to determine whether this observation can be confirmed in patients.

The mechanisms whereby some drugs are more hepatotoxic in NAFLD are complex and not well understood, except for a few drugs[55, 233]. Some drugs could induce more severe ALF in individuals with NAFLD because this disease is associated with altered activity of CYPs and other xenobiotic-metabolizing enzymes (XMEs), which can increase the generation of toxic metabolites or conversely impair detoxification pathways[55, 228, 233]. For instance, human NAFLD is often associated with increased CYP2E1 activity and reduced CYP3A4 activity and also with higher glucuronide formation for some drugs such as APAP and lorazepam[237-239]. For drugs and other xenobiotics triggering the transition from simple fatty liver to NASH, or aggravating pre-existing liver lesions, experimental data strongly suggest a significant role for mitochondrial dysfunction, ER stress and ROS overproduction[55, 233, 240].

In this context, preclinical models of NAFLD can be useful for distinct purposes. First, they can be used to confirm the specific toxicity of some pharmaceuticals in NAFLD, which might have been revealed during clinical investigations. Second, these experimental models can help to decipher the mechanisms whereby some drugs or other xenobiotics are more hepatotoxic in this liver disease. Lastly, these models might also be useful in preclinical safety studies, in particular for drug candidates that would be essentially prescribed in obese patients.

Numerous rodent models of NAFLD have been useful to study drug-induced hepatotoxicity[233, 241, 242]. However, it should be stressed that some of these models do not fully tally with the clinical situation, particularly in the context of NAFLD. For instance, leptin deficiency in genetically obese and diabetic ob/ob mice curbs the development of liver fibrosis[243], and thus these mice are not appropriate to determine whether drugs are able to aggravate liver fibrosis[233]; whilst ob/ob mice do not present augmented hepatic CYP2E1 activity, which limits liver injury induced by APAP[228, 244]. Moreover, mice fed a methionine choline-deficient (MCD) diet consistently lose weight and can develop hypoglycemia[233, 245]. Finally, it should be mentioned that numerous types of energy-dense diets can be used to induce obesity associated with simple fatty liver or NASH, but the degree of the different histopathological lesions can greatly vary between diets[241, 242, 246]. However, the extent of obesity and related metabolic disorders (e.g. insulin resistance and diabetes) as well as the severity of some liver lesions (e.g. steatosis and necroinflammation) are likely to influence the activity of different XMEs such as CYPs and UGTs[55, 239]. Finally, zebrafish larvae fed lipid-enriched diets can also be used to evaluate hepatotoxicity in obesity and NAFLD. Although to the best of our knowledge this model has not been used for pharmaceuticals, recent investigations showed that obese zebrafish larvae were more sensitive to the hepatic toxicity of a mixture of benzo[a]pyrene and ethanol[247]. Interestingly, results collected in this zebrafish model, were reproduced in a cellular model of NAFLD progression, as mentioned below [247].

As with *in vivo* models, numerous *in vitro* NAFLD models have been established for various research purposes, particularly in the field of pharmacology and toxicology[233, 248-251]. These cellular models of NAFLD are based on different types of cells (i.e. primary hepatocytes or cell lines such as HuH7, HepG2, and HepaRG), fatty acids (used individually or in mixture), and duration of lipid overload (from a few hours to 15 days)[233, 248-251]. Interestingly, human iPSC-derived hepatocytes have recently been used in both 2D and 3D format to model NAFLD[252]. Another promising approach is the use of human iPSC-derived hepatocytes from patients with NAFLD including NASH, which might reproduce the inter-individual differences classically observed in DILI [253, 254]. However, some experimental conditions might not be optimal in order to determine whether a drug is more toxic in the setting of NAFLD. For instance, the human hepatoma cell lines HuH7 and HepG2 do not have the full repertoire of XMEs[255, 256], whereas rodent hepatocytes do not have the same profile of drug metabolism as human hepatocytes, as discussed in Section 5. In addition, numerous studies have been performed in cells incubated with fatty acids for only a short duration of time (from a few hours to 2 or 3 days). Thus effects of prolonged or repeat-dose xenobiotic exposure are excluded, while this period may not be long enough to induce NAFLD-related alterations of XME expression and activity[233].

