Genetic and Molecular Factors in Drug-Induced Liver Injury: A Review

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Abstract: The diagnosis of drug-induced liver injury (DILI) is challenging and based on complex diagnostic criteria. DILI falls into two main categories i) intrinsic "dose-dependent" Type A reactions ii) "idiosyncratic" or Type B reaction (which are usually not predictable). Idiosyncratic reactions can be immunoallergic (hypersensitivity), or metabolic, although overlap between categories can occur. The aim of this review is to summarise the general view of underlying mechanisms in DILI and to highlight individual risk factors for developing hepatotoxicity. Polymorphisms of bioactivation/toxification pathways through CYP450 enzymes (Phase I), detoxification reactions (Phase II) and excretion/transport (Phase III) are explored together with immunological factors in hepatotoxicity. The importance of establishing a multidisciplinary and multicentric network to promote the understanding and research in hepatotoxicity is underlined. Challenges such as genetic analyses for association studies and whole genome studies, pharmacogenetic testing and future approaches to study DILI are considered. Knowledge regarding these operational mechanisms could provide further insight for the prospective identification of susceptible patients at risk of developing drug-induced hepatotoxicity.

Keywords: Drug-induced liver injury (DILI), molecular mechanism of DILI, genetic polymorphism, Phase I, II and III enzymes, genetic testing.

1. GENERAL OVERVIEW OF DILI

DILI is a challenge in modern pharmacology and remains the single leading cause of drug withdrawal despite of a rigorous preclinical and clinical review process [1-3]. DILI has been linked to nearly one thousand drugs used in clinical practice [4] and it also nowadays accounts for more than 50% of the cases of acute liver failure in the USA [2].

The main drug classes associated with DILI are: anti- bacterial agents, nonsteroidal anti-inflammatory drugs (NSAIDs) and analgesics [5, 6], perhaps because they represent the most commonly prescribed drugs. Although data to accurately estimate occurrence of DILI are lacking, the frequency of unpredictable hepatotoxicity associated with the use of medication is suggested to be around 1 per 10, 000 to 1 per 100,000 exposed individuals [7, 8].

In addition, the incidence of DILI remains largely under- estimated in general population

because of under-reporting, difficulties in diagnosis, variation of the clinical setting and incomplete examination of exposed individuals. In a French general population, the frequency of DILI was estimated to be about 14 per 100.000 inhabitants per year [9]. Besides this, a recent study suggests that 1% of medical inpatients develop DILI during the course of hospitalization [10]. One of the major challenges on DILI is to understand the environmental and genetic factors that are operating and to identify individual susceptibility to idiosyncratic liver injury. The present review examines current concepts of molecular mechanism related to the pathogenesis of hepatotoxicity.

2. GENETIC MECHANISMS OF DILI

The general view on the pathogenesis of DILI is that parent compounds are rendered hepatotoxic during cytochrome (CYP) 450 metabolism and can exert their action within the target cell [11], although other metabolic pathways can contribute. However, data on CYP polymorphism in DILI are lacking apart from anecdotal reports. Below we will discuss the impact of Phase I, II and III enzymes polymorphism and susceptibility to develop DILI.

Phase I, II, III Reactions

Drug metabolism encompasses three Phases: Phase I, or bioactivation/toxification reactions though CYP450 enzymes, Phase II, or detoxification reactions (synthetic conjugations with glucuronic acid, sulfate, glutathione, acetate, and amino acids) and Phase III (excretion/transport). During toxification (Phase I) reactions, introduction of functional group (-OH, -NH2, -SH or –COOH) take place thus making the chemical compound more water soluble. Indeed, water- solubility of chemical compound further continues in detoxification (Phase II). Ultimately, hydrophilic drug metabolites may be exported by Phase III proteins located at the hepatocyte or cholangiocyte apical canalicular membrane (MRP family, MDR) that shift chemical compounds into the sinusoidal circulation or bile. Slight imbalance of Phase I, II and III Phases might have important chemical consequences, leading to covalent binding, lipid peroxidation, oxidative stress or glutathione depletion. Polymorphic microsomal enzymes appear to play a role in hepatotoxicity with various compounds Phase I Enzymes Cytochrome P-450 is a key enzyme of Phase I metabolism representing the major pathway for drug oxidation. A proximately fourteen CYP families have been described in humans, genetic polymorphism affecting CYP2C9, CYP2C19 and CYP2D6 genes, rendering them relevant to drug metabolism and possibly hepatotoxicity [12-17]. Indeed, several factors such as genetic (SNP, gene duplication) and environmental (drug interactions, underlying disease) are suggested to affect the level and activity of CYP-450 thus leading to altered drug metabolism and formation of toxic metabolites [18]. Furthermore, marked differences regarding CYP isoenzymes have been found between

ethnic groups [19-21]. The ethnic difference between certain isoenzymes may determine differences in drug response across populations, therefore ethnicity would give additional information to clinicians for prospective evaluation of patients at risk.

CYP2C9 and Hepatotoxicity

CYP2C9 is the most abundant among human CYP2C isoform, representing 18% of total hepatic CYPs [22]. This enzyme metabolizes a number of therapeutically important drugs, including nonsteroidal anti-inflammatory drugs (NSAIDs), S-warfarin, phenytoin and losartan [19]. Fluconazol, metronidazol and amiodarone are potent inhibitors of this enzyme, while barbiturate and phenytoin coadministration may induce the CYP2C9 activity. To date, three different allelic variants (CYP2C9*1,*2,*3), has been recognized to be functionally important [19]. In particular, *3 allele, appears to confer the largest reduction in metabolic activity in vitro and clinically significant alterations in the pharmacokinetics of CYP2C9 substrates [20, 22] while the*2 allele produces intermediate reduction in enzyme activity, as compared to wild type *1 [22]. The frequencies of defec- tive alleles CYP2C9*2 and CYP2C9*3 vary between 8-12% and 3-8%, respectively, among Caucasians, while they are lower in Orientals and black Africans [19, 20].

