

Novel clinical biomarkers for drug-induced liver injury

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List of Abbreviations:

Abbreviation	Full names
AcHz	Acetyl-hydrazine
AEDs	Antiepileptic drugs
AILI	APAP-induced liver injury
ALF	Acute liver failure
ALP	Alkaline phosphatase
ALT	Alanine aminotransferase
APAP	Acetaminophen (or paracetamol)
AST	Aspartate transaminase
AUC	Area under the curve
CAT	Catalase
ccK18	Caspase-cleaved K18
C_{min}	Tough plasma concentration
CML	Chronic myeloid leukemia
CNS	Central nervous system
CPIC	Clinical Pharmacogenetics Implementation Consortium Guideline
CYP450	Cytochromes P450
DILI	Drug-induced liver injury
EGFR	Epidermal growth factor receptor
ePCs	Ether-phosphatidylcholines
GIST	Gastrointestinal stromal tumor
GLDH	Glutamate dehydrogenase
GSH	Glutathione
GST	Glutathione S-transferase
GWAS	Genome wide association study
HCC	Hepatocellular carcinoma
HexCer	Hexosylceramide
HLA	Human leukocyte antigen
HMGB1	High mobility group box 1 protein
HPLC-EC	High-pressure liquid chromatography with electrochemical detection
Hz	Hydrazine
IFN- γ	Interferon-gamma
INH	Isoniazid
INR	International normalized ratio
K18	Keratin 18
LPCs	Lysophosphatidylcholines
mBC	Metastatic breast cancer
mCRC	Metastatic colorectal cancer
MHC	Major histocompatibility complex
MOAIs	Monoamine oxidase inhibitors

mRCC	Metastatic renal cell carcinoma
mtDNA	Mitochondrial DNA
NAPQI	N-acetyl p-benzoquinone imine
NAT	N-acetyltransferase
NQO1	NAD (P)H: quinone oxidoreductase
NSAIDs	Nonsteroidal anti-inflammatory drugs
NSCLC	Non-small cell lung cancer
PBMC	Peripheral blood mononuclear cells
PBPK	Population-based pharmacokinetic
PC	Phosphatidylcholine
PLOG	Polymerase γ
PXR	Pregnane X receptor
RCC	Renal cell carcinoma
SMX	Sulfonamide
SMX-HA	Hydroxylamine metabolite of Sulfonamide
SOD2	Superoxide dismutase 2
SSRIs	Selective serotonin reuptake inhibitors
TB	Tuberculosis
TBIL	Total bilirubin
TCAs	Tricyclic antidepressants
TKI	Tyrosine kinase inhibitor
TNF α	Tumor necrosis factor alpha
UGT	UDP-Glucuronosyltransferase
ULN	Upper limit of normal
VPA	Valproic acid

Abstract

Drug-induced liver injury (DILI) remains a critical clinical issue and has been a treatment challenge nowadays as it was in the past. However, the traditional biomarkers or indicators are insufficient to predict the risks and outcome of patients with DILI due to its poor specificity and sensitivity. Recently, the development of high-throughput technologies, especially omics and multi-omics has sparked growing interests in identification of novel clinical DILI biomarkers, many of which also provide a mechanistic insight. Accordingly, in this mini-review, we summarize recent advances in novel clinical biomarkers for DILI prediction, diagnosis and prognosis and highlight the limitations or challenges involved in biomarker discovery or their clinical translation. Although huge work has been done, most reported biomarkers lack comprehensive information and more specific DILI biomarkers are still needed to complement the traditional biomarkers such as ALT or AST in clinical decision making.

Significance Statement

The current review outlines an overview of novel clinical biomarkers for DILI identified in clinical retrospective or prospective clinical analysis. Many of these biomarkers provides a mechanistic insight and are promising to complement the traditional DILI biomarkers. This work also highlights the limitations or challenges involved in biomarker discovery or their clinical translation.

Visual Abstract

None

Introduction

Drug-induced liver injury (DILI) is defined as a predictable or unpredictable adverse reaction to liver induced by drugs or other xenobiotics in either toxic dose or common use (Andrade et al., 2019). DILI is becoming a serious clinical concern with the widespread use of hepatotoxic drugs including nonsteroidal anti-inflammatory drugs, central nervous system disorder drug, anti-microbials, anti-tuberculosis and target anti-tumor agents. The annual incidence of DILI in the general population was 1 to 23.80 cases in 100,000 persons around the world (de Abajo et al., 2004; Vega et al., 2017; Shen et al., 2019). Although most of DILI cases are controllable under intent management, it can lead to acute liver failure (ALF) which can be life-threatening (Chalasani et al., 2015; Stravitz and Lee, 2019).

DILI can be roughly grouped into hepatocellular, cholestatic, or mixed according to the R value defined as serum alanine aminotransferase (ALT)/ upper limit of normal (ULN) divided by serum alkaline phosphatase (ALP) /ULN (Danan and Benichou, 1993). The DILI attribution can be defined as follow: (i) hepatocellular liver injury if R value ≥ 5.0 , (ii) cholestatic liver injury if R value ≤ 2.0 , (iii) mixed liver injury if R value is 2.0 to 5.0. DILI is a complicated process involving a series of factors such as the overload of drug or metabolites, formation of reactive metabolites and the immune status of the host (Weaver et al., 2017). Crude category according to R value is not delicate enough to reflect the complexity of DILI in real world.

Traditional biomarkers such as AST, ALT, ALP or total bilirubin (TBIL) have been introduced to clinic for DILI diagnosis for decades. However, the accuracy is still poor due to their limited sensitivity and specificity. For example, the elevation of transaminase can also be observed when transaminase complexes with immunoglobulins or other proteins which protect the transaminase

from degradation in the physiological state (McGill, 2016). Based on this, the rationale of “Hy’s law” is challenged where patients with AST or ALT >3-fold of the ULN and TBIL >2-fold the ULN tend to have a poor prognosis (Zimmerman, 1968; Chalasani et al., 2015). The specificity of Hy’s law was only 45.7% in predicting the prognosis of ALF (Chalasani et al., 2015). Therefore, identifying appropriate biomarkers for diagnosis, prognosis and prediction of DILI remains a challenge in clinical practice.

Recently, the development of “omics” or “multi-omics” including transcriptomics, genomics, epigenomics, metabolomics and proteomics has sparked a growing interest in novel DILI biomarker discovery. Omics-based studies with high-throughput technology, on the one hand, allow for comprehensive analysis of various biomarkers, and significantly boost the efficiency of biomarker discovery. On the other hand, biomarkers from such screens also provide mechanistic insights for understanding DILI which are usually referred as mechanistic biomarkers. For example, HLA-A*0201 identified from genome wide association study (GWAS) was associated with amoxicillin-clavulanate-induced liver injury suggesting an immune-mediated mechanism (Lucena et al., 2011). Such newly added insights make it more possible for personalized medication in clinical practice. Here, we summarize recent advances in novel clinical biomarkers for DILI and highlight the limitations or challenges involved in biomarker discovery or their clinical translation.

1. Nonsteroidal anti-inflammatory drug-induced liver injury

Nonsteroidal anti-inflammatory drugs (NSAIDs) belong to a class of anti-inflammatory drugs that do not contain a steroid structure. NSAIDs are reported to be well tolerated generally, but adverse effects may occur in a small proportion of patients (Nissen et al., 2016). The incidence of NSAID-associated hepatotoxicity is estimated to be 1-23 cases per 100000 patient per year and NSAIDs were involved for 35.5% at clinical presentation of DILI reports (Bessone, 2010; Bunchorntavakul and Reddy, 2013).

Post-market for decades, acetaminophen (APAP or paracetamol) has been widely used since it was launched in 1995 because of its safe and effective antipyretic and analgesic effects. Although APAP is safe generally under the usual therapeutic doses (1–4 g/d), single overdose ingestion exceeded 15-25 g may cause severe liver injury (Zimmerman and Maddrey, 1995; Larson et al., 2005). In the United States, APAP overdose is the leading reason for connection to the Poison Control Centers and accounts for more than 56000 emergency room visits, 2600 hospitalizations, and 450 deaths per year due to ALF (Lee, 2004). Besides, APAP accounted for 46% of all ALF cases, which was several fold more than all prescription drugs combined (Lee, 2017). By contrast, Asia countries have a lower incidence of APAP-induced ALF because APAP is not recommended to patients with hepatitis viruses, the prevalence of which is higher in Asian countries than the West (Lee, 2017).