Recently, a cellular model of NAFLD was established using differentiated and metabolically competent HepaRG cells incubated with 100 µM stearic acid for 7 days[257] (or with a mixture of stearic and oleic acids (150 µM each) for 14 days[236, 240, 247]. Notably, these *in vitro* models of NAFLD were characterized by enhanced CYP2E1 activity and reduced CY3A4 activity, thus reproducing what has been consistently observed in clinical studies, as previously mentioned. Of note, incubation of HepaRG cells with 100µM stearic acid for 48 hours did not change CYP2E1 and CYP3A4 activities[257], thus underscoring the importance of the duration of fatty acid exposure. Interestingly, a comparison by gene set enrichment analysis (GSEA) between the transcriptome GSE102536 dataset obtained in lipid-laden HepaRG cells[247] and the GSE61260 dataset obtained from biopsies of obese patients with fatty liver[258] revealed a highly significant correlation ($p<0.001$) concerning the up-regulated genes (B. Fromenty and S. Bucher, unpublished data). These models disclosed higher cytotoxicity of APAP[257], troglitazone[236] and a mixture of benzo[a]pyrene and ethanol[240, 247] in NAFLD cells compared with the non-steatotic cells. Regarding APAP toxicity[257], these *in vitro* investigations confirmed previous studies carried out in obese mice and humans with NAFLD[228, 232, 244]. Furthermore, mechanistic investigations showed that higher CYP2E1 activity in lipid-loaded HepaRG cells was, at least in part, responsible for higher APAP cytotoxicity[257]. Finally, it would be interesting to add cholesterol to fat-laden HepaRG cells in order to determine whether this lipid derivative could further enhance APAP cytotoxicity. Indeed, feeding wild-type mice a cholesterol enriched diet (0.5%), which induces microvesicular steatosis and cholesterol accumulation in mitochondria, sensitizes to APAP hepatotoxicity without fasting (JCFC and CGR, unpublished observations). As for NAFLD, there is evidence that older people might be at risk for DILI, at least with specific pharmaceuticals such as antimicrobials and cardiovascular agents [259, 260]. In addition to the role of some specific medications, polypharmacy is deemed to be a risk factor for DILI in old age, although this does not seem to be related to impaired intrinsic drug metabolism [259-261]. Although old mice can be used as a preclinical model [262], cellular models of hepatocyte ageing might also be useful. For instance, by using the senescence β-galactosidase assay, the occurrence of an ageing process has been observed in long-term confluent HepaRG cells [263], and cellular senescence favors lipid deposition in the liver [264, 265]. By using appropriate pre-clinical models, it would thus be interesting to determine whether NAFLD and ageing further increase the risk for DILI with some drugs.

9.2 Application of emerging mechanisms and approaches to human DILI

In the Supplementary Material, we select and briefly highlight a few examples of how some of the emerging mechanisms and approaches described above could be of value to human DILI.

10. Conclusions and future perspectives

Early pre-clinical identification of the toxic events leading to DILI is the primary goal and driver of major efforts in the pharmaceutical industry and academia to develop more realistic human-based models for DILI prediction. DILI represents an unexpected liver injury caused by either prescribed or over-the counter drugs, which entails damage to hepatocytes as well as non-parenchymal cells. Severe DILI is a serious clinical outcome and a major cause of ALF requiring liver transplantation.

Besides its clinical relevance, DILI can be a primary reason for drug withdrawal from the market. Unlike intrinsic DILI, which is predictable, reproducible, and dose-dependent, iDILI is unpredictable, not strictly dose-dependent, and although rare it accounts for 10% to 15% of ALF cases in the United States. Due to its central role in biotransformation (metabolism) of xenobiotics entering the gastrointestinal tract, the liver is the main target of DILI and hepatocyte cell death stands as the major manifestation of DILI. The mechanisms inflicting hepatocellular demise in response to drugs are still not fully understood - representing a multifactorial process in which, often activation of an immune response contributes to overall death of hepatocytes and the spread of the damage to other non-parenchymal cells. The limitation of our understanding of the underlying mechanisms and interplay between different players involved in DILI have hampered the delineation of effective therapies and the ability to predict accurately pre-clinical DILI development. This reflects the use of inadequate models used for DILI research. Indeed, unfortunately most experimental models currently used for DILI mechanistic studies do not adequately reflect the complexity of human biology and barely reproduce the features of DILI described in humans, highlighting the need to establish improved models for preclinical evaluation of DILI. Ideally these improved approaches should include experimental models that exhibit a higher concordance with human outcome through introduction of biological variation and complexity leading to delineation of mechanistic and prediction relevant DILI signals. In parallel, this paradigm shift in approaches to DILI must embrace a technological ‘bioconvergence’ encompassing multidisciplinary approaches across biology, engineering and medicine, such as coupling non-invasive imaging, multi-omics approaches, and conceptual frameworks (AOP) to organize modes and mechanisms of action, combined with microphysiological and other emerging 2D-3D multicellular platforms. Stepwise integration of appropriate human hepatic (acinar unit) cell types within microphysiological devices as well as in novel 2D-3D platforms, and experimental decoupling of the acinar unit could be an important step in enhancing our understanding of DILI pathophysiology from single-cell to organ level - to solve the prediction dilemma in drug development. In principle, this approach may begin to discern what factors are lacking from current models to improve model relevance for DILI and thus unravel better, toxicity mechanisms leading to DILI.

Bioconvergence offers a rich landscape for innovation, and includes the development of highly differentiated iPSC-derived hepatic tissues, which are accepted by regulatory agencies and pharma due to the potential of this cell resource to populate organ-on-a-chip models and to develop multi-cellular organotypic 3D liver models with personalized medicine capability. Importantly, the use of chimeric mice with humanized liver and CYP450 biotransformation potential could offer transformational insights into specific aspects of DILI such as immune signals – and as a comparator system with next generation human-based *in vitro* models. This range of integrative approaches complemented with the development of the state-of-the art of non-invasive imaging methods for screening 2D-3D models within a flexible regulatory acceptance framework could increase the feasibility to better predict DILI and even iDILI with the possibility to identify new targets for intervention and treatment of DILI.

