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Identification of a metabolic biomarker panel in rats for prediction of acute and idiosyncratic hepatotoxicity

Jinchun Sun *, Svetoslav Slavov, Laura K. Schnackenberg, Yosuke Ando [1], James Greenhaw, Xi Yang, William Salminen [2], Donna L. Mendrick, Richard Beger

Division of Systems Biology, National Center for Toxicological Research, US FDA, Jefferson, AR, United States

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ABSTRACT

It has been estimated that 10% of acute liver failure is due to "idiosyncratic hepatotoxicity". The inability to identify such compounds with classical preclinical markers of hepatotoxicity has driven the need to discover a mechanism-based biomarker panel for hepatotoxicity. Seven compounds were included in this study: two overt hepatotoxicants (acetaminophen and carbon tetrachloride), two idiosyncratic hepatotoxicants (felbamate and dantrolene), and three non-hepatotoxicants (meloxicam, penicillin and metformin). Male Sprague–Dawley rats were orally gavaged with a single dose of vehicle, low dose or high dose of the compounds. At 6 h and 24 h post-dosing, blood was collected for metabolomics and clinical chemistry analyses, while organs were collected for histopathology analysis. Forty-one metabolites from previous hepatotoxicity studies were semi-quantified and were used to build models to predict hepatotoxicity. The selected metabolites were involved in various pathways, which have been noted to be linked to the underlying mechanisms of hepatotoxicity. PLS models based on all 41 metabolite or smaller subsets of 6 (6 h), 7 (24 h) and 20 (6 h and 24 h) metabolites resulted in models with an accuracy of at least 97.4% for the hold-out test set and 100% for training sets. When applied to the external test sets, the PLS models predicted that 1 of 9 rats at both 6 h and 24 h treated with idiosyncratic liver toxicants was exposed to a hepatotoxic chemical. In conclusion, the biomarker panel might provide information that along with other endpoint data (e.g., transcriptomics and proteomics) may diagnose acute and idiosyncratic hepatotoxicity in a clinical setting.

1. Introduction

Drug-induced liver injury (DILI) is a major reason for drug failure in preclinical and clinical trials, and drug recall from the marketplace. DILI, therefore, is of major concern to the FDA and consumers [1]. In the USA, more than 50% of all cases of acute liver failure are attributed to DILI, among which idiosyncratic DILI (iDILI) accounts for over 10% of the reported cases [2]. While there is no standard definition, the term "idiosyncratic" generally refers to adverse reactions with a relatively low incidence (0.7–1.3 per 100,000) in patients; iDILI is individual-dependent and cannot be predicted using classical toxicity endpoints in commonly used preclinical testing species [3]. The FDA's Guidance for Industry (http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM174090.pdf) suggests testing blood levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP) and bilirubin to diagnose liver injury during clinical trials. ALT is a biomarker for hepatocyte injury, however, other factors like exercise or muscle injury can also influence its blood levels [4,5] thus it is not necessarily specific for liver injury [6–9]. Furthermore, some drugs (such as fialuridine and troglitazone) do not cause serum ALT elevation at the initiation of treatment but can induce progressive damage in hepatocytes, and the resulting cumulative damage may lead to liver failure [10]. AST is a liver damage biomarker, but it is also present in heart, muscle and red blood cells and is also used as a cardiac marker in the clinic [11]. Bilirubin is a functional liver marker but generally does not increase until severe liver injury occurs [12]. ALP levels in blood increase when bile ducts are obstructed; however, levels are also elevated when active bone formation occurs or the bacteria population changes [13]. The currently used biomarkers do not identify idiosyncratic hepatotoxicants in preclinical species and, as noted above, the standard clinical markers are not specific to liver injury. Therefore, a new biomarker or a panel of biomarkers is needed to provide regulatory agencies information regarding chemicals that may cause drug-induced idiosyncratic hepatotoxicity in humans.

It is known that chemical-induced toxicity is a multiple-step process [14] as shown in Scheme 1. After the chemical compound is absorbed,

* Corresponding author at: Division of Systems Biology, National Center for Toxicological Research, US FDA, 3900 NCTR Road, Jefferson, AR 72079, United States. Tel.: +1 870 543 7556; fax: +1 870 543 7686.
E-mail address: Jinchun.Sun@fda.hhs.gov (J. Sun).
[1] Current address: Medicinal Safety Research Labs, Daiichi Sankyo Co., Ltd., Tokyo, Japan.
[2] Current address: PAREXEL International, Sarasota, FL.