During the past few decades, mechanisms of APAP-induced ALF have been widely and extensively investigated. The generation of toxic metabolite N-acetyl p-benzoquinone imine (NAPQI) by cytochromes P450 (CYP450) is the key event of APAP-induced liver injury (AILI) (Kwon et al., 2020). Glutathione (GSH) is depleted by excess NAPQI and APAP adducts are

formulated by binding NAPQI with intracellular proteins including mitochondrial proteins (Ramachandran and Jaeschke, 2017). APAP can cause mitochondrial oxidative stress which triggers the mitochondrial permeability transition pore opening, resulting in mitochondrial damage and release of intermembrane proteins. The apoptosis-inducing related factor further triggers to DNA fragmentation and cell necrosis (McGill et al., 2012; Woolbright and Jaeschke, 2017). Newly added insights from these studies have shed new light on AILI biomarker discovery (**Table 1**).

1.1 Genetic biomarkers for AILI

Approximately 85% to 90% of APAP at therapeutic doses undergoes phase II metabolic enzymes conjugation *in vivo*, forming sulfated or glucuronidated metabolites which are non-toxic and further excreted through the urine (Larson, 2007). Up to 10% of APAP undergoes phase I metabolic enzymes oxidation via the hepatic CYP pathway (especially CYP2E1) to generate the toxic and reactive intermediate NAPQI (Forrest et al., 1982). The small amount of NAPQI produced from normal doses of APAP is rapidly conjugated by GSH and excreted into the urine (Larson, 2007). NAPQI generation pathway will be favored in the case of GSH saturation or excessive CYPs activity (Yoon et al., 2016). It is well-accepted that AILI is dose-dependent. But APAP can cause liver injury even at therapeutic dose indicating that individual variations in drug disposition gene could have an impact on AILI (Kurtovic and Riordan, 2003).

UDP-Glucuronosyltransferase (UGT) is responsible for APAP detoxication and clearance. Several studies have proposed the influential effect of SNPs in UGT on the glucuronidation status of APAP which possibly explain the individual variation of AILI (Alonso et al., 2015; Court et al.,

2017). But rare studies investigated the association between genetic alterations in corresponding CYPs and AILI. Individuals carrying the CYP3A5 rs776746 A allele had a higher risk to develop ALF in APAP overdose group (n = 78), compared to chronic high dose group (n = 79) and other DILI other than APAP (n = 103) (Court et al., 2014). Given that the metabolic enzymes play critical roles in APAP-induced ALF and reactive metabolite detoxication, APAP disposition gene SNPs are a promising source of biomarker discovery for prediction of AILI even though relevant studies are still limited at present.

MicroRNAs are short non-coding RNAs that regulate protein translation by targeting mRNA. Liver-specific microRNAs such as miR-122, have raised great interest among researchers due to its diagnostic effect of DILI (Liu et al., 2018). In the case of APAP, Philip J et.al first measured miRNAs expression level in serum from patients with APAP-induced ALF. Results showed that serum levels of miR-122 and miR-192 were higher in ALF patients compared to the healthy controls (Starkey Lewis et al., 2011), providing pioneering evidence for miRNAs as potential clinical predictive biomarkers in APAP-induced ALF. Serum level and dynamics of hsa-miR-122-5p can discriminate patients with AILI from those with no toxicity or ischemic hepatitis, another liver injury pattern different from that of APAP (Ward et al., 2014). Serum level of miR-122-5p alone or combined with miR-382-5p were both more sensitive in prediction of APAP-induced ALF compared to ALT (Vliegenthart et al., 2015). Overall, since laboratory assays for quantification of miRNAs are available, circular miRNAs are ideal biomarkers in identifying patients who are susceptible to AILI. But there are also some limitations of microRNAs as predictive biomarker. On the one hand, candidate microRNAs identified from different studies are different and only miR-122 is served as a common candidate. On the other hand, a threshold of

miRNA level also remains to be determined.

1.2 Non-genetic biomarkers for AILI

1.2.1 APAP-protein adducts

APAP-protein adducts are released into blood during hepatocyte lysis and its concentration is much higher in APAP overdose patients compared to the therapeutic dose patients (Heard et al., 2011; Heard et al., 2016). Quantitation of APAP-protein adducts in serum by high-pressure liquid chromatography with electrochemical detection (HPLC-EC) and immunoassays have been well-established in both experimental models and clinical studies allowing clinical measurement of APAP-protein adducts (Davern et al., 2006; Khandelwal et al., 2011). Besides, different from other biomarkers, APAP-protein adducts are considered as APAP specific. The concentration of adducts in serum of overdose patients was correlated with peak AST (but not associated with bilirubin, creatinine, APAP concentration at admission, international normalized ratio for prothrombin time and APAP dose) in a population-based pharmacokinetic (PBPk) study (James et al., 2009). Similar results were also observed in other PBPk studies (James et al., 2008; Alonso et al., 2015). Most of these studies focused on the relationship between the concentration of APAP-adducts and dose or traditional markers like ALT, but failed to provide solid evidence of APAP-protein adducts in prediction of AILI. Recently, a global multi-center prospective observational study was conducted to analyze the predictive power of the initial concentration of APAP-protein adducts in APAP-induced ALF (Chiew et al., 2020). Patients with higher initial APAP-protein adduct concentration were more prone to develop hepatotoxicity compared to those with no hepatotoxicity. More importantly, a threshold of 0.58 nmol/mL APAP-protein adduct

showed 100% sensitive and 91% specific for discriminating patients who subsequently develop AILI (Chiew et al., 2020).

1.2.2 Hepatocyte death-related biomarkers

Drug-induced hepatocyte death through apoptosis or necrosis leads to the release of intracellular contents. Serum level of releasing cell products such as protein, microRNA which has been discussed above are promising mechanistic biomarkers for diagnosis and prognosis of DILI.

The serum level of full-length keratin 18 (K18), a cytoskeletal protein, reflects the cell necrosis and its cleaved form caspase-cleaved K18 (ccK18) reports cell apoptosis. Besides, high mobility group box 1 protein (HMGB1) is reflective of cell necrosis. Clinical observations have associated serum level of these biomarkers to AILI. A small population-based study showed that in patients with normal ALT or international normalized ratio (INR) (n = 99), miR-122, HMGB1, and K18 could differentiate patients who developed AILI (n = 15) from those that did not (n = 84) with a high degree of accuracy. Furthermore, these biomarkers also significantly outperformed ALT, INR and plasma APAP concentration for the prediction of subsequent ALF (n = 11) within 8 h of overdose (Antoine et al., 2013). Similarly, miR-122, HMGB1, and full-length K18 were also associated with APAP-induced ALF in both derivation cohort (n = 985) and validation cohort (n = 202) and a combined model of miR-122, HMGB1, and full-length K18 had a more sensitive predictive power than ALT alone (Dear et al., 2018). Application of such a biomarker panel could improve the efficiency of clinical decision making both in the prevention and the treatment of ALF.

1.2.3 Mitochondrial damage biomarkers

Mitochondrial dysfunction is a pattern of AILI. Serum markers of mitochondrial damage, including mitochondrial DNA (mtDNA), glutamate dehydrogenase (GLDH), nuclear DNA fragment level and circulating acylcarnitines have been investigated as clinical surrogate markers capable of indicating mitochondrial lysis following hepatocyte necrosis during AILI (James et al., 2020). Peak GLDH activity, nuclear DNA fragment level as well as mtDNA were elevated in APAP overdose patients with AILI and serum level of these three biomarkers was increased in mice treated with APAP, but not in mice treated with furosemide which causes a similar pattern of centrilobular necrosis without affecting mitochondria, indicating the essential role of mitochondrial damage in AILI (McGill et al., 2012). More mitochondrial damage was also observed in patients who died of APAP-induced ALF (McGill et al., 2014). However, both mtDNA and GLDH were not identified as powerful predictors for APAP-induced ALF in other studies (Antoine et al., 2013; Dear et al., 2018). Whether mitochondrial damage markers can act as predictors in AILI is still controversial. Therefore, a combination of two or more candidate biomarkers rather than mitochondrial damage markers alone is more reasonable in stratification of patients. Compared to each biomarker alone, a multivariate model with GLDH, K18, and miR-122 was capable of accurately differentiating AILI subjects from healthy volunteers or patients with organ damage indicating that GLDH, K18, and miR-122 are unique biomarker in predicting liver injury (Llewellyn et al., 2021).