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KEY POINTS 1-7:

KEY POINT 1

Gaps in our understanding of DILI and the complexity of underlying mechanisms coupled with interspecies differences have hampered the efforts to develop reproducible animal models.

KEY POINT 2

Currently, none of the existing models are approved by regulatory agencies in Europe and the US, given the limited predictive value of current preclinical systems.

KEY POINT 3

APAP hepatotoxicity is multifaceted and molecular pathways incompletely unknown, although disruption of mitochondrial function is a well-recognized player in APAP mediated liver injury. Novel and emerging mechanisms have been identified in this critical step, although their validation in human DILI remains to be established.

KEY POINT 4

Novel approaches and emerging mechanisms in APAP hepatotoxicity and DILI may be of significance for the discovery of new potential treatments.

KEY POINT 5

To elucidate critical pathogenic features of DILI, including genetic and immune factors, more faithful human in vitro models should include organotypic cultures and more focused investigational studies.

KEY POINT 6

Non-invasive technologies to screen DILI models may improve cell systems' readout and stand as a powerful tool for improving DILI prediction.

KEY POINT 7

More realistic human-based in vitro models and humanized rodent models are urgently required to improve mechanistic understanding in order to de-risk DILI.

FIGURE LEGENDS

Figure 1. **Pathophysiology of APAP-induced liver injury**. Acetaminophen (APAP) toxicity is caused mainly by the excess formation of N-acetyl-*p*-benzoquinone imine (NAPQI). Enhanced NAPQI depletes hepatic glutathione (GSH), covalently binds to proteins and forms protein adducts. Reactive oxygen species (ROS) accumulation oxidizes and removes Trx-1 from Trx-ASK1 complexes, leading to activation of ASK1 and subsequent apoptosis signaling cascade. The activated c-Jun N-terminal kinases (JNK) translocates into the mitochondria and alters the mitochondrial membrane potential, which triggers DNA fragmentation and cell death. Opening of the mitochondrial permeability transition (MPT) contributes to the predominant APAP-induced necrotic cell death, compared to the minor role of the release of cyt c, and apoptosis factors AIF, Smac/endo G. The increase of misfolded or unfolded proteins in the endoplasmic reticulum (ER) lumen triggers ER stress-mediated unfolded protein response (UPR), which has three different effectors: the protein kinase R-like ER kinase (PERK), the activating transcription factor 6 (ATF6) and the inositol-requiring enzyme 1 (IRE1). If UPR are not efficient to restore ER homeostasis, it will ultimately induce the elevated expression of CCAAT-enhancer-binding protein homologous protein (CHOP) and lead to cell death.

Figure 2. **Schematic role of Sab and StARD1 in APAP hepatotoxicity**. Both Sab and StARD1 are induced upon APAP metabolism and act in mitochondria. Whereas Sab functions as a docking site for activation of JNK to mediate mitochondrial dysfunction and ROS generation, StARD1 activation by an APAP-mediated ER stress causes the accumulation of cholesterol in mitochondrial membranes (orange structures), which contributes to the limitation of mitochondrial antioxidant defense and potentiation of ROS generation. Intriguingly, depletion of Sab and StARD1 independently protect against APAP hepatotoxicity, suggesting that both proteins exert complimentary roles in APAP-induced liver injury.

Figure 3. **Role of Extracellular Vesicles in DILI.** Hepatotoxic drugs induce the release of extracellular vesicles (EVs) from hepatocytes carrying a differential cargo which can be isolated from plasma or urine, providing a unique source of low-invasive biomarkers. EVs carry cargo including active enzymes that can modify the microenvironment, participating in drug clearance, but also forming active drug-protein adducts that increase toxic effects, or deplete metabolites from blood. Drug-modified proteins

can trigger an immune response when EVs are presented to dendritic cells. Studies thus far suggest that EVs play an important role in the pathogenesis of DILI, but also offer an opportunity for drug diagnosis (as biomarkers) and therapy.

Figure 4. AOPs in DILI. General representation of the structure of the Adverse Outcome concept, applicable in DILI research. The structure is fed with information obtained from different levels of biological experimentation. It can be considered as a multi-scale data integration tool, helpful in identifying knowledge gaps and prone toward indicating potential biomarkers. (MIE: molecular initiation event; KE: key event; KER: key event relation; AO adverse outcome; adapted from: Vinken et al., 2017[104].

Figure 5. Representation of methodological pathway for the use iPSCs as liver *in vitro* models. Reprogrammed iPSCs can be differentiated following two strategies, directed differentiation to liver cell surrogates or by organogenic induction. Cells resulting from both strategies can be used in 2D or 3D for biotechnological and biomedical applications.

Figure 6. Chimeric mice with humanized liver to model human DILI: Given the scarcity in the availability of PHH for drug toxicity screening, an alternative approach to potentially study human drug metabolism and impact in DILI is the xenotransplantation of primary human adult hepatocytes (PHH) in immunosuppressed mice engineered to selectively kill mouse hepatocytes while PHH gradually repopulation the mouse liver. The FRGN model is amenable for the double humanization with PHH and human hematopoietic cells to model human DILI. On the other hand, the generation of the PIRF mice in which the *Por* gene has been deleted in a hepatocyte specific manner as well as the deletion of *Il2rf, Rag2* and *Fah* genes to allow the xenotransplantion of PHH to study human drug metabolism to predicit human DILI.

