



Mechanisms of drug-induced liver injury

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Purpose of review

Idiosyncratic drug-induced liver injury (iDILI) is a relatively rare condition, but can have serious consequences for the individual patient, public health, regulatory agencies and the pharmaceutical industry. Despite increased awareness of iDILI, its underlying mechanism is still not fully understood. This review summarizes the current understanding of the molecular mechanism behind iDILI.

Recent findings

Genetic variations in drug metabolizing genes are in line with proposed mechanisms based on acetaminophen hepatotoxicity, whereby reactive metabolites covalently bind to cellular proteins and disturb the redox balance. In addition, immune-mediated effects have been reported for flucloxacillin hepatotoxicity, demonstrating both haptization and direct binding between the drug and immune receptors.

Summary

Idiosyncratic DILI development is believed to be orchestrated by multiple events, such as reactive metabolite formations, oxidative stress and signalling pathway inductions, with the mitochondria taking centre stage. Evidence also points towards the immune system (innate and adaptive responses) as important components in iDILI. Interindividual differences in one or more of these events, due to genetic variations and environmental factors, are likely to contribute to the idiosyncratic nature of this condition and subsequently distinguish between patient susceptibility and tolerance.

Keywords

acetaminophen, hepatotoxicity, mitochondrial damage, oxidative stress, pharmacogenetics, signalling pathway activation

INTRODUCTION

Drug-induced liver injury (DILI) is a broad condition that symptomatically can mimic most kinds of acute and chronic liver conditions. The causal agents of this condition were initially restricted to pharmaceutical products, but it is now apparent that many herbal and dietary supplements also can produce hepatotoxicity [1]. Despite stringent requirements for drug development imposed by regulatory agencies, DILI is an increasing health problem and a significant cause for failure to approve drugs, market withdrawal of commercialized medications and adoption of regulatory measures.

DILI is generally divided into intrinsic and idiosyncratic reactions based on dose dependency and predictability. Intrinsic hepatotoxicity is dose dependent and can subsequently be predicted once a specific threshold concentration has been consumed. The best example of this type of DILI is acetaminophen (APAP) overdose. Furthermore, good animal models have significantly enhanced our understanding of the cellular impact of APAP DILI [2].

Idiosyncratic DILI (iDILI), on the contrary, is believed to be dose independent, cannot be explained by the known pharmacological properties of the drug and is subsequently unpredictable. However, the distinction between intrinsic and idiosyncratic DILI in terms of dose dependency may not be so clear cut as initially believed. Idiosyncratic DILI has in fact been shown to be more frequently associated with doses more than 50mg/day [3]. Then again, the majority of today's pharmaceuticals require a dosage of more than 50mg/day to have a desired effect. It is therefore difficult to say with

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KEY POINTS

- Drug-induced liver injury is a complex disease dependent on environmental, chemical, pharmacological and genetic factors. The interplay between these factors and interindividual variations give rise to the idiosyncratic nature of this condition.
- The understanding of the molecular mechanism of iDILI is based on findings from genetic studies of iDILI cohorts and animal models of acetaminophen hepatotoxicity.
- The formation of reactive metabolites during drug metabolism can lead to oxidative stress, mitochondrial damage and initiation of pro-death signalling pathways. This is counteracted by pro-survival mechanisms such as Nrf2 upregulation, mitophagy and cellular repair mechanisms.
- In contrast to acetaminophen hepatotoxicity, adaptive immune responses appear to play a bigger role in iDILI, with evidence of haptenization occurring in specific forms of iDILI being reported.

certainty whether iDILI is associated with higher dosage or whether the higher identification rate of DILI cases due to pharmaceuticals with a recommended dosage of more than 50mg/day, for example antibiotics, is the result of these medications being more frequently used in today's society. Nevertheless, it is believed that some kind of dose threshold needs to be exceeded for iDILI to occur, but that this threshold may vary among individuals [4²²].

Due to the more dose independent nature of iDILI, there are currently no functional animal models to study the underlying mechanism of this

condition. Our understanding to date is therefore based on pharmacogenetic studies performed on collected iDILI cases as well as findings from intrinsic animal models. This review aims to provide a mechanistic picture of iDILI supported by the current research findings.