Long chain acylcarnitines are metabolized in mitochondrial and mitochondrial dysfunction can cause accumulation of circular acylcarnitines. Hence, elevation of acylcarnitines in the serum is considered to be a sign of AILI. Targeted metabolomics in children have observed a significant

increase of acylcarnitines in the APAP overdose group (n = 62) compared to the therapeutic dose group (n = 187) and healthy subjects (n = 23) (Bhattacharyya et al., 2014). Inconsistently, another metabolomic study did not find acylcarnitine increases in overdose patients even though a significant elevation of acylcarnitines in APAP overdose mice was observed (McGill et al., 2014). Overall, studies on the role of acylcarnitines in AILI are limited and whether clinical measurement of circular acylcarnitines can help AILI prediction remains to be validated.

2. Central nervous system disorder drug-induced liver injury

2.1 Antiepileptic drug-induced liver injury

Central nervous system (CNS) drugs, the second most commonly implicated types with liver injury, have substantial reports in DILI rank accounting for 12.5% among 671 drugs (Bjornsson, 2016). The subgroup of antiepileptic drugs (AEDs) gets metabolized in the liver, especially by UGT superfamily. AEDs are shown to be the important cause of DILI worldwide, with an incidence of 11%, 3.9%, 3% and 2.2% of DILI registries from India, Spain, United States and Sweden, respectively (Devarbhavi and Andrade, 2014). Majority of the AED-related hepatotoxicity are attributed to the widely-used first-generation antiepileptic drugs, such as valproic acid, phenytoin, phenobarbital, or carbamazepine while second-generation or newer antiepileptic medications (clobazam, pregabalin, levetiracetam, etc.) are less likely to cause DILI than their older counterparts.

Valproic acid (VPA) is a broad-spectrum antiepileptic drug with specific indications for partial/generalized seizures and bipolar disorders, affecting both adults and children. It is rapidly absorbed after a single dose and has a relatively high bioavailability (96%-100%) and protein

binding rate (87%-95%) (Romoli et al., 2019). VPA is metabolized via three major pathways: glucuronidation, mitochondria β -oxidation (both have been regarded as predominant metabolic routes accounting for 50% and 40%, respectively) and CYP450-mediated oxidation (only 10%) (Romoli et al., 2019). Adequate exposure to ensure antiepileptic effect and minimizing adverse reaction are confirmed as a trough plasma concentration (C_{\min}) of 50-100 ug/L (Vajda et al., 1978). VPA-related liver injury is well-recognized as the third most common cause of drug-induced liver fatalities reported by the World Health Organization in 1808 hepatic ADRs (Bjornsson and Olsson, 2006). Previous data available for analysis illustrated that the incidence of VPA-related liver injury is about 1/35,000 adults, 1/500-1/800 children and less than 1 in 500 in the high-risk population (Perucca, 2002; Zaccara et al., 2007). Currently, VPA has drawn most attention on biomarker discovery compared to other antiepileptic drugs such as carbamazepine (**Table 2**).

2.1.1 Genetic biomarkers for VPA-induced liver injury

Candidate gene studies have been conducted in the setting of clinical trials studying the contribution of the genetic polymorphisms of CYPs and UGTs pathways, including UGT1A6, CYP2A6, CYP2B6 and CYP2C9, which play a vital role in metabolism of VPA and have a potential impact on VPA or its metabolites exposure in patients. For example, UGT1A6 mutations might be responsible for the differences in plasma VPA concentration which results in a dosage adjustment, and might be risk factors for VPA-induced liver injury in infants (Hung et al., 2011; Guo et al., 2012; Ghodke-Puranik et al., 2013). Metabolic pathway of CYPs (CYP2A6, CYP2B6 and CYP2C9) generates hepatotoxic metabolites, such as 4-ene VPA and its β -oxidation

metabolite 2-propyl-2,4-pentadienoic acid, which are responsible for severe hepatic microvesicular steatosis (Kesterson et al., 1984; Zhao et al., 2017). Homozygotes of CYP2C9*2 and CYP2C9*3 could reduce the oxidative biotransformation of VPA to 4-ene-VPA, 4-OH-VPA, and 5-OH-VPA (Kiang et al., 2005). In addition, compared to the WT, the serum level of 4-ene-VPA was decreased by 29% and 61% in patients carrying one and two mutated CYP2C9 alleles, respectively (Ho et al., 2003). Notably, monitoring of VPA and its hepatotoxic metabolites (such as, 4-ene VPA) in plasma can provide a great benefit for avoiding VPA-induced hepatotoxicity (Ghozzi et al., 2011; Chen et al., 2012). However, the mechanisms and underlying signaling transductions of liver injury induced by these metabolites or polymorphisms need further investigations. In addition, we still lack studies to reveal the therapeutic window for VPA with genetic feature (i.e. 50-100ug/L therapeutic window for VPA) and further studies are needed for the determination of therapeutic window of VPA among different populations.

A host of studies have elucidated that the inhibition of mitochondrial β -oxidation or fatty acid transport, and the VPA-metabolic products might contribute to its hepatotoxicity (Guo et al., 2019). Gene mutations involved in mitochondrial metabolic and oxidative stress pathway such as POLG, SOD, GSH and CAT are likely to provide mechanistic insights for VPA DILI.

POLG which codes mitochondrial DNA polymerase γ (pol γ) is associated with an increased risk of VPA-induced hepatotoxicity, and even fatal damage to liver cells (Saneto et al., 2010; Stewart et al., 2010; Sitarz et al., 2014), indicating a mitochondrial-dependent apoptosis to hepatocytes and providing potential biomarkers for VPA-induced liver injury. In a study involving 17 patients with suspect VPA-induced liver injury, heterozygous genetic variations in POLG (c.3708G>T/p.Q1236H and c.3428 A>G/p.E1143G) was strongly related to the hepatotoxicity

with a >20-fold risk elevation (Stewart et al., 2010). When a large retrospective analysis was performed, no association was demonstrated in the VPA DILI patients (Hynynen et al., 2018), suggesting a genetic variation in the population and prospective RCT studies are still invaluable.

Glutathione S-transferases (GSTs) are critical enzymes that catalyze inactivation of various endogenous and exogenous reactive compound by GSH conjugation. Homozygous deletion of the gene comprises null GSTM1 and GSTT1 genotype, leading to deficiency in GST. GSTM1- and GSTM1-/GSTT1- genotypes were associated with elevation of γ -glutamyltransferase levels in VPA-treated patients while no significance was found with ALT or AST (Fukushima et al., 2008). Meanwhile, superoxide dismutase 2 (SOD2) polymorphism was also tested for its potential risk for liver damage, and found that SOD2 Val/Val contributed to VPA-induced liver dysfunction with an elevated γ -glutamyltransferase levels (Saruwataria et al., 2012). Furthermore, catalase (CAT) genotype was reported as a significant genetic risk factor for VPA-induced liver dysfunction, and CAT C-262T carriers exhibited an increased risk of developing abnormal hepatic function compared with the non-carriers (OR=3.968, $P = 0.003$) (Ma et al., 2019). These findings raise the possibility that GSTs, SOD2 and CAT may be implicated in VPA -induced hepatotoxicity for their roles on the liver oxidative stress.

2.1.2 Non-genetic biomarkers for antiepileptic drugs-induced liver injury

Currently, with the development of multi-omics technologies, the discovery and validation of more satisfactory non-genetic biomarkers for VPA therapy-induced hepatotoxicity is further highlighted in the clinical setting. Several studies investigating the metabolic profiles of VPA-induced hepatotoxicity have been conducted to identify the specific indicators of adverse

effects *in vitro* and *in vivo*. For example, a study presented evidence of the early diagnosis of VPA-induced liver injury associated with glycolysis, lipid and amino acid metabolism (i.e. glucose, acetoacetate, lactate, phosphatidylcholines, amino acids, lysophosphatidylcholines, creatine, N-acetyl glycoprotein, choline, uric acid and pyruvate) (Huo et al., 2014). Another metabolic profiling study elucidated that VPA induced alterations in oxidative stress and branched chain amino acid metabolism (Price et al., 2011). Accordingly, several metabolomic studies found that lipid transport/fatty acid in HepG2 cells, hippuric acid in rat urine and disrupted glycine in serum could serve as metabolic biomarkers for VPA -induced liver injury (Lee et al., 2009; Ji et al., 2010; Sun et al., 2010).