Figure 7. Zebrafish to model DILI. Large-scale phenotypic assays in embryos and larvae using zebrafish model for screening of drug- or genetically-induced liver damage using wildtype (wt), mutant or transgenic zebrafish injected with different drugs of interest or morpholinos/CRISPR constructs to knockdown/knockout specific genes in a high throughput screening format. The effects of treatments are scored by microscopy manually or robotically starting from day 3 post-fertilization (3dpf) to observe

differences between the control and treatment groups in terms of differences in liver size and/or lipid accumulation.

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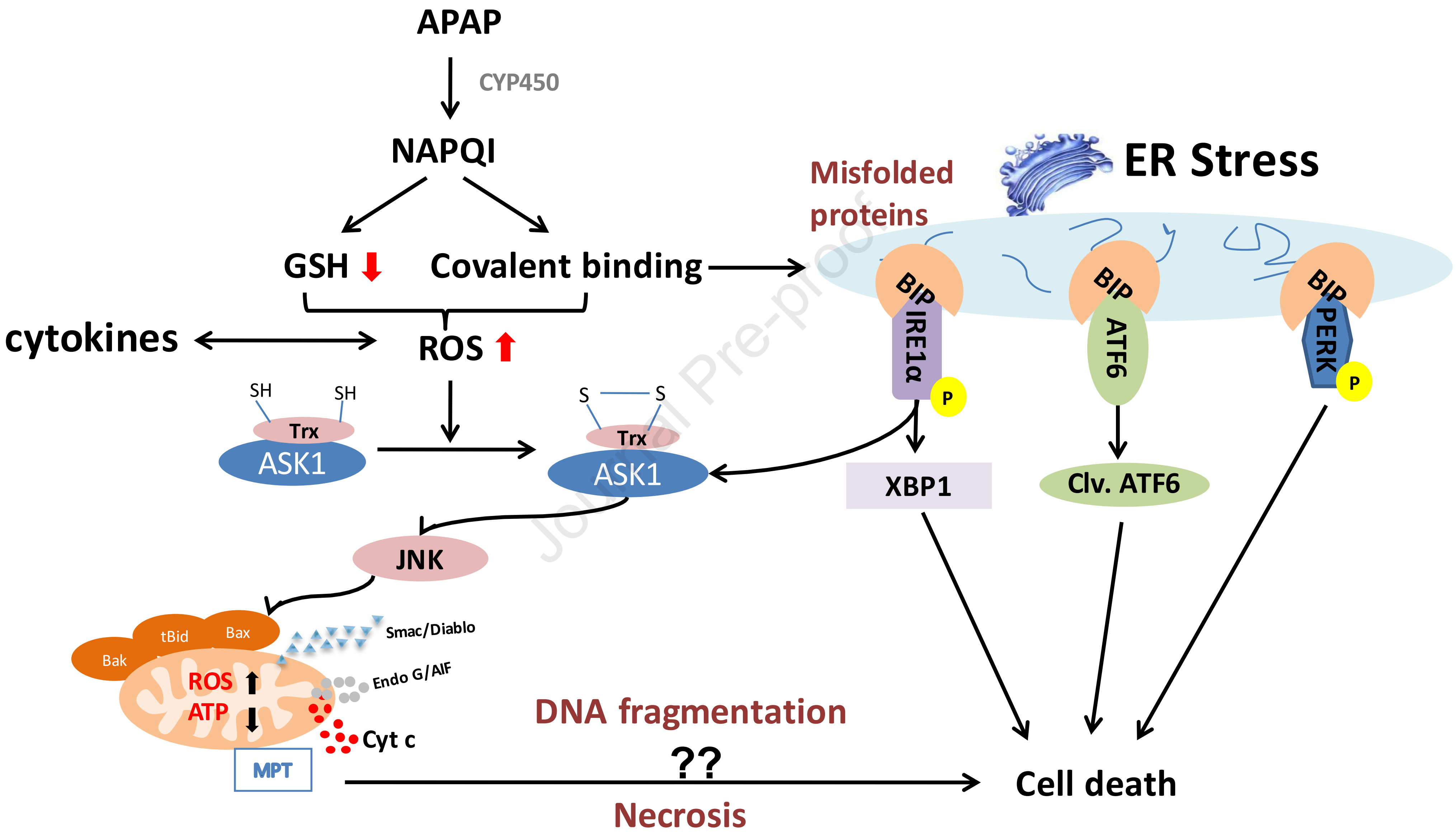
Table 1. Advantages and limitations of complex *in vitro* hepatotoxicity cell culture systems