IDIOSYNCRATIC DRUG-INDUCED LIVER INJURY MECHANISM

The underlying mechanism of iDILI is not fully understood, but is generally believed to be multifactorial, involving environmental, chemical and pharmacological factors. The relatively rare occurrence of iDILI (1/10 000–1/100 000 patients taking a specific drug) and the fact that it often recurs after re-exposure to the culprit drug under different environmental conditions also points towards a major role for genetic variations in the underlying mechanism and disease susceptibility (Fig. 1). The liver plays a central role in drug metabolism with most drugs undergoing some form of biotransformation in this organ. Defects in the interplay between formation of reactive drug metabolites (phase I), detoxification reactions (phase II) and cellular excretion (phase III) could potentially lead to hepatic injury. Pharmacogenetics has therefore been a focal area in iDILI studies over the last decade.

Phase I, reactive metabolite formation

The formation of intermediate reactive drug metabolites is a double-edged sword, as it is a necessary step for many drugs to achieve the desired pharmaceutical effect, but can also lead to cellular damage if not controlled properly. Reactive metabolites are mostly formed through oxidation or reduction by

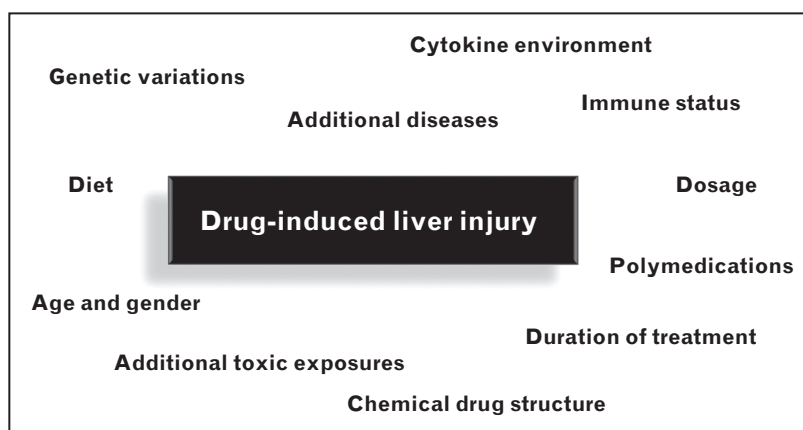


FIGURE 1. Factors potentially involved in drug-induced liver injury development. Idiosyncratic drug-induced liver injury is a complex disease influenced by various elements, such as environmental, chemical, pharmacological and genetic factors. Interindividual variations in these factors lead to the unpredictable nature of this disease.

cytochrome P450 (CYP), making this enzyme family an evident target for genetic DILI studies. Few of these studies have, however, been able to produce strong evidence in favour of specific CYP alleles, with altered enzyme activity, being associated with iDILI. On the contrary, CYP2E1 is now known as an important component in APAP toxicity, as it converts APAP to the toxic metabolite *N*-acetyl-*p*-benzoquinone imine (NAPQI). In addition, CYP3A4 induction in human hepatic cell line HepaRG potentiates lapatinib-induced cytotoxicity via increased reactive metabolite formation [5]. Intermediate reactive drug metabolites are also believed to be an important factor in iDILI development [6²²,7]. The reactive nature of these metabolites enables them to covalently bind to cellular proteins and consequently interfere with protein activity. In addition, reactive drug metabolites can cause enhanced level of reactive oxygen species (ROS) through redox cycling or glutathione depletion, potentially leading to cellular stress, activation of signal transduction pathways and subsequent hepatocyte injury/death. To have a detrimental effect, hepatocyte exposure to reactive metabolites needs to exceed a threshold level. It has been suggested that drug dose and lipophilicity, which when increased require greater metabolism, can affect the ability to reach this threshold [8].

Phase II, detoxification

The ability to detoxify reactive drug metabolites is crucial in order to limit potential cell damage. This is done through phase II conjugation whereby chemical groups, such as glutathione, glucuronate and acetyl, are added to the reactive metabolites to enhance hydrophilicity and subsequent cellular excretion into bile or urine. Genetic variations in glutathione S-transferase (GST) have been associated with iDILI caused by a large variety of drugs, with carriers lacking both the GST M1 and T1 isoforms having higher risk of developing iDILI, suggesting a common mechanism in many forms of iDILI [9]. Similarly, genetic variations leading to reduced enzyme activity of *N*-acetyltransferase 2 have also been associated with hepatotoxicity induced by antituberculosis drugs in specific ethnic populations [10].