Drugs being substrate for CYP2C9 isoforms are known to be hepatotoxic. However, it is not yet known whether variation in CYP2C9 enzyme activity might play a role in determining or predicting risk of hepatotoxicity. To our knowledge only single case reports suggest possible implication of CYP2C9 genotype in DILI [22, 23]. A case of leflunomide-induced severe hepatotoxicity was implicated to a rare CYP2C9*3/*3 genotype [24]. Furthermore, CYP2C9 is known to mediate biotransformation of most non steroidal anti-inflammatory drugs (NSAIDs) such as diclofenac, ibuprofen, indomethacin, naproxen and Cox-2 inhibitors by methyl-hydroxylation. Based on clinical reports, CYP2C9 was thought to be a promising probe candidate for diclofenac hepatotoxicity [25]. However, the pharmacokinetics of diclofenac was found to be independent of CYP2C9 polymorphism [22, 26, 27]. Altered expression of alternative cyto- chromes (e.g., CYP2C8) [28] and cytokines (IL-4, Il-10)

[29] was suggested to be determinant of diclofenac hepatotoxicity. Besides, CYP2C9 is known to mediate biotransformation of HMG-CoA reductase inhibitors such as fluvastatin to toxic metabolites 5-hydroxy-, 6-hydroxy-, and N- deisopropyl-fluvastatin, although the presence of variant genotype did not show to have any influence on fluvastatin kinetics and appearance of adverse drug effect [30].

CYP2C19 and Hepatotoxicity

The deficiency of CYP2C19 is responsible for the g netic polymorphism of S-mephenytoin

4-hydroxylation [31]. Many therapeutically used drugs such as omeprazole, diaze- pam, propranolol, labetalol, nelutamide, tienilic acid, glucocorticosteroids, sexual steroids, ketoconazole and warfarin are subject of S-mephenytoin oxidation [32-35]. Two major mutation in CYP2C19 gene has been identified: CYP2C19*2 has a single pair mutation, producing aberrant splice site in Exon 5 resulting in modification of a reading frame and forming a premature stop codon, and CYP2C19*3 consisting in a G A mutation at position G36 of Exon 4 of CYP2C19 creating a stop codon [36]. It is noteworthy, that CYP2C19*2 mutation account for 75% of the alleles in Orientals [36] and 95% in Caucasians [37] while the CYP2C19*3 accounts for 25% of the alleles in Oriental poor metabolizers only and is not detected in Caucasians [38].

The role of CYP2C19 variant genotype on DILI appearance has not been well established. Study of Horsmans Y et al. suggested that Atrium® (a complex of phenobarbital and derivative of carbamates (febarbamate and difebarabmate)

[39] (already removed from the market) induced hepatotoxicity might be associated with the deficiency of S- mephenytoin although the need of further confirmation was highlighted. Indeed, two out of three patients with Atrium®- induced hepatitis were found to have a deficient phenotype while the third one exhibited intermediate oxidation capacity. A case of severe troglitazone hepatotoxicity (withdrawn from the market) [12] was described in a carrier of partial or complete deficiency of CYP2C19 genotype. However these studies have not been replicated to date.

In sum, the relevance of CYP2C9 and CYP2C19 poly- morphism on the development of DILI has been based on single studies, or small number of patients. In the series of DILI cases (n=60), prospectively collected, the distribution of CYP2C9 (n=28) and CYP2C19 (n=32) allelic variants in DILI patients were similar to those in other European populations, and there were no patients exhibiting very low enzyme activity for CYP2C9 *3/*3 and CYP2C19 *3/*3 alleles. Patients with variant and those with wild-type allele did not differ in regard to clinical presentation of DILI, type of injury and outcome. This data suggest that CYP2C9 and CYP2C19 genetic polymorphisms might not be a predictable potential risk factor for DILI [40].

CYP2D6 and Hepatotoxicity

CYP2D6 represents an average of 2% of hepatic CYP content. The CYP2D6 is responsible for the debrisoquine/dextromethorphan oxidation which exhibit genetic polymorphism [41]. Approximately 40 therapeutically utilized drugs are oxidized by CYP2D6 including b- blockers, tryciclic antidepressants, selective serotonin re-uptake inhibi- tors (SSRI), antipsychotic, anti-arrhythmic and opioids (codeine, tramadol, dextromethorphane) [42].

Contrary to all other CYPs involved in drug metabolism, CYP2D6 is not inducible. Of note, approximately 3-10% of Caucasians are poor metabolizers compared with 1-2% of Orientals [43]. CYP2D6 deficiency largely contributed to perhexiline hepatotoxicity suggesting that perhexiline may accumulate in hepatocytes, which could lead to phospholipidosis and alcohol-like liver disease [44]. Also a study by Watson RGP et al, showed a very high oxidation capacity in subjects with chlorpromazine induced hepatitis [45].