Cell models	Advantages	Disadvantages	
ECM sandwich cultures[266-268][266-268][266-268]	Low complexity Hepatocytes regain polarity, maintain proper basolateral and canalicular transporters localization and functional bile canaliculi Enables estimation of transport clearance, enzyme-transporter interplay, and bile acid mediated hepatotoxicity	Leakage, bile canaliculi damage and development of cholestasis in a time-dependent manner	[266-268]
Stirred Bioreactors	Low complexity Scalable Long-term culture Co-culture of different cell types Enables perfusion Enables online monitoring	Requires specialized equipment Shear stress Variation in size/cell number/shape	[169, 269-273]
Hollow-fiber bioreactors	Moderate throughput Counter-directional flow Scalable Long-term culture Possibility of PBPK studies Real-time monitoring	Complex system Microscopic evaluation is only possible in the end of the experiment Requires high number of cells Cell sampling not possible	[269, 271, 273-276]
Multi-well perfused bioreactors	High throughput Cells form 3D tissue constructs Sustained liver-like cell functionality Physiological shear stress Good correlation with in vivo clearance rates Ability for microscopic examination	Uses greater cell numbers and larger media volumes	[172, 277]
Single-organ OoCs			

HμREL® Biochip	Moderate throughput Allows for multiple cell types and interaction between cell types Preservation of cell viability and metabolic competency Microscopic imaging and oxygen sensing Physiologically relevant ratios of liquid:cells and shear stress Requires less media and cells than traditional culture Good correlation with *in vivo* clearance rates	A complex system to establish and maintain Sample removal difficult No 3D tissue constructs	[278]
Multi-organ OoCs	Long term culture Improved cell functionality More physiologic model Enables tissue communication	Complex system Requires specialized equipment	
Microfluidic devices (*eg. LiverChip system*)	Long term culture Laminar flow of cell culture media mimics the blood flow hemodynamics Stable low shear pressure Possibility to study multiple organs interaction Possibility of PBPK studies Real-time monitoring of metabolic function	A complex system to develop and establish Very low-sample and cell amounts	[278-282]
Liver Bioprinting	Allows to build/design specific structures including endothelial and other cell types	A complex system to establish The printing process induces stress on cells	[279]

Adapted from[272, 279-281, 283]