Oxidative stress induces expression of detoxification as well as antioxidant genes, a process mainly mediated through the nuclear factor erythroid 2-related factor 2 (Nrf2), which binds to the antioxidant response element (ARE) in the target gene promoter [11]. In the absence of cellular stress, Nrf2 is bound to its repressor Kelch-like ECH-associated protein 1 (Keap1) and remains in the cytosol prior to

being degraded by the ubiquitin-proteasome pathway. The many cysteine residues in Keap1 act as redox 'sensors' and bind directly to reactive metabolites during cellular stress, whereby Nrf2 is released and translocated into the nucleus for ARE binding, with continuous studies [12,13] fine-tuning our understanding of the precise Nrf2-Keap1 mechanism. Activation of Nrf2 is believed to play a role in hepatotoxic adaptation in animal models as transgenic *Nrf2*^{-/-} mice are less tolerant to APAP hepatotoxicity, whereas hepatocyte-specific *Keap1*^{-/-} mice tolerate doses that are lethal in wild-type animals [14,15]. Hence, the Nrf2-Keap1 system is a key player in the adaptive response to cellular stress induced by reactive drug metabolites and could subsequently affect interindividual adaptation, progression and outcome of hepatotoxicity.

Manganese superoxide dismutase (SOD2) and glutathione peroxidase 1 (GPX1) are two antioxidant enzymes, whose expressions are regulated by Nrf2. Comparisons between iDILI patients and controls have demonstrated that genetic variations in the corresponding genes could be involved in iDILI development, in particular with drugs known to form reactive metabolites or having mitochondrial hazardous potential. Furthermore, accumulation of *SOD2* and *GPX1* alleles that favour H₂O₂ formation leads to faster DILI onset, which is in line with more rapidly reaching a cellular damage threshold [16]. Both SOD2 and GPX1 are integral components of the mitochondrial antioxidant defence. Mitochondrial dysfunction is believed to be a major component in APAP hepatotoxicity [17,18]. This is due to NAPIQ, which depletes mitochondrial glutathione and forms APAP-protein adducts, triggering initiation of the injury process with mitochondrial oxidative stress, compromised respiratory function and mitochondria permeability transition (MPT) induction. Although glutathione depletion in iDILI may not be as extensive as in APAP overdose, many drugs are associated with increased oxidative stress, which could potentially lead to some form of redox perturbation. Hence, the mitochondria are similarly believed to take centre stage in iDILI development.

Cellular stress and mitochondrial damage can lead to activation of various signal transduction pathways that modulate cell death and survival. The c-Jun kinase (JNK) signalling pathway is an important cellular stress component that with sustained activation leads to cell death. Animal models have demonstrated that glutathione depletion and covalent binding of NAPQI are not sufficient to cause hepatocyte death with hepatotoxic APAP doses, but that JNK is required to actively induce what has been termed 'programmed' necrosis [19,20]. JNK is now known to translocate from the

cytoplasm to redox-modified mitochondria, wherein it induces MPT and subsequent release of apoptotic factors, such as cytochrome c during APAP hepatotoxicity. Nevertheless, necrosis rather than apoptosis being the main form of hepatocyte 'execution' was recently demonstrated by measuring serum levels of full length and caspase-cleaved fragment of keratin-18 in APAP overdose patients [21,22[¶]]. It has been suggested that extensive glutathione depletion and redox changes induced by NAPQI could prevent caspase activity, leading to programmed necrosis rather than apoptosis [4^{¶¶}]. The extent of JNK activation and programmed necrosis in iDILI development remains to be determined, though it seems plausible that JNK could play an important role.

In addition to prodeath signalling pathways, such as JNK, cellular regeneration and repair pathways are also initiated during APAP hepatotoxicity and similarly assumed to occur in iDILI. Although these pathways can modulate the outcome, the effect will most likely depend on the cellular environment and possibly treatment dose. Similarly, prosurvival pathways activated by redox changes are thought to be involved in distinguishing between iDILI susceptibility and tolerance. As a means of protection, cells can also remove damaged mitochondria by inducing mitophagy (mitochondrial autophagy) in order to restrict necrotic areas and promote tissue regeneration. Lysosomal degradation of damaged mitochondria enables proteins to be recycled and used in the synthesis of new macromolecules or to generate ATP for cell survival. Although the complete signalling pathway of autophagy/mitophagy is to be identified, it holds promise as a potential therapeutic target for APAP hepatotoxicity treatments [23].