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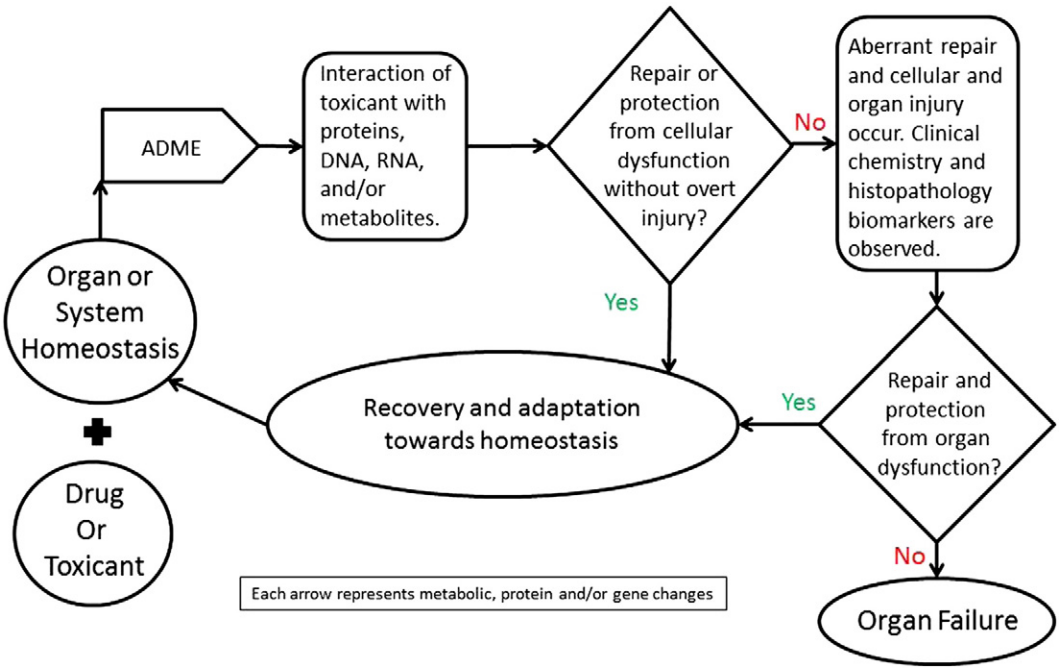


Scheme 1. Scheme of the subsequent response stages after drug/toxicants exposure to a biosystem. As end-point products of genes and proteins, metabolic profiles can represent its corresponding response status. ADME is an abbreviation for chemical absorption, distribution, metabolism and excretion.

distributed, and metabolized within an organism, the parent compound or its reactive metabolites can cause cellular damage. Once toxic injury occurs, a cell can try to recover before apoptosis or necrosis, or it may go through apoptosis or necrosis if the injury is beyond repair. We hypothesize that perturbations in biochemical pathways may occur with or without observable injury and those biomarkers could be exploited to provide more specific indicators of potential for toxicity. Similar to transcriptomic biomarkers [15], these metabolite biomarkers might be defined as "prodromal," i.e., changing prior to liver injury unlike classical biomarkers. Most importantly, the metabolic profile (end-point products of genes and proteins) of a biosystem is highly sensitive to drug/toxicant exposure. The goal of these studies is to discover a metabolite biomarker panel in blood which is able to reflect animal responses to a toxicant prior to or during overt liver injury. The hypothesis is that drugs of an idiosyncratic nature cause disruption in some of these same biological pathways yet the rats, and most humans, can reverse the effect before classical signs of injury are seen.

Efforts have been undertaken to discover novel genetic [16,17], microRNA [18] and proteomic biomarkers [19,20] for DILI prediction and/or diagnosis.Most recently, Mattes et al. predicted DILI potential using a database of rat plasma metabolite profiles – MetaMap®Tox developed by metanomics GmbH and BASF SE [21]. Yang et al. [18] evaluated microRNA profiles in rat urine 24 h after a single oral dose of 1250 mg acetaminophen (APAP)/kg, 2000 mg carbon tetrachloride (CCl_4)/kg, or 2400 mg penicillin (PEN)/kg. It was reported that the urinary levels of ten microRNAs were increased by APAP and CCl_4 (hepatotoxicants), whereas for the group treated by PEN, they remained unchanged. The potential diagnostic microRNA biomarkers of hepatotoxicant-induced liver injury are involved in a range of biological functions including cell death, lipid metabolism, and drug metabolism. The present metabolomics study was designed with the intent to identify metabolic biomarkers of idiosyncratic toxicity in the blood of rats dosed with compounds that have been shown to be overt hepatotoxicants, idiosyncratic hepatotoxicants, and non-hepatotoxicants. The overt hepatotoxicants were APAP and CCl_4. Five additional compounds were studied and included the idiosyncratic compounds, felbamate (FEL) and dantrolene (DAN), and the non-hepatotoxicants, meloxicam (MEL), PEN and metformin (MET). In the present study, results from the semi-targeted metabolomics study of the overt hepatotoxicants, APAP [22] and CCl_4 [23] and non-hepatotoxicant PEN [24], were extended to identify a panel of biomarkers for idiosyncratic hepatotoxicity prediction based upon analysis of the blood samples collected after dosing with the seven compounds listed above.