Seybold U et al. reported the first case of senna causing hepatitis at a low dose in a 28-year-old woman with known homozygosity for the CYP2D6 variant [17]. Furthermore, Maurer HH et al. suggested that the possible hepatotoxic effects of "designer drugs" including amphetamine derivatives, piperazine drugs and pyrrolidinophenones might be due to CYP2D6 polymorphism [46]. Several reports of hepatotoxicity recorded were due to antidepressant drugs that are substrate of CYP2D6 enzyme [47, 48]. For mianserin hepatotoxicity, an immunologically-mediated mechanism has been proposed. Mianserin is converted by microsomes (CYP2D6) into a reactive metabolite, desmethylmianserin, which exhibits cytotoxicity [47]. However, routine monitoring of concentrations of the parent drug or the demethylated metabolite is not useful since liver injury has not been re- lated to the plasma concentrations of these compounds, al- though in certain cases, improvement was achieved after a dose reduction [48].

Trazodone hepatotoxicity is related to its toxic metabolite m-chloro,4-phenylpiperazine (mCPP) generated by CYP3A3/4 dependent. Subsequent mCPP metabolism is mediated by CYP2D6, which shows genetic polymorphism. Therefore, steady-state concentrations of mCPP are markedly higher in poor metabolisers than in extensive metabolisers [47]. Inhibition of trazodone metabolism mediated by thioridazine (a CYP2D6 inhibitor) in humans produced an increase in the plasma concentrations of mCPP of 50% [49]. In fact, the combination of trazodone and trifluoperazine (a neuroleptic drug) was used in the only case reported as lead- ing to fatal liver failure [50].

CYP3A and Hepatotoxicity

The human CYP3A subfamily consists of 3 isoforms, 3A4, 3A5 and 3A7, encoded by gene located in the chromo- some 7 [51]. This isoform catalyzes the biotransformation of a large number of structurally diverse and endogenous com- pounds [52]. CYP3A4 drug metabolizing activity has been reported to vary more than 20-fold among individuals and plays important roles in the metabolism of a wide variety of drugs, such as immunosuppressants, calcium channel blockers, cancer chemotherapeutic agents, antihistamines, sedatives, and synthetic estrogens [53]. CYP3A4 is the most abundant isoform in the human liver, accounting for approximately 30% of total CYP liver contents

and for the majority of CYPs in the human small bowel [54]. According to experimental and clinical data, CYP3A4 induction occurs in acute cholestasis and/or elevated concentration of secondary bile acids via the pregnane X receptor (PXR) [55, 56]. Moreover CYP3A has been reported to play a major role in ethanol-mediated increases in acetaminophen hepatotoxicity [57].

Several therapeutic drugs used in clinical practice are catalyzed selectively by CYP3A4 enzyme. Therefore genera- tion of reactive metabolites through CYP3A4 mediated metabolism might contribute to the drug induced liver injury such as flucloxacillin induced cholangiopathies [58], trioleandomycin induced cholestasis [59] and troglitazone induced liver injury [60]. Recently severe hepatotoxixicity due to unfavourable interaction between amiodarone-simvastatin[61] and raloxifene-fenofibrate [62] were possibly related to CYP3A4 inhibition.

Phase II Enzymes

Phase II enzymes are involved in conjugation of various Phase I compounds or in direct metabolic activation. Com- pared to Phase I, phase II reaction generally proceed faster resulting in increase drug water-solubility [63]. Several hepatic no enzymatic and enzymatic pathways of detoxification have been identified, including glutathione conjugation of quinones by glutathione S-transferases (GSTs) and hydration of arena oxides to dihydrodiols by epoxide hydrolases [63]. However, reactive metabolites may not undergo detoxific tion, either because they are poor substrates or because of failure of detoxification enzyme function (genetic polymorphism) [64]. Phase II enzymes are known to be polymorphically expressed. The major Phase II enzymes implicate in hepatotoxicity are N-acetyltransferase 2 (NAT2), the glutathione M and T (GSMT and GSTT) and the thiopurine S- methiltransferase (TPMT) (Table 2).

N-Acetylation and Hepatotoxicity

The N-acetylation is controlled by a pair of allele at a single gene locus. The frequency of rapid acetylators is 70% in Asians while in Western Europe and North America ranges from 30 to 60% [65, 66]. Many therapeutically used drugs such as isoniazid, sulfonamides, procainamide, hydra- lazine, dapsone, phenelzine, acebutolol and caffeine are sub- strates of N-acetylation [67]. Adverse clinical reactions have been related to the presence of N-acetylation polymorphism [68].

N-acetylation polymorphism is due to genetic deficiency of N-acetyltransferase 2 (NAT2) activity. In human populations, 27 alleles have been reported for NAT2. Of these, allele *5, *6, *7 are of main importance exhibiting marked differences in metabolic activity of NAT2.

The gene frequency of *5, *6, and *7 are about 44.2%, 25.6%, and 1.2%, respectively, in Caucasians [69, 70] and 6.0%, 30.5%, and 11.2%, respectively, in Chinese [71]. Subjects who carry two defective NAT2 alleles exhibit slow acetylator capacity, whereas rapid acetylators are homozygous or heterozygous for wild-type NAT2.

An association between slow N-acetylation and sulfonamides hepatotoxicity leading to enhanced production of chemically reactive metabolites such as hydroxylamines, has been described [23]. Moreover, the risk of hepatotoxicity might be enhanced in patients with poor N-acetylation capacity as suggested for dihydralazine [72-74]. Regular moni- toring of serum aminotransferase was suggested in slow NAT-2 acetylators receiving isoniazid treatment [72]. Isoniazid is metabolized to acetylisoniazid via hepatic N- acetyltransferase (NAT) [75]. Further acetylisoniazid is hydrolised to acetylhydrazin, that is oxidised by CYP2E1, forming hepatotoxic intermediates [75, 76]. However acetyl- hydrazine is further acetylated by NAT into non-toxic me- tabolite, diacetylhydrazin [73, 74].