Lipid metabolism disruption was proposed to be a key event in VPA-treated patients and several lipidomic studies have already identified lipid biomarkers for VPA-induced liver injury. Un-targeted lipidomics analysis revealed that LPCs, Cers and SMs decreased markedly in patients with abnormal liver function (Sun et al., 2010), in which peroxime proliferators-activated receptor pathway played an essential role in activating the uptake of long-chain fatty acid and TAG synthesis. The details of lipid dysmetabolism were described and metabolite molecules, such as phosphorylcholine, sphingomyelin, triglyceride, phenolic phthiocerol, ceramides, as well as topped reductions of diradylglycerols, 1 α ,25-dihydroxy-24-oxo-22-oxavitamin D₃, phosphoethanolamines, dolichyl-4 phosphate, 2-deoxy-20-hydroxy-5 α -ecdysone 3-acetate were also identified as potential biomarkers in epileptic patients with VPA-induced dyslipidemia (Li et al., 2019). In addition, lipidomics analysis in rodents with VPA-induced hepatotoxicity found compelling evidence that ether-phosphatidylcholines (ePCs) were altered significantly in the liver and plasma though receiving a low dose level (Goda et al., 2018). Overall, current

comprehensive information on effective biomarkers for VPA-induced hepatotoxicity still lack. Further validation studies should be conducted to confirm these results.

2.2 Antidepressant drug-induced liver injury

The prevalence of antidepressant usage increases with years worldwide. Almost all antidepressants can trigger hepatotoxicity, even under a therapeutic dose. Such injuries are usually unpredictable, idiosyncratic, dosage unrelated and generally occur as early as within a few days or up to 6 months after initial drug administration (de Gage et al., 2018). Antidepressant-induced liver injuries have been reported 5% and 4% of cases in Spanish registry and DILIN (Andrade et al., 2005), respectively. A cross-sectional analysis of the cases reported in a registry (n = 387) presented that hepatotoxicity induced by antidepressants constituted 5% of all cases. The traditional antidepressants, such as tricyclic antidepressants (TCAs) and monoamine oxidase inhibitors (MOAIs), have higher risk for DILI than the next generation counterparts (Spigset et al., 2003). Selective serotonin reuptake inhibitors (SSRIs), the second-generation antidepressants, accounted for the incidence of SSRIs-induced liver injury of 4/100,000 patient-years (Voican et al., 2014). Life-threatening or severe DILIs has been reported for duloxetine, bupropion, trazodone, venlafaxine, nefazodone, etc (Voican et al., 2014).

Antidepressant-induced liver injury is generally hepatocellular, and less frequently cholestatic or mixed. Regarding the mechanisms for antidepressant-induced liver injury, immune-allergy or reactive metabolite formation was usually considered as the main pathophysiology (Voican et al., 2014). A major concern regarding of risk factors is dependent primarily on CYPs pathway, because the antidepressant drugs are mainly metabolized in liver by CYP system. Antidepressant-induced liver injury is generally deemed to follow a dose-independent pattern

(Voican et al., 2014; Vukotic et al., 2021). However, inter-individual variabilities of antidepressant drugs in treatment response and adverse effect have been related to the variability of tricyclic or corresponding metabolites concentration in plasma, and thereby potentially increasing the risk of liver injury (Rudorfer and Potter, 1999).

Amitriptyline, a commonly used tricyclic antidepressant with tertiary amines, exerts both the antidepressant and antinociceptive activities due to, but not exclusively, its ability to combine with serotonin and noradrenaline at central sites (Mico et al., 2006). Liver test abnormalities occurred in 10%-12% of patients who administered amitriptyline. A high administration dose of amitriptyline and prolonged exposure can cause steatosis (Sahini et al., 2014), but the patho-mechanism to liver damage is still incompletely understood. It is assumed that the toxic metabolites of amitriptyline result in hepatotoxicity (Wen et al., 2008). Amitriptyline is mainly metabolized by CYP2C19 to desmethyl-metabolites, including nortriptyline which is also regarded as secondary amines. And then both the amitriptyline and its desmethyl-metabolites are metabolized by CYP2C6 to 10-hydroxy metabolites with low activity. There is substantial information relating CYP2C19 and CYP2D6 genotypes to phenotypic variability in tricyclic pharmacokinetic profiles and side effects. Clinical Pharmacogenetics Implementation Consortium Guideline (CPIC) (Hicks et al., 2017) published dosing recommendations for tricyclic antidepressants based on CYP2C19 and CYP2D6 genotypes, in which patients were assigned as ultrarapid metabolizers, normal metabolizer, intermediate metabolizer and poor metabolizer determined by their CYP2D6, CYP2C19 genotypes alone or combination. It is noted that CYP2D6 poor metabolizers exhibited higher than expected plasma concentrations at usual dosage. The FDA-approved drug label for amitriptyline recommended that monitoring of plasma

concentration is necessary whenever it is going to be co-administered with an inhibitor of CYP2D6

[https://www.accessdata.fda.gov/drugsatfda_docs/label/2014/085966s095,085969s084,085968s096,085971s075,085967s076,085970s072lbl.pdf; <https://www.ncbi.nlm.nih.gov/books/NBK425165/>].

However, whether CYP2C19 and CYP2D6 are specific to DILI is still unknown.

Regarding the results from metabolomics studies, lysophosphatidylcholines (LPCs) (16:1, 18:1, 18:2, and 20:4) and 42:1 hexosylceramide (HexCer) could be served as biomarkers by analyzing the molecules separating control rats and rats with amitriptyline-induced hepatotoxicity (Saito et al., 2014). Another study was conducted to describe the spatial abundances of lipids and bile acids in amitriptyline treated rat livers, as a result, several lipids were found to be upregulated and nine of them were classified as distinct phosphatidylcholine (PC) species illustrating phospholipidosis (Kampa et al., 2020). The useful genetic or non-genetic biomarkers for amitriptyline-induced hepatotoxicity are poorly known and have not been validated in depressant patients.

So far, no genetic polymorphisms related with greater susceptibility to idiosyncratic DILI from antidepressant drugs have been depicted. More efforts have to be made in the research of anti-depressant drug-induced liver injury.

3. Antimicrobial-induced liver injury

Antimicrobials are the one of most common types of drugs leading to liver injury with an incidence of 20% - 40% of all drugs that cause DILI in western countries (Sgro et al., 2002; Bjornsson et al., 2013; Chalasani et al., 2015). In a recent retrospective study of 46,266 cases for the assessment of causality of idiosyncratic DILI, amoxicillin-clavulanic combination is the most

leading cause of DILI (Shen et al., 2019). Different from the West, according to a recent study conducted in mainland China, the proportion of antimicrobial-induced liver injury in China is 6.08%, ranking fourth among all the implicated DILI drug classes (Teschke, 2019). Anti-microbial-induced liver injury is normally mild and moderate but it can also lead to severe consequences such as liver-related death or ALF which needs liver transplantation. The reason why antimicrobials are so prone to DILI is still not fully elucidated. Several retrospective studies have found that antimicrobials are the main cause of idiosyncratic DILI which develops within several weeks after treatment due to metabolic or and immuno-allergic drug reaction (Garcia-Cortes et al., 2011; Teschke, 2019; Teschke and Danan, 2020). These studies indicate that pathways involving the generation of immunoreactive intermediate metabolite or host immune response are possibly the main mechanism of antimicrobial-induced liver injury. Biomarkers participating in generation of immune reactive compound like NATs polymorphisms or immune allergic pathway like HLA polymorphisms are promising to predict the risk of antimicrobial-induced hepatotoxicity (**Table 3**).

3.1 Genetic markers for antimicrobial-induced liver injury

Antimicrobials-induced liver injury is usually considered idiosyncratic for the reason that it usually occurs in the recommended dosage and thus dose-unrelated. However, researches shown that idiosyncratic DILI could be dose-dependent. Higher daily dose of oral prescription medication and longer duration of exposure were associated with higher risk of idiosyncratic liver injury (Lammert et al., 2008). These observations suggest that polymorphisms in drug metabolism enzymes or transporters have an effect on the risk of anti-microbial induced liver injury.

Sulfonamide (SMX) is a common cause of hypersensitivity related hepatotoxicity which is attributed to its hydroxylamine metabolite (SMX-HA) (Cribb et al., 1991; Cribb and Spielberg, 1992). SMX-HA can undergo an autoxidation and become a hyperreactive derivative. One of the main detoxication pathways of sulfonamide is the N-acetylation by N-acetyltransferases (NATs), leading to the generation of an inactive form of the patent drug and further excretion through urine. Patients carrying SNPs in NAT2 leading to a slow phenotype of N-acetylation are more likely to develop sulfonamide-induced hepatotoxicity. In a study from Poland, NAT2 slow acetylator genotype NAT*5 (481 C > T) was found related to cotrimoxazole associated idiosyncratic reactions in 20 infants (Zielinska et al., 1998). Besides, in another study of 18 patients developing sulfonamide induced toxic epidermal necrolysis and Stevens-Johnson syndrome, 17 were identified as slow acetylator genotype (NAT2*5, *6, *7) (Wolkenstein et al., 1995). Conversely, no association of NAT2 coding alleles with sulfonamide hypersensitivity was observed in another study of 99 Caucasian adults (Sacco et al., 2012). Association between NAT2 SNPs and sulfonamide-induced hepatotoxicity needs further validation.