Phase III, cellular excretion

Cellular excretion of drugs and drug metabolites (phase III) is executed by ATP-binding cassette (ABC) transporters. Although altered ABC transporter activity gives rise to specific liver conditions, such as intrahepatic cholestasis and Dubin–Johnson syndrome, its role in hepatotoxicity is still unclear. The bile salt export pump (BSEP) is a selective bile salts transporter, and although not involved in drug transport directly, specific drugs can inhibit BSEP activity. This has led to a heightened alert for BSEP activity as a hepatotoxicity potential in drug development [24]. Furthermore, a genetic variation in *ABCB11* (encoding BSEP), assumed to reduce the transporter activity, has been associated with an enhanced risk of iDILI, with the degree of BSEP inhibition being dependent on the chemical

structure of the causal agent [25]. It was hypothesized that enhanced hepatocyte exposure to toxic bile salts due to drug-mediated BSEP inhibition enhances the risk of iDILI. Reduced BSEP activity has also been demonstrated in primary rat hepatocytes after 24 h exposure to simvastatin [26]. A genome-wide association study (GWAS) [27] of 783 DILI cases of European ancestry detected allelic differences in the *ABCC2* transporter gene (encoding the MRP2 transporter) between cases and control, although the differences did not reach genome-wide significance. In a recent study of 10 polymorphisms in the ABC transporter genes *ABCB1*, *ABCB4* and *ABCC2* in our laboratory, we did not find any indications of the selected polymorphisms being significantly associated with iDILI in Spanish patients. However, a rare variant in the *ABCC2* promoter was found to be restricted to iDILI cases and could potentially contribute to enhanced iDILI susceptibility [28]. Additional *ABCC2* polymorphisms have also been associated with different forms of iDILI in ethnically different cohorts [29,30].

Role of the immune system in idiosyncratic drug-induced liver injury

The introduction of GWAS brought great expectations to the revelation of genetic components responsible for iDILI susceptibility. Unfortunately, the outcome has yet not reached the anticipated results. Despite the wide coverage of variants across the entire genome, none of the previously identified drug metabolizing genes has been confirmed as a risk factor using this technique. On the basis of the hypothesis that some cellular components, such as drug-metabolizing enzymes, drug transporters and stress responses could incur an overall risk of DILI and consequently might be shared across multiple drugs, a GWAS including 487 patients with various forms of iDILI (not previously GWAS analysed) was performed, but was unable to detect any genome-wide significant differences [27]. As suggested by the authors, strong genetic determinants of DILI being largely drug-specific or rare genetic variants not assessed could explain the inability to find associations. On the other hand, genetic heterogeneity is common in complex diseases. The implication of multiple genes and pathways in DILI could possibly vary to some degree between individuals. Different genetic variations within the same gene may have the same cellular effect. However, the effect of the individual polymorphism could be 'diluted' in a GWAS, not reaching genome-wide significance and subsequently go unnoticed. Moreover, the genetic redundancy existing in the human genome may very well neutralize the disruption of

a single gene. A combination of genetic variants affecting several genes simultaneously may therefore be required to produce a specific cellular environment in which the presence of a drug could lead to iDILI.