2. Materials and Methods

2.1. Chemicals

Optima LC/MS grade acetonitrile and water were purchased from Fisher (Pittsburgh, PA). Penicillin V potassium tablets (1600 units/mg) were purchased from Teva Pharmaceuticals (Sellersville, PA), MEL tablets (15 mg) were from BI Pharmaceuticals (Ridgefield, CT), FEL tablets (600 mg) were from Meda Pharmaceuticals (Somerset, NJ), MET tablets (1000 mg) were from Bristol-Myers Squibb (Princeton, NJ), and DAN capsules (100 mg) were purchased from JHP Pharmaceuticals (Rochester, MI). All the tablets were ground to a fine powder for dosing. APAP, methylcellulose, CCl_4, formic acid, leucine-enkephalin, imidazole, pentadecafluorooctanoic acid, *L*-tryptophan and all the MS standards were obtained from Sigma-Aldrich (St. Louis, MO). Fig. S1 displays structures of APAP, CCl_4, FEL, DAN, MEL, PEN and MET.

2.2. Animal Care and Treatment

Six- to seven-week-old male Sprague–Dawley rats were obtained from the FDA National Center for Toxicological Research (NCTR) breeding colony. Animals were housed individually at a room temperature between 19 and 23 °C, 40–70% relative humidity, and with a 12 h dark/12 h light cycle. Animals accessed feed ad libitum for all compound studies except APAP. Rats in the APAP study were fasted overnight for at least 12 h prior to dosing, and feed returned 4 h post-dosing. All experiments were conducted in accordance to the National Institutes of

Health (NIH) guidelines and reviewed and approved by the Testing Facility's Institutional Animal Care and Use Committee (IACUC).

Groups of rats were orally gavaged with the vehicle or a single dose of the compound at the following dose levels: 100 or 1250 mg APAP/kg, 50 or 2000 mg CCl_4/kg, 300 or 1920 mg FEL/kg, 100 or 1000 mg DAN/kg, 100 or 1500 mg MET/kg, 0.4 or 12 mg MEL/kg, or 100 or 2400 mg PEN/kg. Corn oil was used as the vehicle control in the CCl_4 study and 0.5% methylcellulose was used as the vehicle control for all other compound studies. For the APAP study, there were 4 rats in the control groups and 7 rats in the treated groups. For all other studies, 5 animals were used in both the control and treated groups. The total number of animals used in this study as well as the group size and number of groups is the minimum required to properly characterize the response to individual chemicals. This determination was based on statistical power calculations using SigmaStat v. 3.1, Build 3.11.0. The "Difference in Means" and "Standard Deviation" were set based upon historical data obtained from similar animal experiments. Based on the calculation, a group number of 4, 5 or 7 provided adequate power for the study. The dose levels were selected based on the results of range-finding studies (data not shown). The high doses of all 7 compounds were chosen to induce mild to moderate adverse effects, while the low doses were chosen because these doses are similar to the maximum human doses when scaled on a body surface area basis.

2.3. Sample Collection, Clinical Chemistry and Histopathology Analyses

Rats were anesthetized with carbon dioxide; blood was withdrawn via cardiac puncture. Rats were then euthanized with carbon dioxide asphyxiation. Terminal blood was collected at 6 and 24 h after dosing into serum separator tubes for the APAP study and EDTA tubes for the other compound studies. The blood samples were centrifuged (10 °C, 2000 ×*g*, 10 min) and the serum as well as the EDTA plasma were removed and frozen at −80 °C until analysis. Analytes in the clinical chemistry panel included ALT, AST and ALP. Sections of liver were fixed in 10% neutral buffered formalin, routinely processed and embedded in paraffin, sectioned at 5 μm, stained with hematoxylin and eosin, and examined by light microscopy. Lesions were scored on a 4-point scale [25] (1 = minimal, 2 = mild, 3 = moderate and 4 = marked) by a board-certified Veterinary Pathologist. Food consumption was recorded for all animals.