The current view of predisposition to hepatotoxicity is that acetylation of the moiety to a non-toxic derivate is im- paired in slow acetylators thus favouring alternative CYP450 mediated pathway to form a toxic-metabolite. Thus slow acetylators might develop severe hepatotoxicity as it was acase for isoniazid-induced liver injury [72] compared to a rapid acetylators.

Sulfoxidation and Hepatotoxicity

Sulfoxidation polymorhism has been implicated in chlorpromazine hepatitis [45]. Chlorpromazine induced hepatotoxicity has been strongly correlated with sulfoxidation defi- cient phenotype [45]. Hypothetically, larger fraction of the drug in sulfoxidation deficient subjects undergoes biotrans formation through the alternative pathway of CYP450 sys- tem leading to reactive metabolite formation thus provoking immunoallergic hepatitis. Moreover, primary biliary cirrhosis has been described to be associated with sulfoxidation deficiency [77]. Other examples where the reactive metabolite was thought to be responsible for idiosyncratic reactions through deficiency of epoxide hydrolase has been reported in the case of the anticonvulsants, although this hypothesis has been recently placed in doubt [78].

Phase III Enzymes

Under physiological conditions, hepatocyte actively con- verts all exogenous and endogenous substances into anionic conjugates with glutathione, glucuronate, sulfate, or other negatively charged molecules that lead to drug detoxification [8, 79]. Thus, these chemical substances formed by drug detoxification may become substrate for export pump of the multidrug resistance protein (MRP) family that mediated ATP dependent secretion

across the canalicular membrane into the bile [80] and multidrug resistance 3 gene (MDR3) [81].

MRP Family and Hepatotoxicity

In humans, the best characterized members of the MRP family are MRP1 and MRP2, which share similar substrate specificity [82]. MRP 1 gene is located on chromosome 16

[83] and MRP2 gene is located on chromosome 10 [84, 85]. Genetic predisposition represented by nonsense or missense (including amino acid polymorphism) MRP family mutations could manifest under the pressure of xenobiotic intake.

MRP1 is localized generally in the basolateral hepatocyte membrane [86]. High affinity substrates for MRP1 are: mono/bis glucuronosyl bilirubin, endogenous glutathione S-conjugate leukotriene C4, 17b-glicuronosyl estradiol [87]. Much lower affinity exhibit the following substrates: glu- tathione S-conjugates, sulfoconjugates, glucuronides of drugs and other xenobiotics. MRP and its related transporters (Pgp) has been reported to play an important physiologic role in defending the cellular environment from the endo- and/or xenobiotic toxins [88]. MRP2 is expressed in the apical (canalicular membrane) of hepatocytes. Inhibitor of MRP2 include cyclosporine A [89]. Consequently, xenobiot- ics that inhibit conjugation export pump could induce cholestasis in susceptible individuals. Indeed, the cholestatic type of liver injury produced by sulindac, flucloxacillin and terbinafine is attributable to its inhibition of canalicular bile salt transport [58, 90, 91]. Besides, Mrp-2 deficient rats do not develop cholestasis after the exposure of bile ductular toxins [92].

MDR3 and Hepatotoxicity

Recently, the role of multidrug resistance 3 gene (MDR3) in developing drug-induced cholestasis was discussed [81]. MDR3 gene is located on chromosome 7 and belongs to ATP-binding cassette transporters [93]. These transporters are generally expressed in the canalicular membrane of the hepatocytes. It has been reported, that heterozygosity of MDR 3 gene lead to defective protein trafficking [94] and susceptibility to cholestasis of pregnancy [95] as well as genetic predisposition to certain biliary diseases [95-97]. It is thought that non genetic factors, such as female sex or reactive metabolites may also modify MDR 3 heterozygouse state expressivity by decreasing normal allele expression

[95] thereby altering drug transport. However, only limited information of Phase III proteins and the role in predicting risk of hepatotoxicity is available.

3. IMMUNOLOGICAL MECHANISMS

Individual susceptibility to idiosyncratic hepatotoxicity is a complex and multi-step process

determined by the interaction of multiple metabolic pathways as well as immunological factors that might influence immune responsiveness and tissue injury. Furthermore we will discuss a possible contribution of immunological factors such as genetic variations of human leukocyte antigen (HLA) molecules and inflammatory cytokines in the appearance of immunoallergic drug hepatotoxicity.

Mechanism of Immune-Mediated Idiosyncratic Liver Injury

Generally, immune mediated hepatotoxicity appears to involve the generation of reactive metabolites that undergo covalent binding with hepatocytes/carrier proteins, also known as "haptenization" [79], however the pattern of the immune response to adduct is likely to vary among individuals [98, 99]. Besides it is not yet clear weather the association of immune mediates liver injury and reactive metabolite formation are coincidental or consequential.

Hapten formation leading to major histocompatibility complex class II (MHC II) presentation of haptenized peptides by antigen-presenting cells (APCs) along with co- stimulation of APC by "danger" signals promote helper T- cell activation (clonal antigen-recognizing cytotoxic cell expansion) and B-cell mediated antibody production. In response to cellular stress/death signal, innate immune system leads to the production of protective and/or injurious cytokines [100]. Such a "danger" signals may occur as a consequence of toxic drug metabolites, viral infections or systemic inflammatory conditions. This may explain why AIDS patients more frequently develop immune mediated liver injury (cytokine imbalance) [101].