The genetic associations detected in iDILI GWAS to date are all located in the human leukocyte antigen (HLA) region on chromosome 6, suggesting a prominent role for the immune system in iDILI [31–35]. Interestingly, some HLA associations appear to coincide between iDILI induced by pharmacologically and structurally different medications. HLA genotyping of 75 Spanish amoxicillin-clavulanate hepatotoxicity cases has also demonstrated phenotype-specific HLA associations [36]. A justification for the detected HLA associations would be the involvement of haptenization, whereby reactive drug metabolites covalently bind to carrier proteins to form adducts that can function as neoantigens. Neoantigens are then presented in HLA grooves on antigen-presenting cells and subsequently mislead the immune system into mounting an immune response against, in this case, healthy hepatocytes [37]. A GWAS on flucloxacillin hepatotoxicity has revealed a strong association with the HLA-B*57:01 allele [33] and hapten formations between flucloxacillin and albumin were recently confirmed [38[■]]. This study was able to demonstrate flucloxacillin-stimulated cytokine proliferation in peripheral blood mononuclear cells from B*57:01 carrying DILI patients, but not from flucloxacillin-tolerant or drug-naïve B*57:01 carriers. However, CD8⁺ clones obtained from naïve CD3⁺ T cells (CD45RA⁺CD8⁺) in HLA-B*57:01 positive controls were found to secrete similar cytolytic molecules when primed with dendritic cells and flucloxacillin. The CD8⁺ clones from DILI patients and drug-naïve controls were only activated in response to B*57:01 and B*58:01 antigen-presenting cells, but additional reactivity against alternative β -lactam antibiotics forming similar adducts with albumin was also detected. Although this study very elegantly demonstrates that haptenization in fact appears to be an important factor in flucloxacillin hepatotoxicity, the role of haptenization in flucloxacillin DILI patients not being B*57:01 or B*58:01 carriers remains. One might hypothesize that adduct formations with other proteins that are presented by different HLA molecules could be possible. A recent study [39] in amoxicillin-elicited allergic reaction has demonstrated haptens formed between this β -lactam antibiotic and human serum albumin, transferrin as well as immunoglobulin. Alternatively, in circumstance of heightened cellular stress, the adaptive immune system might play a less important role in iDILI

development. Interestingly, Willemin *et al.* [40[■]] recently demonstrated that flucloxacillin haptens were able to stimulate CD8⁺ T cell lines generated from individuals not having the B*57:01 allele. This group also demonstrated T cell stimulation due to pharmacological interaction with immune receptors, so-called p-I based T-cell reactivity, which was restricted to B*57:01 carriers and concluded that the presence of B*57:01 drives CD8⁺ T cell responses to flucloxacillin towards a nonhapten mechanism [40[■]].

Haptenization by itself may not be sufficient to induce liver injury, as many drugs known to form reactive metabolites that bind to endogenous proteins are not prone to induce DILI [41]. It has been hypothesized that endogenous molecules, so-called damage-associated molecular patterns (DAMPs) or ‘danger signals’ released after tissue injury, may also be required (danger model) [42]. The DAMPs can promote immune activation and triggering of inflammation that consequently affect DILI progression. An example of a potential DAMP molecule is high mobility group box 1 (HMGB1), which is translocated from the nucleus to the cytoplasm and the extracellular spaces during oxidative stress. Increased HMGB1 serum level as a reflection of protein release due to necrosis has also been demonstrated in APAP overdose patients, rendering HMGB1, similar to keratin-18, a promising new iDILI biomarker [22[■]].

Cytokines are known to regulate both innate and adaptive immune responses and might even act as potential ‘danger signals’. These messenger proteins, released from most cells, bind to specific target cell receptors in order to stimulate or suppress cell participation in immune responses. The ensuing intensity and immune response is the result of a plethora of different cytokines with synergistic or antagonistic effects on the target cell. Although cytokine release is generally tightly regulated, the cytokine environment may vary between individuals due to different cellular circumstances, such as additional infections, and this could influence the susceptibility to iDILI. A potential contribution of the antiinflammatory cytokine interleukin (IL)-10 in iDILI severity has been reported, whereby reduced IL-10 expression, due to promoter variants, and low levels of serum eosinophils were found in iDILI patients with a serious outcome [43]. The absence of eosinophils in severe DILI has recently been confirmed in iDILI biopsy samples [44].

CONCLUSION

The complete molecular mechanism of iDILI is still to be deciphered. Pharmacogenetic studies in iDILI