2.4. Open Metabolic Profiling by LC/QTof-MS

A quality control (QC) sample comprised of 40 common chemicals for LC/MS open profiling was evaluated. Pooled blood for each study and the QC sample were run every 10 sample runs by LC/MS to monitor the analytical equipment variability, while the samples from each study were run in a randomized manner.

A 3 μL aliquot of blood supernatant after methanol precipitation was introduced into a Waters Acquity Ultra Performance Liquid Chromatography (UPLC) system (Waters, Milford, MA) equipped with a Waters bridged ethyl hybrid (BEH) C8 column with a dimension of 2.1 mm × 10 cm and 1.7 μm particle size. The separation methods were the same as our previous reports [22–24]. The mass spectrometric data were collected with a Waters QTof Premier mass spectrometer (Waters, Milford, MA) operated in positive and negative ionization electrospray modes as reported previously [26,27]. Briefly, MS^E analysis was performed on a QTof mass spectrometer set up with 5 eV for low collision energy and a ramp collision energy file from 20 to 30 eV. Full scan mode from m/z 100 to 900 and from 0 to 22 min was used for data collection for serum analysis in both positive ion and negative ion modes.

Raw UPLC/MS data were analyzed using Micromass MarkerLynx XS Application Version 4.1 (Waters, Milford, MA) with extended statistical tools. The same parameter settings for peak extraction from the raw data were used as previously reported [26,27]. The aligned data from MarkerLynx analysis for QTof-MS data was filtered using the pooled QC samples based on the following criteria: i) ions with % RSD less than 30% in the pooled QC samples were included; ii) ions present in ≥70% of QC samples were included. In total, 41 metabolites (overlapping metabolite biomarkers from toxicity studies of the overt hepatotoxicants, APAP [22] and CCl_4 [23], that were also detected in spectra from the other 5 studies) were semi-quantitated and their intensity was exported from MarkerLynx for normalization detailed below.

2.5. Statistical and Modeling Analysis

For the clinical chemistry, histopathology and metabolomics data, the values in the treated groups were compared to their respective control group and analyzed by a Student's *t*-test (MS EXCEL). A value of $p < 0.05$ was considered statistically significant. The intensity data for the 41 metabolites was exported from MarkerLynx and were further normalized in the same dynamic dimension scale. By doing so, all of the detected ion features are on the same "scale", which had a variety of intensity dynamic dimension ranging from 0 to 2000. Data was normalized using the formula: $x\prime = \frac{x}{Control_{avg}}$; where x' is the normalized data, x is original data, and $Control_{avg}$ is the average value in the corresponding control rats. Normalizing by the $Control_{avg}$ reduced the noise and errors associated with the samples of the seven studies being collected and analyzed over a three-year period. The resulting normalized data for all 41 metabolites was further subjected to partial least squares discriminant analysis (PLS-DA) using SIMCA v. 13 (MKS Umetrics AB, Sweden). Further, SIMCA PLS was used to build models for the modeling sets, which were then used to predict the samples in the external test sets (high dose). At 6 h, the modeling set was comprised of a total of 36 animals (3 of the controls and high dose samples from each non-hepatotoxicant study (PEN, MEL, and MET) and all controls and high dose samples from the APAP and CCl_4 studies). The prediction set consisted of a total of 30 animals (the remaining controls and high dose samples from each study (PEN, MEL and MET) as well all the control and high dose animals in the DAN and MEL studies). For 24 h, the modeling set contained a total of 38 animals (3 control and 3 high dose PEN and MEL samples and 2 control and 3 high dose samples for MET as well all the control and high dose APAP and CCl_4 samples). The external test set (high dose) consisted of 29 animals (the remaining control and high dose PEN, MEL and MET samples as well all the control and high dose DAN and FEL samples). Details about these two subsets for each time-point are shown in Table 1. The variable importance in projection (VIP) values generated by SIMCA using 41 metabolites were used to select subsets of metabolites explaining most of the variance in the experimental data.