Therefore, modulating of innate immune response might be crucial in the determining severity and extent of liver in- jury (Fig. 1). Consequently, factors affecting expression of protective cytokines (genetic polymorphisms) or an underlying disease might favour liver toxicity. The balance between protective (Interleukine (IL)-10, IL-6, monocyte chemoat-tractant protein (MCP)-1, MCP-2) and pro-inflammatory cytokines (Interferon (IFN)-y, Fas ligand (FasL), Tumor- necrosis factor (TNF) in the liver has been suggested to determine the extent of organ damage and the mode of cell death (apoptosis and necrosis) [79]. Studies in IL-10 knock- out mice reported that IL-10 is protective in APAP toxicity by controlling NO and iNOS formation [102]. Furthermore, knockout mice of IL-10 and IL-6 was demonstrated to be associated with increased susceptibility to paracetamol hepatotoxicity [103] and increased levels of TNF-a and IFN-y [104]. According to the results of small study an association between IL-10 and IL-4 polymorphism and diclofenac hepatotoxicity was also found [29]. Thus, it may be that proinflammatory cytokines contribute to the toxicity, and that they are regulated by anti-inflammatory cytokines, such as IL-10 and others. Interestingly, the analysis of three polymo phisms -1082G/A, -819C/T, and -592C/A in the IL-10 gene promoter were performed in a cohort of 146 DILI patients. In this exploratory

study no association between IL-10 polymorphism and the development of DILI was found [105].

HLA Genotype and Idiosyncratic Drug-Induced Liver Injury

Major histocompatibility complex molecules (i.e. class I and II HLA molecules) participate in antigen presentation to immunocompetent cells and in the regulation of the immune response. An association of particular HLA molecule with the susceptibility to suffer liver injury from several immuno- genic drugs has been reported using serological studies. For example, HLA-A11 in hepatitis induced by halothane, amineptin, amitriptiline and diclofenac, HLA-B8 in clometacin hepatitis, HLA-DR6 and DR2 in nitrofurantoin and chlo-promazine hepatitis [12]. All these studies, however, were small and hence the associations have become inconclusive. Additional immunological determinants in predisposingDILI are given in the Table 3. Significant association was found between DRB1*1501-DRB5*0101-DQB1*0602 haplotype and cholestatic hepatitis related to amoxicillin- clavulanate [106] and DRB1*0201-associted DRB1*0301, DRB1*0701 haplotype and cytotoxic hepatitis related to antituberculous therapy [107]. Conversely, when using sero- logical methods no association was found between general propensity to DILI (regardless to causative drug) and HLA class I and II molecule in a large group of patients (n=71) [108]. In a recent study using genomic techniques to assess HLA-class II molecules in the largest cohort of DILI patients analysed to date (n=140) [109], no association between any specific HLA allele and the propensity to develop DILI was demonstrated. In this study, on the other hand, a significant association between HLA-DRB1*15 and –DQB1*06 alleles and cholestatic/mixed pattern of DILI was found while DRB1*07 and DQB1*02 alleles appeared to be protective from this particular expression of liver injury. These findings suggest that HLA class II allele may explain in part why a given drug provokes different types of liver damage among patients, and also supports the general notion on the allergic based mechanism of cholestatic/mixed hepatotoxicity.

4. MOLECULAR MECHANISM

Mechanism of Apoptosis and Necrosis

Hepatocyte death normally follows one of two patterns: necrosis or apoptosis. However, apoptosis and necrosis are usually consequence of the same initiating factors and signaling pathways [110]. It is currently not known, which mode of cell death pre- dominates in various type of liver injury.

Apoptosis is a programmed cell death often initiated by specific stimuli, including DNA damage (ionizing radiation or chemotherapy) and death receptor ligands (TNFa, Fas ligand)

[111]. These events lead to an increased leakage of proapoptotic cytochrome c, Smac/Diablo, etc [112] from the mitochondrial intermembrane space into the cytosol, thereby carrying out the cellular disassembly. Apoptosis leads to an individual cell resorption and distinctive nuclear changes (chromatin condensation, nuclear fragmentation and internu-clear DNA degradation) and ultimately, Councilman (apototic) body's formation subjected to phagocytosis by adjacent cells and macrophages. On the other hand, necrosis is a consequence of acute metabolic perturbation leading to severe ATP depletion, irreversible breakdown of plasma mem- brane barrier, mitochondrial depolarization, lysosomal breakdown, collapse of electrical gradient and leakage of cytosolic compounds and reactive metabolites [113]. Mem- brane blebbing due to ATP depletion-dependent cytoskeleton alteration is an essential characteristic of necrosis [114, 115], while loss of membrane integrity is a typical sign distinguishing apoptosis from necrosis [116].

Modulators of Apoptosis

The disassembly process in apoptosis is executed by ATP dependent death ligand/death receptor interaction, which leads to caspase (cysteine-dependent aspartate specific pro- teases) activation cascade. The activation of caspase cascade is orchestrated by two types of signals: caspase initiators (caspases 8 and 9) and caspase terminators (caspases 3, 6 and 7) that execute cell to apoptosis. The signal of caspase initiators converge to produce mitochondrial permeabilization by activating proapoptotic Bcl2 family members (tBid, Bax, Bak) that might form specific cytochrome c release channels in the mitochondrial outer membrane [111, 117] and/or mi- tochondrial permeability transition (MPT) pores in the inner mitochondrial membrane. Consequently opening of these pores leads to mitochondrial swelling and release of inter- membrane proteins (cytochrome c, Smac, poly(ADP-ribose) polymerase (PARP) and other apoptosis inducing factors) [118]. Once cytochrome c is released, it activates the sequence caspase 9 in ATP-requiring reaction that in turn acti- vates caspase 3 and the final stage of apoptosis.