APAP
CYP450
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Misfolded proteins
ER Stress
BIP
IRE1α
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ATF6
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PERK
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XBP1
Clv. ATF6
tBid
Bax
Bak
Smac/Diablo
ROS
ATP
Endo G/AIF
Cyt c
MPT
DNA fragmentation
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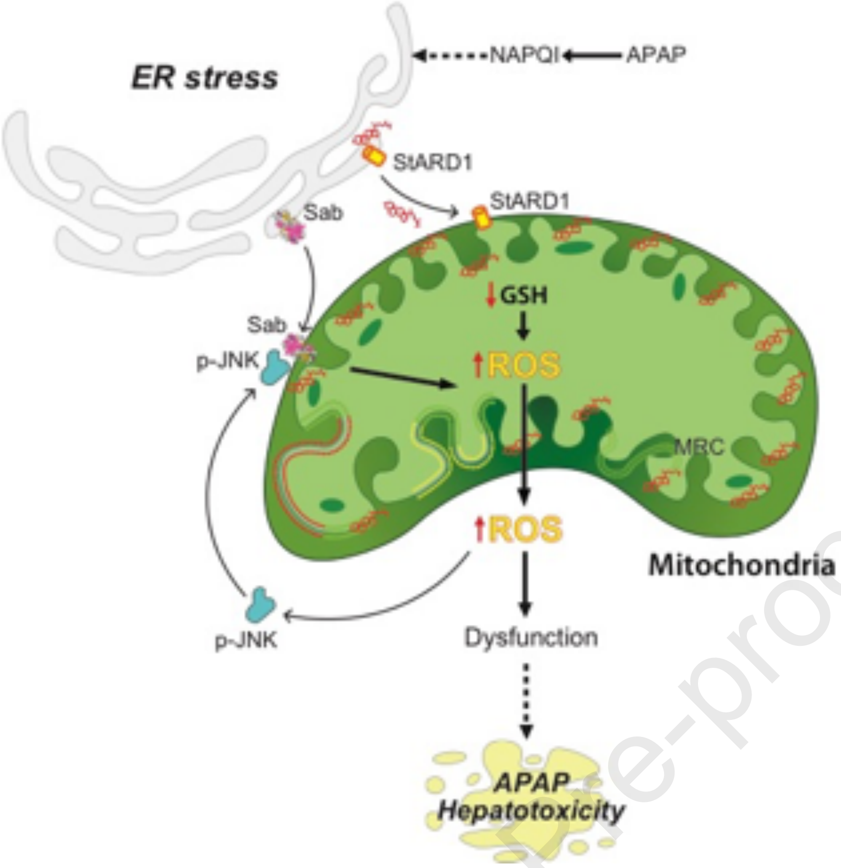
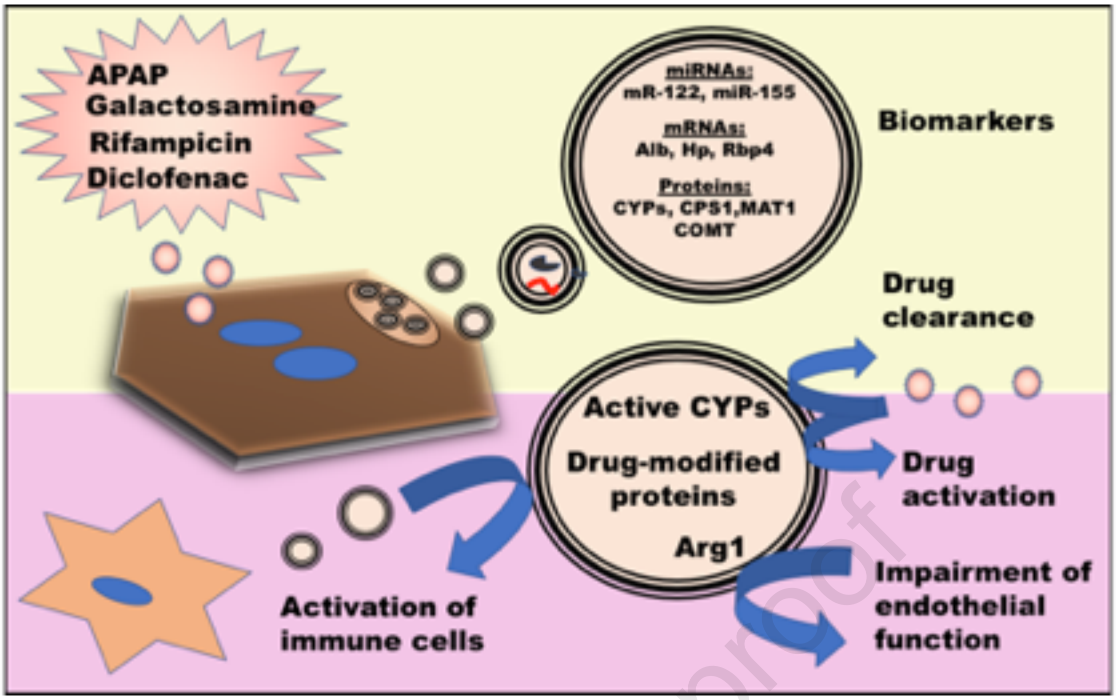
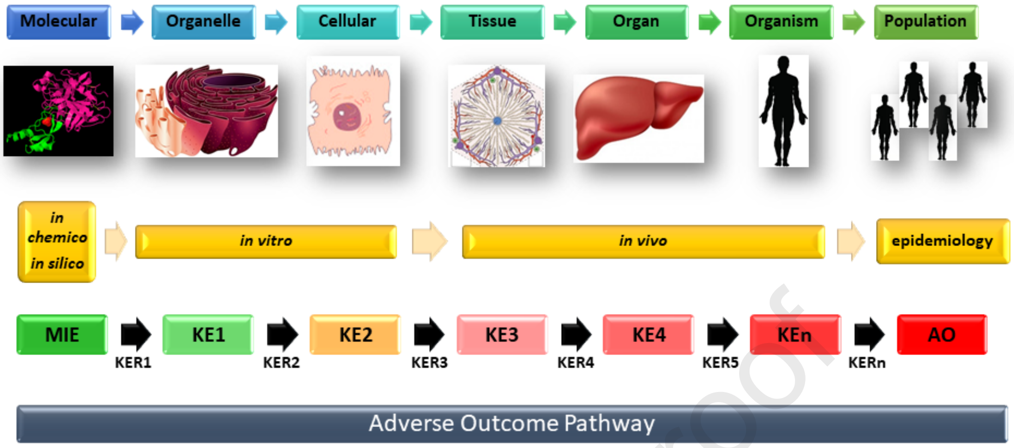
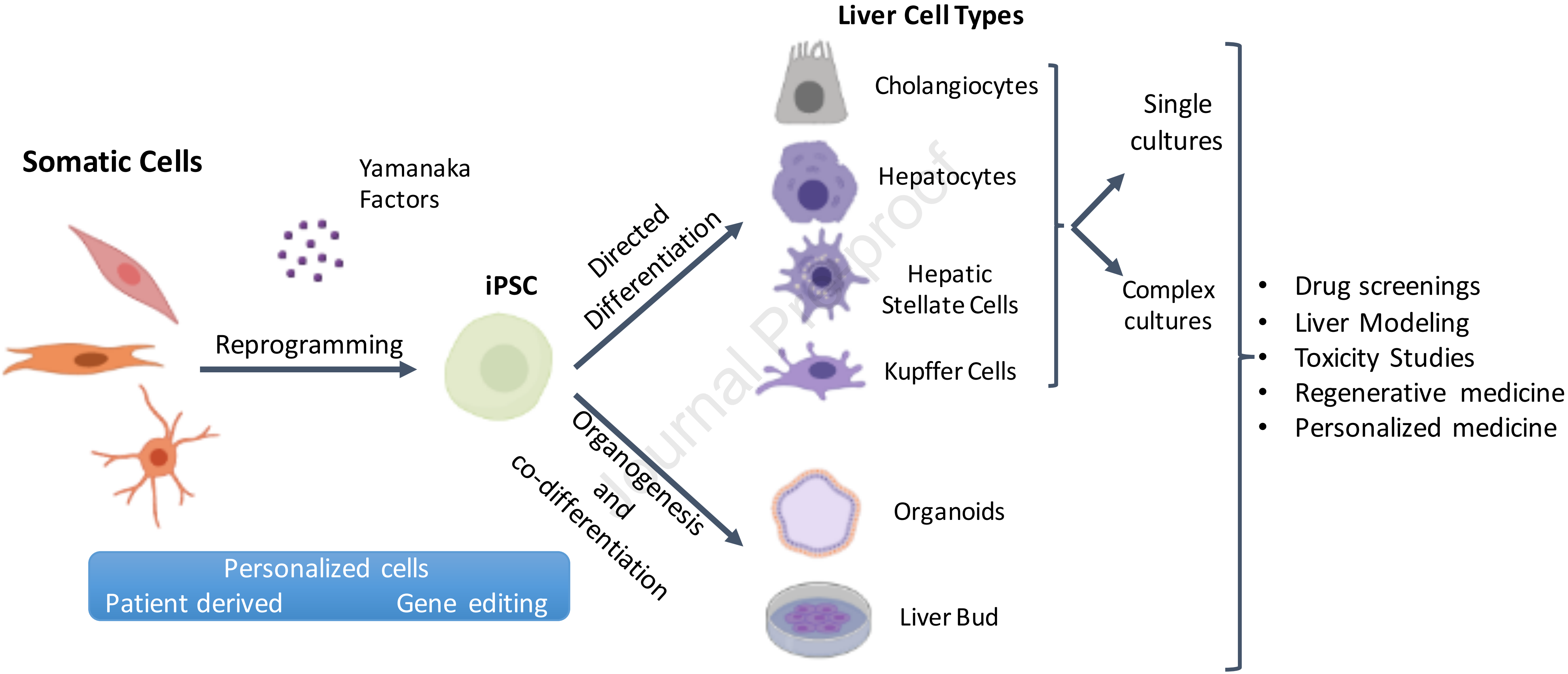
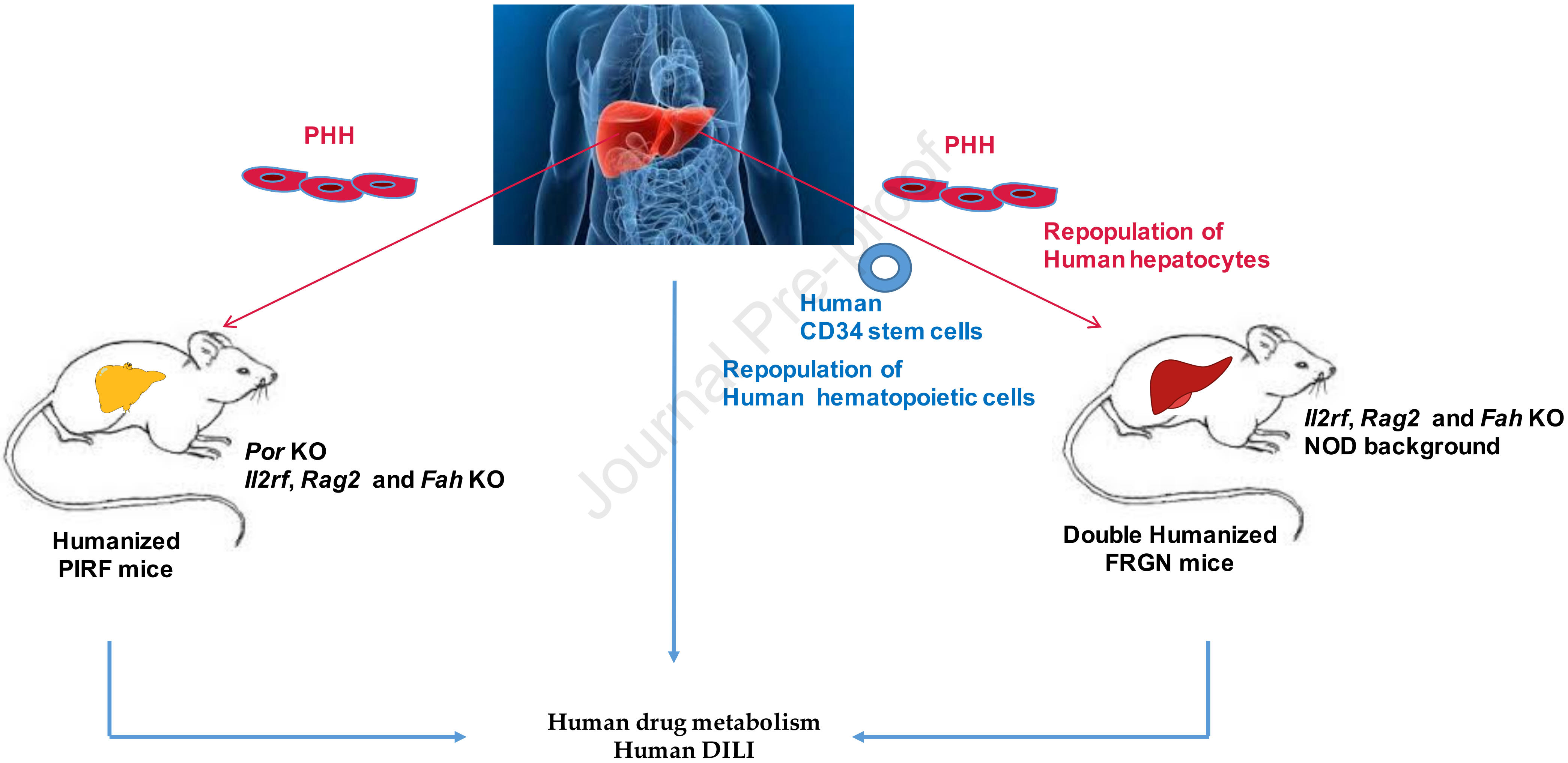
Necrosis
Cell death