Apart from metabolic pathways, immunological responses of the host also play an important part in idiosyncratic hepatotoxicity which is a complex procedure involving numbers of factors and multiple steps. Human leukocyte antigen (HLA) molecules are crucial proteins in antigen presentation, the initial process of the adaptive immune system which regulates the immune response. HLA class I molecules (HLA I) present endogenous peptides processed in the proteasomes while HLA class II molecules can grasp peptides outside the cell. HLA genotype has been reported to be associated with idiosyncratic liver injury of several types of drugs including antimicrobials (Grove and Aithal, 2015). A pioneering study in Belgium (n = 39) first

reported the strong association between HLA-DRB1*15:01-DRB1*01:01-DQB1*06:02 haplotype and amoxicillin-clavulanate-induced cholestatic or mixed hepatitis (Hautekeete et al., 1999). Another independent association was observed between HLA-A*0201 and amoxicillin-clavulanate-induced liver injury in a GWA study of 201 white European and US cases and 532 matched controls. More importantly, HLA-A*0201 was highly associated with DQB1*06:02 located in HLA II genotyping in 177 cases and 219 controls, suggesting that both HLA I and II genotype contribute to the susceptibility of amoxicillin-clavulanate-induced liver injury (Lucena et al., 2011). HLA-B*57:01 was related to flucloxacillin DILI in an GWA study of 51 cases with flucloxacillin DILI and 60 controls and this association was repeated in another 23 cases, first providing insights for flucloxacillin-induced hepatotoxicity (Daly et al., 2009). Several GWA studies proved repeatedly that HLA-B*57:01 was the major genetic risk factor specific to flucloxacillin with no association found in other isoxazolyl penicillins or amoxicillin (Nicoletti et al., 2019).

Pregnane X receptor (PXR) is the key modulator of drug metabolism by regulating multiple metabolic enzymes in the liver. Numerous studies have illustrated that how PXR functions in DILI (Wang et al., 2020). Flucloxacillin is a PXR agonist and PXR polymorphism rs3814055 C>T was associated with flucloxacillin-induced liver injury in 51 flucloxacillin DILI cases, 64 flucloxacillin controls and 90 community controls (Andrews et al., 2010).

Similar as APAP, studies have identified several circulating hepato-specific microRNAs as the predictive biomarkers for DILI such as miR-122 (Kagawa et al., 2018). A multi-omics research from Korea found that miR-122 level was elevated prior to and correlated to the increase of ALT level in 32 healthy volunteers received amoxicillin-clavulanate suggesting that miR-122 could be

a better biomarker than the traditional serum marker ALT (Lee et al., 2017). Disappointedly, patient-based studies focusing on the relation between microRNA and anti-microbial DILI still lack.

3.2 Non-genetic biomarkers for antimicrobial-induced liver injury

Given that HLA genotype is proposed to be associated with amoxicillin-clavulanate and flucloxacillin-induced liver injury, it is reasonable that further immune response should be activated in patients carried specific HLA mutations when the antigen presentation process is completed. Lymphocytes from patients developing amoxicillin-clavulanate-induced liver injury were able to proliferate and/or secrete interferon-gamma (IFN- γ). And interestingly, both amoxicillin-responsive and clavulanate-responsive CD4⁺ or CD8⁺ clones from patients with or without risk alleles could secrete certain immune mediators such as IFN- γ , suggesting that both amoxicillin and clavulanate are responsible for DILI rather than clavulanate alone (Kim et al., 2015). Likewise, CD4⁺ and CD8⁺ T cells isolated from patients with flucloxacillin live injury are responsive to flucloxacillin. Besides, flucloxacillin responsive CD8⁺ cells could be stimulated to proliferate only by antigen presenting cells expressing either HLA-B*57:01 or the structurally related HLA-B*58:01 which explained the immune rationale of the genetic association of flucloxacillin (Monshi et al., 2013). However, these studies are exploratory and based on a few cases with DILI. Whether the T cell profile is significantly different between patients developing DILI and not developing DILI is poorly defined. Given that antimicrobial-induced liver injury develop within a few weeks after treatment and delayed liver injury is also observed, identification and characterization of drug-responsive T cell at early treatment stage could be a promising

diagnosis biomarker for antimicrobial-induced immune allergic hepatotoxicity in a way.

4. Anti-tuberculosis (TB) drug-induced liver injury

The most frequent adverse event of anti-TB agents is hepatotoxicity. About 7.9% of patients receiving first-line anti-TB therapy develop treatment-related liver injury characterized by the elevation of serum level of ALT and/or AST and hyperbilirubinemia (Molla et al., 2021). In China, anti-TB agent are also the second leading cause of liver injury in China accounting for 21.99% of all cases (Shen et al., 2019). In addition, anti-TB treatment-related liver injury can interrupt patients' adherence to the regimen contributing to relapse or drug resistance. Standard treatment of tuberculosis at present is the 6-month rifampicin-based therapy containing with a 2-month regimen containing isoniazid, rifampicin, pyrazinamide, and ethambutol followed by a 4-month regimen containing isoniazid and rifampicin according to WHO guideline (World Health Organization, 2017). Among the first-line anti-TB agents, isoniazid, rifampicin and pyrazinamide are considered potentially hepatotoxic while no hepatotoxicity has been described for ethambutol (Tostmann et al., 2008). Mechanism under isoniazid-induced liver injury is well-documented which is associated with the generation of the toxic metabolite of the parent drug while the reason for rifampicin- and pyrazinamide-induced liver injury are under investigation (**Table 4**).

4.1 Genetic biomarkers for anti-TB drug-induced liver injury

Isoniazid (INH) is metabolized both by phase I and phase II enzymes. INH itself and its two metabolites, hydrazine (Hz) and acetyl-hydrazine (AcHz) are considered as hepatotoxic and therefore contribute to liver injury. In liver, INH undergoes hydrolysis by CYPs or acetylation by

NAT2 and is transformed into acetyl-INH and Hz respectively. Hz can be further hydrolyzed, generating another hepatotoxic metabolite AcHz which is detoxicated through a second acetylation by NAT2 (Wang et al., 2016).

Acetylation rate in liver is determined by a pair of alleles of NAT2 and individuals can be divided into rapid acetylators and slow acetylators based on their haplotype of NAT. Up to date, there are 40 SNPs in NAT2 locus reported among which NAT2*5, *6 and *7 are regarded as clinically important for INH since they greatly reduce the activity of NAT2 enzyme compared to the reference allele NAT2*4 (Andrade et al., 2009; Hein and Millner, 2021). Individuals carrying any two of the mutants are slow acetylators. And more importantly, the frequencies of these alleles vary remarkably among different races with approximately 44, 26 and 1% of Caucasian population carry NAT2*5, *6 and *7 respectively in while 6, 31, 11% in Chinese population (Lin et al., 1994; Xie et al., 1997). Area under the curve (AUC) of Ac-Hz was 1.7-fold higher in slow acetylators, suggesting that slow acetylators are more susceptible to develop INH-induced liver injury (Lauterburg et al., 1985). The first study on the association between NAT2 polymorphisms and INH-induced hepatotoxicity was carried in 224 Chinese tuberculosis patients and showed that patients carrying two NAT2 mutants (slow acetylator) had higher risk to develop INH-induced hepatitis (26.4% vs. 11.1%, $P = 0.013$). Compared with rapid acetylators, slow acetylators were more likely to have severe liver injury characterized as a higher incidence to have 3 times of ULN of serum ALT when rechallenged with INH. (Huang et al., 2002). In Japanese population, the association of NAT2*6A and anti-TB-induced hepatotoxicity was observed but no relation was found between NAT2*7B and hepatotoxicity (Higuchi et al., 2007). Similar results were also reported in Southwestern Indian (Singh et al., 2009) and Brazilian population (Santos et al., 2013).

Two independent studies on dose optimization of INH based on NAT genotyping in Japanese and Korean population proved the rationale of a dose reduction in slow acetylators in East Asian population (Azuma et al., 2013; Yoo et al., 2021). However, in a diverse population-based study, no significant association was observed between NAT2 polymorphism and INH-induced liver injury (Yamada et al., 2009).