Acetaminophen (APAP) is a powerful inducer of oxida- tive stress, DNA fragmentation, and apoptosis. The C-jun (NH2) terminal kinase (JNK) signal transduction pathway was suggested to be involved in the pathogenesis of paracetamol induced liver failure [121, 122]. C-jun (NH2) terminal kinase (JNK) is a member of the mitogen activatedprotein kinase family and is a key intracellular signalling molecule involved in the control of cell fate. Inhibition of JNK has been reported to protect hepatotoxicity in ischemia- reperfusion model [121]. Recently knockout and knockdown approaches have provided evidence that APAP induces a prolong activation of JNK resulting into hepatocellular necrosis [122]. This study further suggested that JNK inhibition markedly protects against paracetamol induced liver

injury, despite of glutathione (GSH) depletion/covalent binding. Therefore JNK inhibition might find clinical application in the group of patients that present late after overdose or in which timing of the overdose is unclear

Drug Induced Mitochondrial Injury

Mitochondrial DNA may be particularly vulnerable to injury and act as a sensitizer to hepatotoxicity with drugs such as valproate, possibly be mediated through effect on mitochondrial fatty acid beta oxidation [123-125]. Other studies suggest anti mitochondrial antibodies may follow the intake of iproniazid [126].

In addition, mitochondria may exhibit functional and/or acquired defects (for example infection or diabetes); leaving then susceptible to drug toxicity (aspirin, NSAIDS or reactive metabolites) although some effect of mitochondrial mutations are still unclear. Recent evidence suggests that mitochondrial injury may be progressive and a result of increased/cumulative oxidative stress [127].

5. CLINICAL IMPLICATIONS Clinical Pattern of DILI

In general, liver injury caused by drugs is known to be either Type A "dose dependent" (intrinsic toxicity) or Type B idiosyncratic [128, 129]. Perhaps with the main exception of single high dose of paracetamol-associated liver injury,most DILI cases evaluated in clinical practice are considered as idiosyncratic [4].

As the rule, predictable reactions can be detected at the preclinical and clinical stage of drug development. In gen- eral, these reactions are dose related (intentional or acciden- tal). Predictable reactions have short latency period usually several hours to a few days (e.g. acetaminophen or chemo- therapy drugs). Although idiosyncratic allergic hepatotoxic- ity is considered unrelated to dose, however this reaction has been observed when drugs were given at a daily dose of 100 mg or higher, being the likehood of hepatotoxicity greatly reduced with potent drugs and it is very rare to see an exam- ple when a drug is administered at a dose below 10 mg per day [130].

The mechanism of hepatotoxicity is poorly understood. It can be accompanied with 1) immunoallergic features such as eosinophilia, rash, antibody titer and fever having variable, usually short latency period (1-6 weeks) or 2) proceed with- out immunoallergic manifestations and delayed latency period (up to 1 year) [12, 13]. However, the absence of the common features of hypersensitivity does not exclude an immune mediated toxicity. These features are only present in 23 % of the patients with DILI [6]. Many independent co-stimulatory factors may determine idiosyncratic DILI such as environment, age, sex,

infections and pharmacogenetic variation in drug metabolising polymorphisms between individuals (Table 1).

Risk factors and DILI

Gender

It is generally accepted that women are more vulnerable than men to the toxic effects of drugs in the liver, however gender differences have not always become apparent when large case series were analyzed [6]. Regarding the clinicopathological expression of hepatotoxicity, the variety of chronic autoimmune hepatitis that is induced by drugs is seen almost exclusively in women. Hepatotoxicity with cer- tain medications such as nitrofurantoin, chlorpromazine, tetracycline, halothane, and diclofenac has been reported more frequently in women [59]. Female sex along with hepatocellular liver damage and increase total bilirubin levels on admission is suggested to be a risk factor for development of fulminant liver failure [6].

Age

Analysis of a cohort of patients with hepatotoxicity, considered all drugs collectively suggested older age to be a risk factor to develop hepatotoxicity [6]. Recently a large Spanish cohort study reported the age-related pattern of liver damage resulting from amoxicillin-clavulanate (AC) treatment. According to this study older age is related to cholestatic/mixed type of damage while younger age is associated with cytolitic damage [131]. Hepatocellular damage in the whole population was inversely correlated with age and had the worst outcome [6].

Alcohol

Alcohol is capable of modulating the hepatotoxic potential of other drugs through CYP induction, inhibition, or substrate competition. Alcohol seems to have a dual effect on CYP2E1. During chronic regular intake, ethanol enhances acetaminophen hepatotoxicity by inducting CYP2E1, as well as susceptibility to liver damage from isoniazid, methotrexate, halothane, and cocaine, and perhaps to other drugs that are substrates for this microsomal isoform. Duringacute intake, however, substrate competition with acetaminophen occurs, actually decreasing the speed of metabolism of this drug to its toxic intermediate. However, this latter effect is partially counteracted by the ability of alcohol to slow the degradation of the CYP2E1 fraction, thus enhancing again the formation of the harmful metabolite once alcohol intake is interrupted. Alcohol also contributes to acetaminophen hepatotoxicity by the direct inhibition of gluathione synthesis and through the malnutrition that frequently accompanies chronic alcoholism [59].

Smoking

Cigarette smoking was reported to be a risk factor for the development of hepatotoxicity [132, 133]. Cigarette smoke contains thousands of structurally diverse chemicals that possess cytotoxic, genotoxic, and tumorigenic activity. A toxic air pollutant formed by smoking such as acrolein was reported to induce hepatotoxicity through a direct mitochondrial damage [132]. Moreover, smoking may induce CYP isoform (CYP2E1) and could contribute to acetaminophen hepatotoxicity and alcohol-induced liver disease [133].