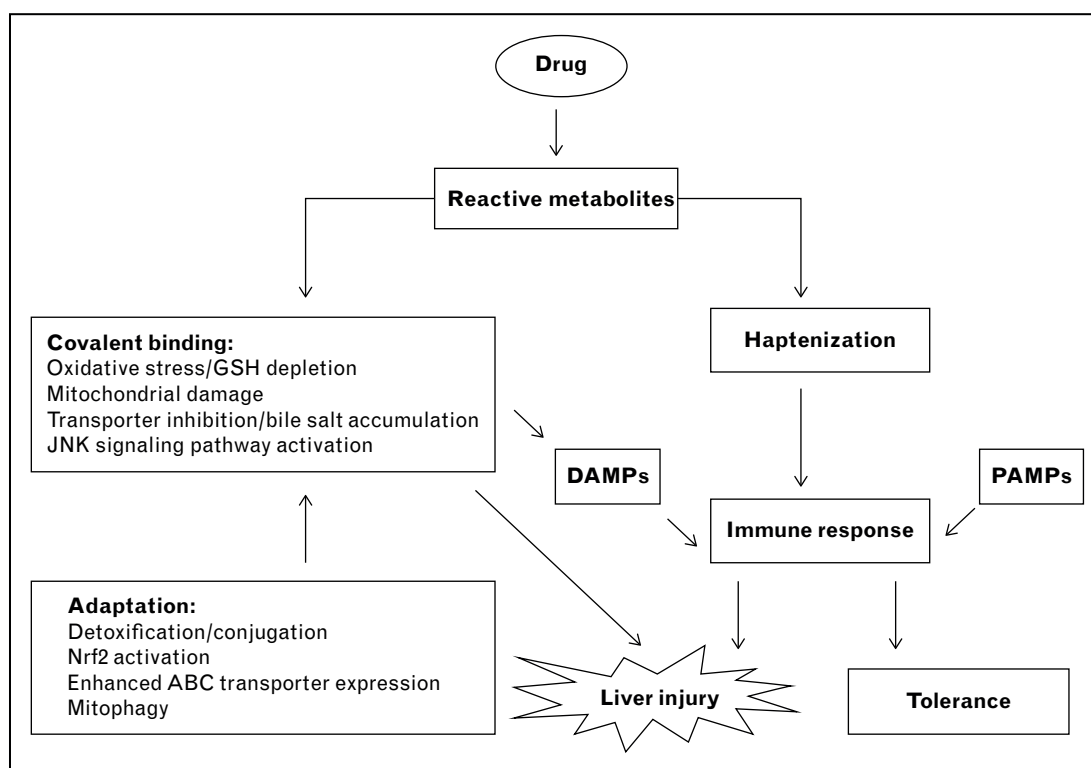


FIGURE 2. Model of molecular mechanisms in idiosyncratic drug-induced liver injury. Drug metabolism often leads to the formation of intermediate metabolites (phase I), which are conjugated (phase II) for increased hydrophilicity and facilitated cellular excretion (phase III). Covalent binding of reactive metabolites may lead to oxidative stress, mitochondrial damage and subsequent initiation of prodeath/prosurvival signalling pathways. Adducts formed between reactive metabolites and cellular proteins (haptenization) can also function as neoantigens and induce immune responses dependent on damage/pathogen-associated molecular pattern (DAMP/PAMP) signals. The balance between stress induction, adaptation and immune responses will determine the degree of injury and subsequently distinguish between iDILI tolerant and susceptible individuals.

cohorts and animal models for APAP hepatotoxicity, however, have provided important leads towards a mechanistic perception. Idiosyncratic DILI development is now believed to be orchestrated by multiple events, such as reactive metabolite formations, oxidative stress and signalling pathway inductions, with the mitochondria taking centre stage (Fig. 2). Evidence also points towards the immune system (innate and adaptive responses) as important components in iDILI. Interindividual differences in one or more of these events, due to genetic variations and environmental factors, are likely to contribute to the idiosyncratic nature of this condition and subsequently distinguish between patient susceptibility and tolerance.

Although intrinsic animal models can be useful in highlighting mechanistic areas of interest, they do not fully reflect the molecular occurrence in iDILI. Hence, patient cohorts have become a focal point in iDILI research. Due to the relative rarity of iDILI, a single centre is unlikely to obtain sufficient cases for successful studies. Collaborative efforts are

therefore needed to reach a high number of prospective cases. The concerted action of such multidisciplinary and multicentre networks enables enhanced case recruitments and subsequently the design of more powerful pharmacogenomic and mechanistic DILI studies.

Genome-wide association studies have a clear advantage due to their ability to screen a large number of variants simultaneously. However, the statistical side of this technique is somewhat lagging behind, as single polymorphisms are generally considered individually, controlling the overall type I error rate by correcting for multiple testing. Considering biological pathways and variant interactions in GWAS analyses could potentially lead to more meaningful results [45*]. This could lead us towards the ultimate goal in iDILI studies: the identification of genetic risk factors to provide a better insight into the underlying pathogenesis and enable the development of new diagnostic tools along with new safer treatment strategies.

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Conflicts of interest

There are no conflicts of interest.

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