Another possible detoxication pathway of INH-derived reactive metabolite is glutathione conjugation to Hz or Ac-Hz by GSTs even if the conjugation form has not been identified (Boelsterli and Lee, 2014). Higher serum level of Hz was observed not only in NAT2 slow acetylators but also in patients carried GSTM null genotype indicating that Hz tend to accumulate in GSTM null carriers thus contributing to hepatotoxicity (Fukino et al., 2008). At present, there are four main classes of GSTs, alpha (A), mu (M), pi (P) and theta (T) among which the expression of GSTs T1 and M1 are polymorphic. Complete loss of GSTT1 (null genotype) occur in 57%, 20% and 22% and in Chinese, Caucasian and African American population respectively while 58%, 53% and 21% for GSTM1 null genotype in those races (Ginsberg et al., 2009). The homozygous null GSTM1 genotype was predisposed to anti-TB-induced hepatotoxicity in 33 Indian patients (Roy et al., 2001). Furthermore, GSTM1 but not GSTT1 null genotype were found to significantly increase the risk of hepatotoxicity in sub-category of anti-TB drugs in Chinese population (adjusted OR: 2.47, 95% C.I.: 1.13-5.39, $P = 0.02$) (Huang et al., 2007). In addition, GSTT1 null carriers showed higher risk of anti-TB DILI in small Brazilian population ($n = 43$) (Santos et al., 2019).

Different from NAT2 and GSTs which are responsible for detoxication of INH or its toxic metabolites, CYP2E1 is involved in the generation of Ac-Hz thorough hydrolyzation of Ac-INH

which confers liver toxicity. Individuals homozygous for CYP2E1 c1/c1 have higher enzyme activity than those with one or two CYP2E1 c2 alleles (Huang et al., 2003). A number of studies have investigated the role of CYP2E1 genotype in INH-induced hepatotoxicity whereas the results remain in controversy. CYP2E1 c1/c1 genotype was first reported associated with higher risk of INH-induced liver injury compared with c1/c2 or c2/c2 genotype in 318 TB Chinese patients. The risk of hepatotoxicity increased significantly in CYP2E1 c1/c1 combined with rapid acetylator compared to slow acetylators indicating that CYP2E1 c1/c1 is a biomarker independent of NAT status and CYP2E1 and NAT2 genotypes both contributed to the predisposition to INH DILI possibly through their roles in the production or detoxication of hepatotoxic mediators (Huang et al., 2003). Consistent with this study, CYP2E1 c1/c1 was also associated with TB DILI in Tunisian (Ben Fredj et al., 2017) and Brazilian (Santos et al., 2013) patients. Nevertheless, there were some studies with different results. A study conducted in 2244 Xinjiang Uyghur TB patients found no association between CYP2E1 SNP and ALT elevation (Xiang et al., 2014). Besides, INH caused no liver injury in wild-type and CYP2E1-null mice suggesting that CYP2E1 may not involve in INH-induced hepatotoxicity (Cheng et al., 2013). Overall, the role of CYP2E1 genotype in INH-induced hepatotoxicity are controversial and further evidence is still needed.

Nearly 20% and 15% of patients developing INH-related liver toxicity have symptoms of fever or eosinophilia respectively, for which an immunological mechanism was speculated in INH-related liver injury. INH-peptide conducts were detected in vitro when INH was incubated with human liver microsomes which may result in liver injury in an immune allergic manner (Metushi et al., 2012). Furthermore, anti-INH antibodies and anti-P450 autoantibodies were detected in patients diagnosed with INH-induced liver failure providing solid evidence for an

immune-related mechanism under INH -induced liver injury (Metushi et al., 2014). HLAII molecules are essential in the regulation of normal function of immune system and they are polymorphically expressed, SNPs of which may explain, to some extent, why an acute immune reaction was observed when rechallenged with INH. In North Indian patients (n = 346), absence of HLA-DQA1*0102 (OR 4.0) and presence of HLA-DQB1*0201 (OR 1.9) as risk factors were predictive in INH-induced hepatotoxicity (Sharma et al., 2002). Tumor necrosis factor alpha (TNF α), a cytotoxic cytokine produced by monocytes, is also involved in drug-induced immune reaction which mediates direct attack to hepatocytes. A TNF- α genetic polymorphism -308G/A was found significantly associated with anti-TB-induced hepatitis in Korean population (Kim et al., 2012).

MicroRNAs are also potential biomarkers for the early diagnosis of DILI including anti-TB-induced hepatotoxicity. MiR-122 could affect oxidative stress in mice and thus participating in INH-induced liver injury (Song et al., 2015). Furthermore, DNA methylation has been reported to regulate the expression level of miR-122, miR-125b, and miR-106b in rats during INH-induced liver injury (Li et al., 2018). Recently, the serum level of miR-122 and miR-192 in Indian subjects was measured. Compared to the healthy control, naive tuberculosis patients and anti-TB tolerant subjects, serum level of both miR-122 and miR-192 was decreased significantly in the DILI subjects (Bakshi et al., 2021), providing evidence for selecting microRNA as potential biomarkers for anti-TB-induced hepatotoxicity. However, studies on the clinical associations of microRNAs and DILI induced by anti-TB drugs are rarely reported, further investigations are needed.

4.2 Non-genetic biomarkers for anti-TB drug-induced liver injury

There are few studies on non-genetic biomarkers for anti-TB agents related hepatotoxicity. INH as well as its conjugates with albumin could stimulate the lymphocyte transformation and patients whose lymphocyte transformation test was positive were more prone to suffer INH-induced hepatitis compared to negative subjects (Warrington et al., 1978; Warrington et al., 1982). An increased number of Th17 cells and T cells producing IL-10 was observed in 35 Canadian patients with mild liver injury who underwent INH prophylaxis treatment (Metushi et al., 2014). Peripheral blood mononuclear cells (PBMC) from 6 patients with anti-TB agent-related liver injury (n = 4) or skin adverse reaction (n = 2) were all positive for lymphocyte transformation test and/or ELISpot. Besides, INH-specific CD4⁺ T-cell clones were identified in patients with liver and skin injury which suggests that the adaptive immune system is involved in INH-induced adverse event (Usui et al., 2017). In spite that these studies are based on small sample size, characterization of lymphocytes are reasonable to prevent severe liver injury when INH is rechallenged in patients whose immune system already develop immune memory.

5. Small molecular targeted anti-tumor drug-induced liver injury

Tyrosine kinase inhibitors (TKIs) have improved survival dramatically in non-small cell lung cancer (NSCLC) patients carrying epidermal growth factor receptor (EGFR)-activating mutation and in breast cancer patients, including gefitinib, lapatinib, crizotinib, etc. TKIs associated DILI is the major concern in clinical practice with high prevalence, making it one of main reasons for drug discontinuation (Shah et al., 2013). In clinical trials, approximately ~60% patients developed TKIs-induced liver injury, ~30% of which suffered grade \geq 3 DILI that led treatment interruption (Mitsudomi et al., 2010). Thus, several TKIs including lapatinib, gefitinib, pazopanib, regorafenib, crizotinib and sunitinib require routine monitor of liver function during treatment recommended by FDA box labels (<https://www.accessdata.fda.gov/scripts/cder/daf/>).

TKIs-induced liver injury is common and severe whereas the mechanism of DILI remains poorly investigated. It is assumed that CYPs polymorphisms are responsible for TKIs-induced liver injury since these mutations contribute to individual variations in pharmacokinetics, formation of reactive metabolites and further mediated immune dependent liver injury (Sugiyama et al., 2015; Paludetto et al., 2019). Most TKIs are mainly metabolized via CYP3A4, partially by CYP2C19 (gefitinib), CYP1A2 (erlotinib), CYP2C8 (lapatinib) and CYP2D6 (imatinib), implying that variants in CYPs are promising to predict TKI-induced liver injury (Hartmann et al., 2009). Meanwhile, TKIs exert vast individual variation in plasma exposure. For example, the median area under the concentration-time curve (AUC_{0-24}) was 10.086 $\mu\text{g}\cdot\text{h}/\text{mL}$ (3.247-24.726 $\mu\text{g}\cdot\text{h}/\text{mL}$) for gefitinib (Kobayashi et al., 2015), 79.518 $\mu\text{g}\cdot\text{h}/\text{mL}$ (34.665-182.409 $\mu\text{g}\cdot\text{h}/\text{mL}$) for lapatinib (Inoue et al., 2015) and the trough plasma concentration was 334 ng/mL (77.9-813 ng/mL) for gefitinib (Kobayashi et al., 2015), 821 ng/mL (633-1064 ng/mL) for lapatinib (LaBonte et al.,

2016), respectively. Differences in drug exposure provide the rationale of identifying metabolomic biomarkers for DILI. In addition, recent publications reported distinct liver injury manner among different kinds of TKIs such as autophagy-dependent liver injury for gefitinib (Luo et al., 2020), mitochondrial toxicity for regorafenib (Akamine et al., 2015), crizotinib (Guo et al., 2021), and immune-mediated DILI for lapatinib (Spraggs et al., 2011), indicating that possible mechanistic biomarkers could be specific to each case (Table 5).