Clinical Presentation of DILI and Treatment

At presentation, DILI may mimic all forms of acute and chronic hepatobiliary disease [134]. Therefore, the possibility of DILI should be considered in every patient with liver dysfunction and especially in older patients presenting with a non-obstructive cholestatic/mixed pattern of damage [131]. Use of non- prescription, concomitant treatment, herbal or alternative medication may be important undereported risk factors [135, 136]. Indeed, numerous herbal compoundsincluding weight-loss preparations (ma-huang, hydroxycut) [137, 138], kava [139], chaparral leaf [140] amongst others- have been found to induce severe liver injury. Other causes of liver dysfunction, such as hepatitis B, C, HIV, biliary tract, alcohol-induced liver disease or non-alcoholic fatty liver disease may act as contributing factors. In addition, the temporal relationship, the response to dechallenge and, in some instances, to inadvertent rechallenge are important considerations in assigning causality [18].

Finally, because of effective therapy for DILI does not exist, the most important intervention is the prompt discontinuation of the drug. Specific antidotes are available only for acetaminophen intoxication (N-acetylcystein) [141] and valproate induced mitochondrial injury (intravenous carnitin) [142]. If severe allergic reaction appears, corticosteroid could be of benefit; however no controlled trials have been performed to establish its efficacy. Ultimately, if acute hepatic failure ensues, patients may require liver- transplantation.

Clinical Significance of DILI

Individual susceptibility to idiosyncratic hepatotoxicity is determined by the interaction of multiple metabolic path- ways and immunological factors that might influence im- mune responsiveness and tissue injury (Fig. 3). Production of reactive metabolites due to disbalance of toxification/detoxification pathways is a critical step to trigger hepa- tocellular apoptosis/necrosis or produce immunoallergic hepatitis. Generally for the majority of treated patients the drug administered is entirely safe. Only few numbers of patients develop elevated level of alanine aminotransferase (ALT) and /or direct biliubin (well known markers of liver injury) [143] however rest will suffer adaptation/tolerance with continued treatment [144] as has been shown with some drugs (statins, isoniazid) [145]. Those who are unable to adapt to

the injury may become susceptible to develop drug- induced liver injury or progress into severe liver toxicity.

It is important to stress that genetic studies seeking associations with diseases that do not exhibit a classical inheritance attributable to a single gene locus are subject to a variety of potential pitfalls, especially the risk of type II errors (failure to reject a false null hypothesis) if the sample size is too small, which often limits the significance of the data re- ported [59].

6.CHALLENGES FACING GENETIC ANALYSES FOR ASSOCIATION STUDIES AND WHOLE GE- NOME STUDIES

Association studies can generally be classified as "direct" or "indirect" approaches. Direct approaches rely on identification of functional polymorphisms of interest using bioinformatics tools. Indirect studies may identify a variant or marker that is close to the functional polymorphism in the same gene (termed linkage disequilibrium or "LD Where allelic heterogeneity is low (only one or two disease- associated alleles in each of the genes that influence risk), anindirect approach is more efficient [146]. The "haplotype map" project initiated by the US National Institutes of Health has identified "haplotype-tagging" SNPs (Interna- tional HapMap Project, http: //www. hapmap. org/), based on African, European and Asian populations, in which 4-10 single-nucleotide polymorphisms (SNPs) in each gene are used to "tag" the haplotypes and capture most of the com- mon variation in the gene [147]. Currently almost 6.4 million SNPs have been identified, with further detail on LD values, recombinations and hotspots for reference available from the website. Although tag SNPs can be used for several populations, this may fail to detect an association if the ADR is caused by rare haplotypes [148].

Whole genome association studies (WGA) are now possible using SNP chips such as Illumina or Affymetrix, rendering technology platforms more efficient and affordable, provided there is adequate statistical power to detect associations at stringent levels of significance that allow for multiple testing [149, 150]. One cause of spurious associations is population stratification, which can arise where populations differ in the frequency of the trait prevalence and the frequency of the polymorphism and should be adjusted for. Although technology has vastly improved in recent years; error rates are still problematic. Using the same genotyping platforms for cases and controls can help to reduce differential error rates in genotype scoring which can bias results, unless adjusted for [151].

ADRs such as DILI show complex phenotypic variation; and there are likely to be several different genetic mechnisms underlying the classification of liver injury. As DILI and other idiosyncratic adverse drug reactions are typically extremely rare, initiatives such as Spanish Registry of Hepatotoxicity, DILI network in EEUU, HepaTox and Eudragene may be useful

519 in providing biological collections for hypothesis testing of underlying genetic variants [152].

520 Determination of genes whose products are involved in the development and regulation of
521 inflammation, apoptosis and tissue repair may yield further clues to molecular mechanism of
522 DILI. Identification of critical proteins modified by reactive metabolites and their functional
523 consequences represent important task for future understanding of the role of metabolic
524 activation in DILI [8]. Determination of conventional cytotoxic marker for apoptosis (e.g.
525 activation of caspases), cytolisis (e.g. LDH release, membrane impereable DNA stain), loss
526 of critical macromolecules (ATP or glutathione (GSH), mitochondrial effects (e.g. tetra-
527 zolium salt essays, Alamar blue assay) and anti-proliferative effects (e.g. inhibition of DNA
528 synthesis or protein synthesis) might be helpful to identify multifaceted phenomenon of
529 DILI.