2.5.1. Matlab PLS modeling

An ensemble modeling PLS algorithm written in Matlab (see Scheme 2) was used to build 100 fully randomized models for the training set (90% of the modeling set size), each of which was then used to predict: i) the hold-out test set (10% of the modeling set), ii) the external high dose test set of non-DILI and iDILI treated animals and iii) the external test set comprised of the low dose treated animals from all seven studies. Details of the modeling and external test sets (high and low dose) are listed in Table 1. At the end, all aggregated predicted values were averaged and a threshold of 0.5 was used to convert the quantitative predictions to categorical (predicted values ≥ 0.5 were defined as hepatotoxic, while these <0.5 were defined as non-hepatotoxic). To improve the flow of the manuscript, predicted "hepatotoxic" is sometimes referred to hepatotoxicant or hepatotoxicity and non-hepatotoxicant or non-hepatotoxicity for predicted "non-hepatotoxic".

Probability density functions describing the distribution of the quantitative averaged predicted values for the hold-out and the external test sets (Matlab) were used for risk analysis.

Table 1
Number of animals used for model building and prediction at 6 and 24 h.

Treatment		Modeling set		External test set (high dose)		External test set (low dose)*		
		Non-liver toxicants: MEL, MET, PEN + controls	*Liver toxicants*: APAP, CCl4	*Non-liver toxicants*: MEL, MET, PEN + controls	*Idiosyncratic toxicants*: DAN, FEL	*Non-liver toxicants*: MEL, MET, PEN	*Liver toxicants*: APAP, CCl4	*Idiosyncratic toxicants*: DAN, FEL
Time point	6 h	9 + 18	9	6 + 15	9	15	10	10
	24 h	9 + 17	12	5 + 15	9	15	10	9

* External test set (low dose) was modeled by Matlab PLS.

The 41 metabolites and the panels of metabolites were subjected to the pathway analysis (http://www.metaboanalyst.ca). In addition, the effect of food consumption on the metabolome changes was elucidated through correlation analysis. Correlations between metabolite levels and food consumption were calculated using Pearson's correlation ranking analysis in Statistica v. 9 (StatSoft Inc, OK, USA).

3. Results and Discussion

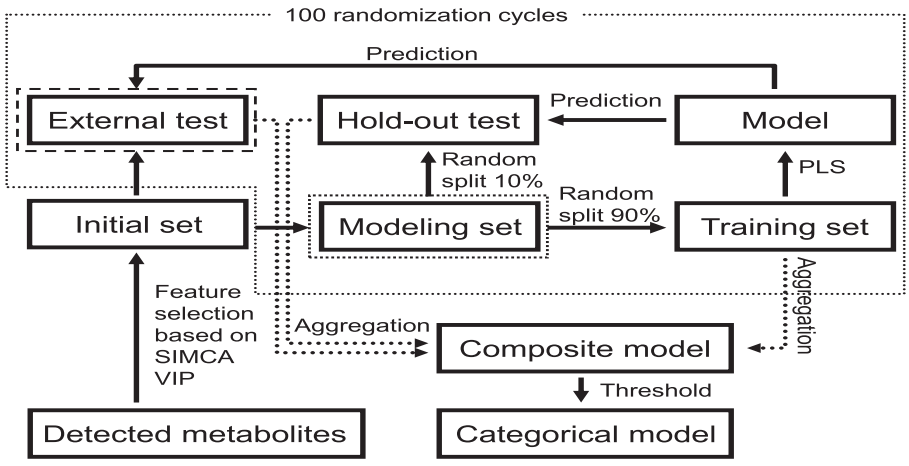
An overdose of APAP, a widely used over-the counter analgesic and antipyretic drug, frequently leads to acute liver failure associated with hepatic centrilobular necrosis [28]. However, it has been reported that a therapeutic dose of APAP can cause idiosyncratic acute hepatic injury [29]. CCl_4, a highly toxic chemical agent, is commonly used to elicit experimental liver damage [30]. FEL, an anti-epileptic drug, has been reported to cause hepatic failure with a risk of 1 in 18,500 to 1 in 25,000 [31]. This has resulted in a black box warning label by the FDA to limit its use only to patients whose epilepsy is so severe that the benefit of therapy outweighs the risk of liver failure [31]. DAN, a muscle relaxant, has a potential for hepatotoxicity at the incidence of 0.35% [32,33]. FEL and DAN are classified as idiosyncratic hepatotoxicants based on the fact that the drugs are safe at therapeutic doses for the majority of patients while a small subset is susceptible to DILI. There are no reports indicating that MEL (a nonsteroidal anti-inflammatory drug) and PEN (an antibiotic widely used for bacterial infection treatment) alone can cause liver injury. However, other side effects can be induced by these drugs including gastrointestinal toxicity by MEL [34] and hypersensitivity and diarrhea by PEN [35]. MET is an oral anti-diabetic drug to suppress glucose production in the liver, but MET-induced hepatotoxicity is extremely rare. By 2012, fewer than 10 cases of liver injury have been reported to be associated with MET and these cases involved concomitant intake of MET with other potential hepatotoxicants; only one hepatotoxicity case has been related to MET alone [36]. Therefore, one must be cautious when using MET as one of the non-hepatotoxicants in the study.