5.1 Genetic markers for TKIs- induced liver injury

As mentioned above, most TKIs are metabolized via CYPs. CYP polymorphisms could be potential predictors for TKIs-induced liver injury. Low activity of CYP2D6 could predict gefitinib-induced hepatotoxicity in several cases (Kijima et al., 2011). Another independent study validated this finding in a large cohort in Japanese (Sugiyama et al., 2015). These studies implied that gefitinib-induced liver injury is mediated by desmethyl-gefitinib, the main metabolite of gefitinib in plasma generated by CYP2D6 (McKillop et al., 2005). However, neither CYP2D6 variations nor exposure of desmethyl-gefitinib was found to be associated with gefitinib-induced liver injury (Kobayashi et al., 2016). In addition, a pharmacogenomics study showed that CYP2D6 polymorphisms was unrelated to gefitinib-induced liver injury, which was similar to H. Kobayashi's finding (Kobayashi et al., 2015). Although FDA recommend to detect CYP2D6 polymorphisms before treatment, it fails to prevent patients from liver injury and large individual variation is still inevitable in clinical practice. Studies on the role of CYPs polymorphisms in both drug and metabolite exposure is in extreme shortage.

Since the chemical structure of TKIs vary a lot, the mechanism of liver injury could be

idiosyncratic. An immune mechanism for lapatinib-induced liver injury in breast cancer patients was observed in a case-control study (Spraggs et al., 2011b). A two-stage (exploratory data set and confirmatory data set) pharmacogenetic investigation was conducted by genome-wide screening HLA polymorphisms within the major histocompatibility complex (MHC) in a retrospective study. The results showed that HLA-DQA1*02:01, TNXB rs12153855, HLA-DQB1*02:02, and HLA-DRB1*07:01 were associated with liver injury-induced by lapatinib. HLA-DQA1*02:01 allele was further validated as a clinically useful predictor of lapatinib-induced hepatotoxicity with negative predictive values of 0.97 and positive predictive values of 0.17. Furthermore, Daniel J. Schaid and Colin F. Spraggs conducted a study to validate these MHC polymorphisms as predictors for hepatotoxicity of lapatinib in a prospective randomized controlled trial, and the results showed that HLA-DRB1*07:01 was a risk factor in lapatinib-induced liver injury (Schaid et al., 2014). More importantly, these results were introduced and validated in an international multi-centered clinical trial (Spraggs et al., 2018; Xiang et al., 2019; Tangamornsuksan et al., 2020). These findings imply that the underlying mechanism of lapatinib-induced hepatotoxicity is based on an immune-related pathology. The binding of the ligand peptide to HLA-DRB1*07:01, not HLA wild type, was supposed to be enhanced by lapatinib, which allowed the formation of a tightly closed binding groove structure thus stimulating the immune response leading to liver injury (Hirasawa et al., 2015). However, drug lymphocyte stimulation test was negative in lapatinib treated patients despite presence of HLA-DRB1*07:01 (Faulkner et al., 2016), challenging the assumption that lapatinib induced liver injury in an immune-related manner.

5.2 Non-genetic markers for TKIs- induced liver injury

The off-target effect of targeted drug is considered as one of the possible mechanisms for adverse reactions due to the physiological expression of target in normal tissues or organs, especially the liver. Thus, excess activity of the parent drug or generation of reactive metabolites may lead to hepatocytes damage and liver injury (Paludetto et al., 2019). Recently, the trough steady-state plasma concentration (C_0) of gefitinib was reported associated with gefitinib-induced liver injury in Japanese population and a cutoff of 334 ng/mL was determined with a satisfied sensitivity (82.4%) and specificity (85.7%) (Kobayashi et al., 2015). However, the results reported by Takashi Hirose et al., T. Kawamura et al. and our team revealed that neither the AUC nor the C_0 of gefitinib correlated with gefitinib-induced hepatotoxicity (Xin et al., 2015; Hirose et al., 2016; Kawamura et al., 2020). Taken together, the relationship between gefitinib exposure in plasma and gefitinib-induced liver injury is still in controversy, large sample studies with appropriate subgroup analysis based on pharmacogenetics are needed in the future investigations.

Compared to the parent drug exposure, studies on the relationship between metabolites of TKIs and DILI are rare. The reactive aldehyde derivative is the toxic metabolite of sunitinib and pazopanib *in vitro* (Paludetto et al., 2018). These electrophilic metabolites were also successfully detected in plasma of several patients even though their roles in DILI were not fully studied (Paludetto et al., 2020). Meanwhile, using targeted metabolomics approach, we established a quantification method of plasma gefitinib and its four metabolites in NSCLC patients (Guan et al., 2019) and found that the exposures of metabolites were not associated with gefitinib-induced liver injury (data unpublished). Although several metabolites of TKIs could be detected by LC-MS/MS method, we still lack metabolite profiling method for the structure characterization of reactive metabolites of TKIs *in vivo*.

6. others

As the prevalence of cardiovascular disease increases globally, cardiovascular drugs including antithrombotic, antihypertensive agents, antiarrhythmics and lipid-regulating agents are prescribed more often with a rising concern of cardiovascular drug-induced liver injury. The cardiovascular drug induced liver injury may not be fully observed during the clinical trials due to limited number of patients. However, the DILI may emerge after reaching the market with a large number of patients and long-term consumption of medicines. Cardiovascular drugs were the main group (28.5%) leading to DILI (9.8%) in a hepatotoxicity registry with a long-term follow-up study (n = 40) (Andrade et al., 2006) but most of the DILI cases were mild to moderate and rare of them developed to severe liver injury (Chalasani et al., 2004; Licata et al., 2018). Therefore, identifying clinical biomarkers for cardiovascular agents is extremely difficult due to relative rarity of incidences. Several researchers have concerned on clinical biomarkers for DILI induced by cardiovascular agents, we summarized these results in **supplementary materials**.

Discussion

DILI is a highly complex process involved many factors such as host liver function, immune status, amount of drug exposure and so on. It tremendously and negatively influences the safety of medication and quality of patients' lives. Application of multi-omics technology and insights into pathophysiological mechanisms provide biomarkers like HLA polymorphisms, microRNAs, GLDH, HMGB1, mtDNA, certain lipids, etc. Compared to traditional biomarkers, these novel biomarkers appear to be more specific and sensitive in some extent. Nevertheless, there are some limitations of these biomarkers which remain a huge hurdle blocking their clinical translation. First of all, rare incidence of severe DILI, racial and individual variations make it difficult for a candidate biomarker to be validated in limited population before application. Secondly, for genetic biomarkers, due to the low prevalence of mutations such as HLA SNPs for amoxicillin-clavulanate or lapatinib DILI, positive predictive value of routine test in clinic is disappointed. Finally, even though biomarkers indicated by mechanistic studies are promising, conclusions drawn from human subject-based validation studies often contradict to each other.

Definitely, studies on DILI biomarkers are insufficient and more effort should be involved to clarify DILIs and their specific biomarkers. These include i) application of advanced analytical methods and tools such as a deep machine learning for fully data mining; ii) international efforts and cooperation to identify and validate clinical biomarkers for DILI with rare incidence, i.e. Translational Safety Biomarker Pipeline, TransBioLine; iii) conduction of well-designed prospective DILI clinical studies for investigation of clinical biomarkers and so on.

Conflict of interest

The authors declare that they have no conflict of interests.

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Authorship Contributions

Participated in research design and revised the manuscript: Huang and Bi

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Footnotes

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Tables

Table 1. Clinical biomarkers for ALL.

Category	Biomarker	Population (n)	References
Genetic biomarkers	CYP3A5 rs776746 A allele	American (260)	(Court et al., 2014)
	miR-122 and miR-192	European (53)	(Starkey Lewis et al., 2011)
	An 11-miRNA panel including hsa-miR-122-5p	American (49)	(Ward et al., 2014)
	miR-122-5p, miR-382-5p	European (135)	(Vliegenthart et al., 2015)
	miR-122, HMGB1, and full-length K18	European (129)	(Antoine et al., 2013)
	miR-122, HMGB1, and full-length K18	European (1187)	(Dear et al., 2018)
	GDH, mtDNA, nuclear DNA fragmentation	American (42)	(McGill et al., 2012)
Non-genetic biomarkers	GDH, mtDNA, nuclear DNA fragmentation	American (74)	(McGill et al., 2014)
	GLDH, K18, and miR-122	American (175)	(Llewellyn et al., 2021)
	Circular acylcarnitines	American (272)	(Bhattacharyya et al., 2014)
	APAP-protein adducts	Australian (240)	(Chiew et al., 2020)

Table2. Clinical biomarkers for antiepileptic drug-induced liver injury.