530 The use of "humanized" transgenic mice represents a potentially important tool for further
531 identification of molecular mechanism of DILI. The use of metabolically- competent
532 hepatocytes that express phase I and II and III enzymes are highly encouraged. Current
533 advances in high- throughput technologies allow the cataloguing of genetic variants for
534 common serious pharmacogenetic traits. Technologies such as transcriptomics, proteomics
535 and metabolomic profiling of hepatotoxic drugs and non- hepatotoxic counterparts might
536 result in the discovery of new biomarkers for liver toxicity [153].

537 Identification and Nomenclature of Drug Metabolising Polymorphisms

538 Classification of drug metabolising enzymes has been attempted on all members of the P450
539 family from wholegenome sequence data. Nelson et al. were the first to attempt a systematic
540 classification of CYP450 polymorphisms fol- lowing an official nomenclature proposal
541 [155]. Further in- formation on drug metabolising polymorphisms is available through
542 OMIM (Online Mendelian Inheritance in Man http://www.ncbi.nlm.nih.gov/entrez)

543 **Approaches to Studying Complex Traits**

544 Several approaches are likely to be required to study complex traits such as DILI particularly
545 as phenotypes show considerable variation and pharmacologically relevant genes may be up
546 and down regulated in response to other gene expression and drug administration.
547 Furthermore projects such as HapMap that characterize genetic variation in Afrian, European
548 and Asian populations will require extensions to other populations including South
549 Americans [157].

550 Alternative approaches to clinical pharmacogenetics include extreme discordant phenotype
551 (EDP) methodology which studies patient pairs in drug treated populations for example
552 comparing those who exhbit and adverse events against those who do not taking low and

high extremes of drug dosage [158]. The EDP aims to correlate extreme phenotype (for example drug sensitivity) with genotype, how- ever this is limited by the numbers identified in each group and requires prior identification of the causative genetic variant.

Ethnicity, and population stratification including admix- ture are also important in relation to study ADRs and methods to controls for this have been developed [159]. However drug metabolising genes may display large frequency differentials even within Europe which may act as confounders, unless adjusted for [160].

Pharmacogenetic Testing

Clinical trials could be a potentially useful source of collecting data in drug metabolising polymorphisms in individuals; however even large clinical trials may fail to detect rare adverse events such as hepatotoxicity. Pharmacogenetic tests may be considered as a pre-treatment screening tool before prescribing medications, or amongst first degree rela- tives who share increased risk of pharmacogenetic traits. Screening chips for common variants are already commercially available : Amplichip CYP450 produced by Roche diagnostics and EU approved allows analysis of 29 polymorphisms and mutations for the CYP2D6 gene and 2 polymorphisms for the CYP2C19 gene, with predicted phenotype (poor, intermediate, extensive, or ultra rapid metabolizer); although current costs are high (450 euros). Rapid advances in technology are now leading the way for custom built SNPs with the potential to test thousands of multiple drug metabolising poymorphisms. Further challenges lie in the interpretation of complex pharmacogenetic data (including sensitivity, specificity and positive predictive value) in assessing an individual's risk for drug safety.

Problems Associated with Pharmacogenetic Testing and Predicting Drug Response

More practical difficulties include selection and counsel- ling of patients, and the increased workload and economic costs for physicians and laboratories in undertaking these tests. Transition from medical research to practical implementation is slow and ability to interpret results of genetic tests is difficult even amongst trained staff. Rare genetic variants associated with adverse drug reactions may also fail to be detected via haplotype strategies, or haplotype approaches may simply identify SNPs in LD with causal genetic markers. Therapeutic monitoring (for example CYP2C9 polymorphisms in the case of warfarin sensitivity) may provide a quicker method of adapting the individual's drug dose to reach the target concentrations than conventional pharmacogenetic testing [154]. Furthermore a pharma-cogenetic test may have both high sensitivity and specificity yet fail in predictive value if prevalence of ADR is low.

7. FUTURE PERSPECTIVES

Creation of large prospective database on DILI through collaborative multidisciplinary and multicentric networks focused on the identification of bona fida cases following the same structural report form has been the very first step to provide insights into epidemiology and pathogenesis of DILI. This has allowed creating a pharmacoepidemiological culture in the attending physicians that become more alert in the detection of DILI and consequently the recruitment of well characterised cases that would promote understanding of complex mechanism of DILI. This has been accomplished by Spanish Registry set up in 1994 [6] and in 2002 by Drug- Induced Liver Injury Network (DILIN) in USA along with collaboration of the National Institute of Diabetes and Diges- tive and Kidney Diseases (NIDDK) and National Institute of Health (http: //dilin.dcri.duke.edu/web).

DILI in paediatric patients is an orphan field and there is obvious need to develop strategies to accomplish implementation of a specific network in this age group [161-163].

EUDRAGENE and Hepatotoxicity

Further collection of cases of drug induced hepatotoxicity are underway with the EUDRAGENE project, funded by the European Commission 5th Framework programme. This project is establishing a freely-shared case-control collection of DNA samples, as a multi-centre European collaboration. A multi-centre collaboration is required as no single centre is able to generate enough cases within a reasonable period of time. This will act as a resource for studying genetic predictors of adverse drug reactions, using the existing system for spontaneous reporting of suspected adverse drug reactions to national or regional pharmacovigilance centres. Currently this project is collecting clinical data and DNA on all identified cases of drug induced hepatotoxicity as defined by CIOMS international standards [146] from several European centres. Classification of liver injury will collect data on laboratory tests and allow classification into hepatocellular; cholestatic and mixed categories, and severity of hepatotoxicity based on ratio of enzymatic abnormalities to standardised normal range values. Analyses of the determinants of hepatoxicity will be undertaken based on drug class and hepatoxicity classification. Further detail on the EUDRAGENE project is given form the website [146].

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