ER stress
NAPQI
APAP
StARD1
StARD1
Sab
Sab
p-JNK
↓GSH
↑ROS
↑ROS
MBC
Mitochondria
p-JNK
Dysfunction
APAP
Hepatotoxicity


APAP
Galactosamine
Rifampicin
Diclofenac
miRNAs:
mR-122, miR-155
mRNAs:
Alb, Hp, Rbp4
Proteins:
CYPs, CPS1,MAT1
COMT
Biomarkers
Drug clearance
Active CYPs
Drug-modified proteins
Arg1
Drug activation
Activation of immune cells
Impairment of endothelial function


Molecular
Organelle
Cellular
Tissue
Organ
Organism
Population
in chemico
in silico
in vitro
in vivo
epidemiology
MIE
KER1
KE1
KER2
KE2
KER3
KE3
KER4
KE4
KER5
KEn
KERn
AO
Adverse Outcome Pathway


Somatic Cells
Yamanaka Factors
Reprogramming
iPSC
Directed Differentiation
Organogenesis and co-differentiation
Personalized cells
Patient derived
Gene editing
Liver Cell Types
Cholangiocytes
Hepatocytes
Hepatic Stellate Cells
Kupffer Cells
Organoids
Liver Bud
Single cultures
Complex cultures
Drug screenings
Liver Modeling
Toxicity Studies
Regenerative medicine
Personalized medicine



PHH

PHH

Repopulation of
Human hepatocytes

Human
CD34 stem cells

Repopulation of
Human hematopoietic cells

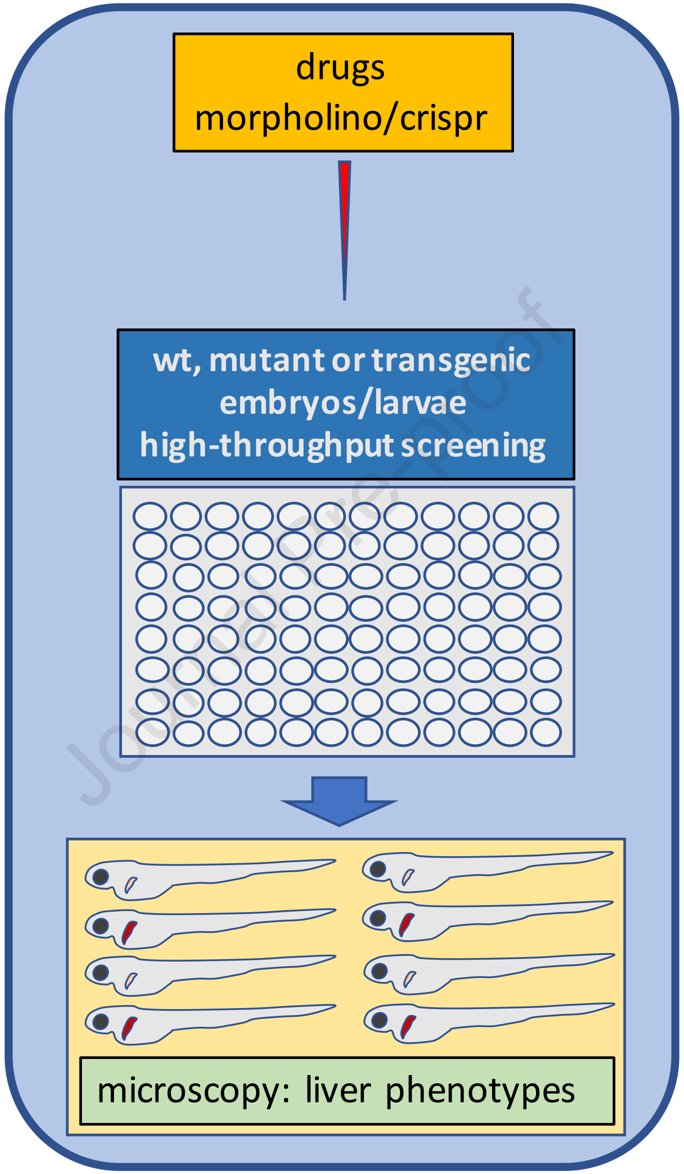
***Por* KO**
***Il2rf*, *Rag2* and *Fah* KO**

Humanized
PIRF mice

***Il2rf*, *Rag2* and *Fah* KO**
NOD background

Double Humanized
FRGN mice

Human drug metabolism
Human DILI



drugs
morpholino/crispr

wt, mutant or transgenic
embryos/larvae
high-throughput screening

microscopy: liver phenotypes