3.1. Clinical Chemistry and Tissue Observations

No gross pathology was observed in kidneys from rats treated with any of the agents at 6 h or 24 h. Histopathology findings (hepatocyte vacuolization, hepatocyte necrosis) and clinical chemistry data (ALT, AST and ALP) specifically related to liver injury are reported in Table 2.

As previously noted for rats in the APAP study [22], there was heterogeneity in the degree of hepatic necrosis at the 24 h timepoint in the high dose group (1250 mg APAP/kg); three animals showed no evidence of hepatic necrosis, one animal had minimal hepatic necrosis, one had mild hepatic necrosis and two had moderate hepatic necrosis. ALT and AST levels increased to >1000 U/L in the two rats with moderate hepatic necrosis, while the other 5 animals had ALT and AST levels similar to controls. Hepatocyte glycogen depletion was observed in all seven rats 24 h after dosing. The severity ranged from minimal to moderate. No hepatocyte vacuolization was observed in any of the animals treated with APAP. The low dose APAP animals had a minor decrease in ALT at 24 h.

Clinical chemistry data and histopathology results after dosing with CCl_4 have been published previously [18]. All 5 animals in the high dose treated group had hepatic vacuolization (ranging from minimal to mild) and hepatic necrosis (ranging from mild to marked). ALT, AST and ALP were increased in the treated group (>2 fold). All 5 high dose animals had mild to marked hepatic necrosis (average score of 2.6) and minimal



Scheme 2. Flowchart of the PLS modeling process. The initial set contained APAP, CCl_4, MET, PEN, MEL and their corresponding controls, while the external test set included DAN, FEL and their corresponding controls.

Table 2
Clinical chemistry and liver histopathology data (mean ± SD) in rats 6 h and 24 h after a single oral dose of APAP, CCl_4, FEL, DAN, MEL, PEN or MET. Bold numbers denote significant changes at $p < 0.05$ level relative to their corresponding control.