Category	Biomarkers	Population (n)	Reference
Genetic biomarkers	POLG (c.3708G>T/p.		
	Q1236H and c.3428	European (17)	(Stewart et al., 2010)
	A>G/p.E1143G)		
	VPA POLG	Unknown (4)	(Saneto et al., 2010)
	GSTM1, GSTT1	East Asians (149)	(Fukushima et al., 2008)
	SOD2 Val16Ala	East Asians (207)	(Saruwataria et al., 2012)
	CAT C-262T	East Asians (267)	(Ma et al., 2019)
	CYP2A6*4, CYP2C9*3	East Asians (279)	(Zhao et al., 2017)
	CMZ GSTM1, GSTT1	Tunisian (129)	(Chbili et al., 2018)
	GSTM1, GSTT1	East Asians (192)	(Ueda et al., 2007)
HLA-A*31:01	European (26)	(McCormack et al., 2011)	
SCN1A	European (425)	(Tate et al., 2005)	
Non-genetic biomarkers	4-ene-VPA,2,4-diene-VPA	East Asians (279)	(Zhao et al., 2017)
	4-ene-VPA	East Asians (64)	(Chen et al., 2012)
	VPA Glycolysis, lipid and amino acid metabolism	East Asians (34)	Huo et al., 2014)
	Oxidative stress, branched chain amino acid metabolism	Unknown (50)	(Price et al., 2011)
	LPCs, Cers, SMs	East Asians (23)	(Xu et al., 2019)

	Lipid mediators	East Asians (3)	(Li et al., 2019)
CMZ	CBZ plasma or 3-hydroxy CBZ concentration	Unknown	(Higuchi et al., 2012)

Table 3. Clinical biomarkers for antimicrobial-induced liver injury.

Category	Drug	Biomarker	Population (n)	References
	Cotrimoxazole	NAT2 *5	European (20)	(Zielinska et al., 1998)
	Cotrimoxazole, Sulfasalazine, Sulfadiazine	NAT2 *5, *6, *7	European (18)	(Wolkenstein et al., 1995)
		HLA-B*57:01	European (74)	(Daly et al., 2009)
Genetic	Flucloxacillin	HLA-B*57:01	European (197)	(Nicoletti et al., 2019)
biomarkers		PXR rs3814055 C>T	European (51)	(Andrews et al., 2010)
		DRB1*15:01-DRB1*01:01- DQB1*06:02	European (39)	(Hautekeete et al., 1999)
	Amoxicillin-cla vulanate	HLA-A*02:01	European and American (201)	(Lucena et al., 2011)
		miR122	East Asian (32)	(Lee et al., 2017)
Non-genetic	Flucloxacillin		European (6)	(Monshi et al., 2013)
biomarkers	Amoxicillin-cla vulanate	T cell profile	European (7)	(Kim et al., 2015)

Table 4. Clinical biomarkers for anti-TB drug-induced liver injury.

Category	Drug	Biomarker	Population (n)	Reference	
Genetic biomarkers	INH	NAT2 *5, *6, *7	East Asian (224)	(Huang et al., 2002)	
		NAT2 *6A	East Asian (100)	(Higuchi et al., 2007)	
		NAT2*5B, *6A	South Asian (201)	(Singh et al., 2009)	
		CYP2E1 c1/c1, NAT2	East Asian (318)	(Huang et al., 2003)	
			African (71)	(Ben Fredj et al., 2017)	
		*5 *6 *7	South American (270)	(Santos et al., 2013)	
			Absence of		
		HLA-DQA1*0102,	presence of	South Asian (346)	(Sharma et al., 2002)
				HLA-DQB1*0201	
		Combination therapy ^f	INH	GSTM1 null	South Asian (33)
GSTM1 null	East Asian (63)			(Huang et al., 2007)	
GSTT1 null, CYP2E1	South American (43)			(Santos et al., 2019)	
c1/c1					
MiR-122, miR-192	South Asian (50)			(Bakshi et al., 2021)	
Non-genetic biomarkers	INH	Th17 and T cell	Canadian (35)	(Metushi et al., 2014)	
		expressing IL-10			
		Isoniazid-specific CD4+	Caucasians (5)	(Usui et al., 2017)	
		T-cell			

^f Combination therapy = Isoniazid, rifampicin, pyrazinamide, and ethambutol

Table 5. Genetic biomarkers for TKI-induced liver injury.

Drug	Cancer type ^f	Biomarker	Population (n)	References
Erlotinib	NSCLC	CYP2D6 *5 or *10	East Asians (25)	(Kobayashi et al., 2015)
	NSCLC	CYP3A5 *3/*3	East Asians (60)	(Sugiyama et al., 2015)
Gefitinib	NSCLC	FOXO3 rs75544369/rs4946935	East Asians (172)	(Guan SX, 2020)
	NSCLC	STAT1 rs10208033	East Asians (42)	(Xin et al., 2021)
Crizotinib	NSCLC	STAT1 rs10208033	East Asians (42)	(Xin et al., 2021)
Imatinib	CML/GIST	UGT1A1 *28	Caucasians (2)	(Saif et al., 2016)
Lapatinib	mBC	CYP3A5 *3	Global (12)	(Bissada et al., 2019)
	mBC	HLA-DQA1 *02:01	Caucasians (24)	(Spraggs et al., 2011)
Nilotinib	CML	UGT1A1 *6/*6, *6/*28	East Asians (34)	(Abumiya et al., 2014)
	mRCC	UGT1A1 *28	Caucasians (261)	(Henriksen et al., 2020)
Pazopanib	mRCC	UGT1A1 *36, *37 or *6	Global (62)	(Motzer et al., 2013)
	mCRC	UGT1A9 *22	Caucasians (3)	(Sacre et al., 2016)
	Unknown	SLCO1B1 *1b	East Asians (37)	(Maeda et al., 2017)
Regorafenib	mCRC	CCL3 rs1130371	East Asians (79)	
	mCRC	CCL4 rs1634517	and Caucasians (150)	(Suenaga et al., 2018)
Sunitinib	RCC	UGT1A1 *28, *37 or *6	Global (246)	(Motzer et al., 2013)
Vemurafenib	Melanoma	CYP3A4 *22	Caucasians (97)	(Goey et al., 2019)
	Melanoma	ABCB1 3435C>T		
Sorafenib	Unknown	SLCO1B1 *1b	Caucasians (114)	(Bins et al., 2016)
	HCC	UGT1A1 *28	Caucasians (1)	(Meza-Junco et al., 2009)

HCC

CYP3A5 *3

East Asians (51)

(Guo et al., 2018)

^f CML=chronic myeloid leukemia; GIST=gastrointestinal stromal tumor; mBC= metastatic breast cancer;
mRCC=metastatic renal cell carcinoma; mCRC=metastatic colorectal cancer; RCC=renal cell carcinoma;
HCC=hepatocellular carcinoma

SUPPLEMENTARY MATERIALS

Novel Clinical biomarkers for drug-induced liver injury

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Table S1. Clinical biomarkers for cardiovascular drugs-induced DILI

Category	Drug	Biomarker	Race (n)	PMID
Genetic biomarkers	Atorvastatin	ABCB1 G2677T/A	Asian (162)	31574240
		ABCB1 C3435T	Asian (30)	27296832
	Fenofibrate	HLA *33: 01	Caucasian (7)	28043905
	Perhexiline (withdrawn)	CYP2D6*2	Caucasian (4)	6479680
		Ticlopidine	CYP2B6*1H, *1J	Asian (114)
		HLA-A*3303	Global (862)	20610889
		HLA-B*4403, -Cw*1403, -DRB1*1302 or -DQB1*0604	Asian (107)	28043905
Non-genetic biomarkers	Ximelagatran	3-hydroxybutyrate, pyruvic acid, colony-stimulating factor 1 receptor(CSF1R), Gc-globulin, L-glutamine, protein S and alanine	Unknown (n=134)	19780643
	Heparins	Sorbitol dehydrogenase (SDH), glutamate dehydrogenase (GLDH), miR-122, high-mobility group box-1 protein(HMGB1) and full-length keratin 18	Unknown (n=48)	22739141
		Amiodarone	Caspase-cleaved keratin-18	Unknown (n=28)
	Amiodarone	Desmosterol	Unknown (n=56)	28861933