Compound	Dose (mg/kg)	Hepatocyte vacuolization	Hepatocyte necrosis	ALT (U/L)	AST (U/L)	ALP (U/L)
APAP						
6 h	0	0	0	32.5 ± 4.04	110.2 ± 17.0	195.3 ± 42.7
	100	0	0	36.4 ± 4.0	106.2 ± 24.9	21.4 ± 77.7
	1250	0	0	36.8 ± 0.96	92.8 ± 8.42	184.3 ± 24.2
24 h	0	0	0	67.5 ± 3	98.0 ± 14.1	353.0 ± 89.2
	100	0	0	**55.4 ± 5.0**	102.8 ± 19.6	309.6 ± 47.5
	1250	0	**1.3 ± 1.4**	459.9 ± 673.5	3006.6 ± 5166.3	365.4 ± 63.2
CCl_4						
6 h	0	0	0	56.4 ± 6.1	109.0 ± 25.3	416.8 ± 49.1
	50	0	0.4 ± 0.5	52.2 ± 4.5	122.2 ± 46.5	385.8 ± 39.8
	2000	0	**1.6 ± 0.5**	78.8 ± 32.3	164.0 ± 50.2	331.8 ± 97.3
24 h	0	0	0	59.6 ± 3.1	92.4 ± 7.8	341.2 ± 44.6
	50	0	0	**68.6 ± 7.8**	111.2 ± 53.9	390.8 ± 96.6
	2000	**1.8 ± 0.4**	**2.6 ± 0.9**	391.0 ± 494.2	1166.6 ± 1612.0	**608.0 ± 120.2**
Felbamate						
6 h	0	0	0	43.8 ± 5.6	96.8 ± 32.1	350.8 ± 50.0
	300	0	0	45.2 ± 8.8	88.8 ± 7.9	359.2 ± 78.8
	1920	0	0	38.2 ± 5.4	85.4 ± 5.0	**254.4 ± 19.3**
24 h	0	0	0	49.4 ± 8.2	88.8 ± 14.5	404.6 ± 82.0
	300	0	0	51.4 ± 5.6	90.0 ± 2.9	359.0 ± 53.7
	1920	0	0	48.0 ± 10.1	95.6 ± 12.8	387.0 ± 69.0
Dantrolene						
6 h	0	0	0	48.0 ± 6.2	86.4 ± 7.1	351.4 ± 76.6
	100	0	0	**40.6 ± 2.4**	79.8 ± 14.7	254.6 ± 67.5
	1000	0	0	**40.6 ± 3.6**	79.4 ± 22.7	**214.0 ± 55.3**
24 h	0	0	0	52.2 ± 11.6	95.2 ± 13.8	333.2 ± 38.2
	100	0	0	42.4 ± 5.0	75.6 ± 16.3	**269.2 ± 47.7**
	1000	0	0	**31.2 ± 7.5**	**68.0 ± 5.7**	**252.6 ± 51.4**
Meloxicam						
6 h	0	0	0	54.6 ± 9.6	96.2 ± 15.7	499.8 ± 98.7
	0.4	0	0	56.6 ± 10.9	95.2 ± 9.4	396.0 ± 84.6
	12	0	0	63.6 ± 4.5	101.0 ± 5.0	427.8 ± 71.5
24 h	0	0	0	52.4 ± 3.8	90.8 ± 9.6	421.6 ± 66.1
	0.4	0	0	60.6 ± 10.3	**106.6 ± 9.5**	455.8 ± 80.0
	12	0	0	**81.0 ± 16.2**	111.8 ± 22.8	421.0 ± 92.2
Penicillin						
6 h	0	0	0	43.2 ± 4.1	94.2 ± 6.4	309.0 ± 102.0
	100	0	0	52.0 ± 7.5	95.8 ± 14.6	397.0 ± 29.3
	2400	0	0	51.4 ± 4.0	107.2 ± 12.1	371.6 ± 45.7
24 h	0	0	0	59.4 ± 10.8	103.4 ± 10.2	371.8 ± 58.8
	100	0	0	64.4 ± 15.9	102.6 ± 15.8	352.8 ± 100.4
	2400	0	0	51.4 ± 9.1	96.0 ± 13.8	326.8 ± 96.3
Metformin						
6 h	0	0	0	34.8 ± 16.5	81.2 ± 5.1	254.2 ± 32.2
	100	0	0	47.6 ± 11.7	92.6 ± 12.9	292.0 ± 88.4
	1500	0	0	**70.4 ± 29.8**	89.6 ± 13.6	215.0 ± 26.4
24 h	0	0	0	54.4 ± 7.6	125.6 ± 57.1	393.4 ± 127.0
	100	0	0	49.8 ± 10.5	92.0 ± 10.6	341.6 ± 134.3
	1500	0	0	**37.2 ± 6.4**	84.6 ± 9.3	**237.6 ± 43.7**

to mild hepatic vacuolization (average score of 1.8) at 24 h. There was a low degree of hepatocyte necrosis reported for animals given the low dose of CCl_4 at the 6 h timepoint.

Low and high doses of FEL, DAN, MEL, PEN and MET caused neither hepatic vacuolization nor hepatic necrosis at 6 or 24 h. However, for rats treated with MEL for 24 h, inflammation was observed in the capsule of kidney (1 out of 5), liver (2 out of 5) and other organs including spleen, stomach, intestine and abdominal cavity with different percent incidences. No other significant increases were observed in other treated animals at 6 h. Low dose FEL, PEN and MET rats did not have any clinical chemistry changes at 6 or 24 h. High dose FEL caused a decrease in ALP at 6 h. Dosing with MEL resulted in elevated ALT at 24 h in the high dose group (<2 fold increase of the ALT value of the control group), and elevations in AST at 24 h in the low dose group. High dose DAN treatment induced significant decreases in ALT and ALP at 6 and 24 h and AST at 24 h, while low dose DAN caused a significant decrease in ALT at 6 h and a significant decrease in ALP at 24 h. High dose MET caused significant decreases in ALT and ALP at 24 h and a significant increase in ALT (<3 fold increase of ALT value in control group) at 6 h.

3.2. SIMCA PLS-DA Analysis

It has been recognized that the diagnosis of certain diseases based upon one individual marker might yield false prediction due to low sensitivity or low specificity [37,38]. The level of serum ALT is a routinely used clinical biomarker for hepatocellular injury. However, ALT can markedly increase (>20 fold of the upper limit of normal) in drug treatments with no liver cell injury, for example after treatment with tacrine [39] and statins [40]. Tacrine and statins have induced transient

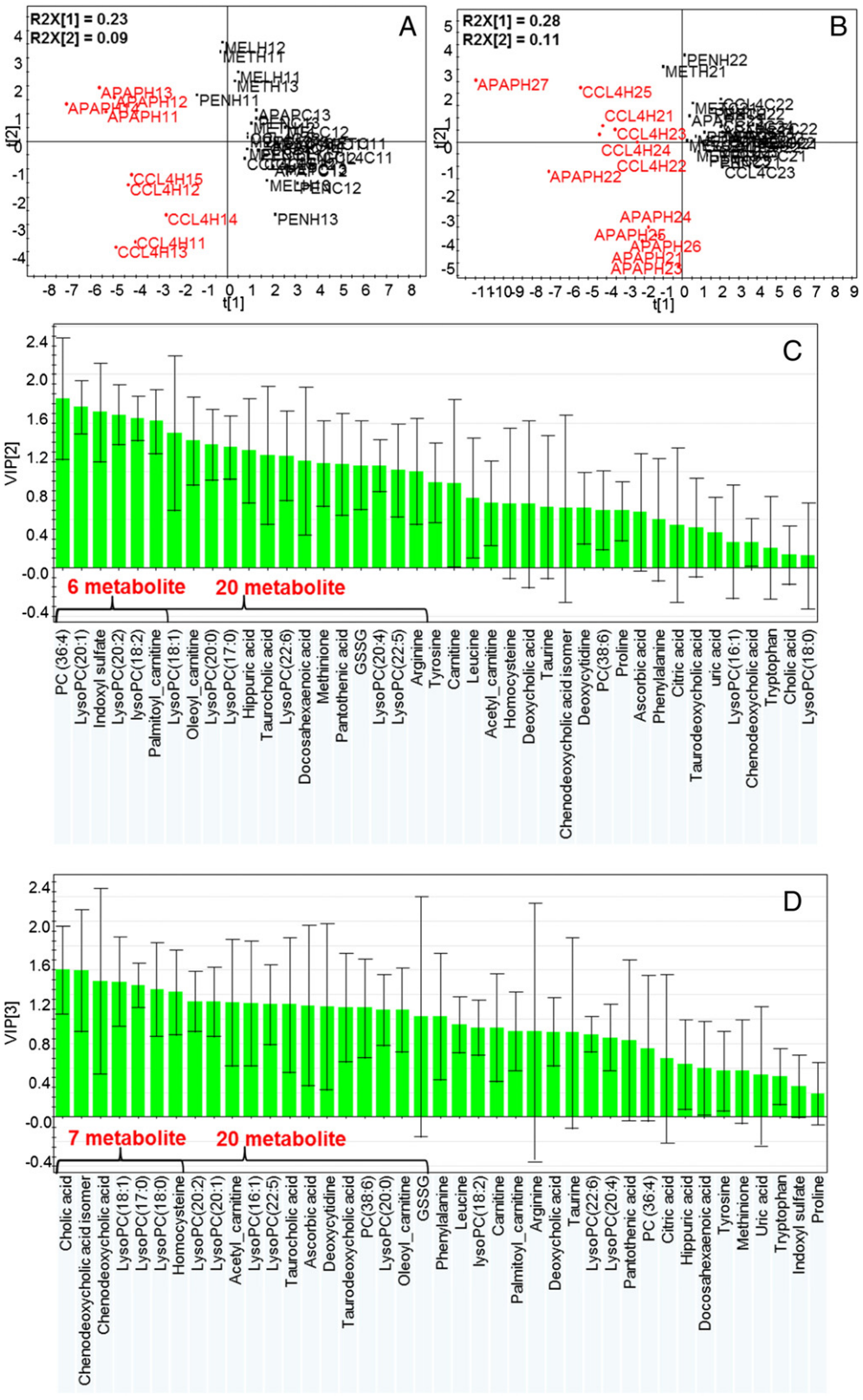


Fig. 1. The scores plot at 6 h (A) and 24 h (B) from the SIMCA PLS training set modeling using the 41 metabolites. VIP plots display the contribution of each metabolite to the modeling at 6 h (C) and 24 h (D). Each dot in the score plots represents one animal labeled as dose compound followed by high dose (H), time point (1 for 6 h and 2 for 24 h) and animal number, for example APAPH14 is animal #4 in the APAP high dose group at 6 h; while APAPH27 is animal #7 in the APAP high dose group at 24 h.

elevations in ALT but the liver injury can be self-resolved during chronic treatment. Thus, although ALT is a sensitive liver injury biomarker, it alone is impractical for differentiating drugs that cause ALT elevations where liver injury is self-resolved from drugs that cause liver injury that progresses to hepatocellular injury. A biomarker panel based on pathways or common mechanisms of hepatotoxicity would

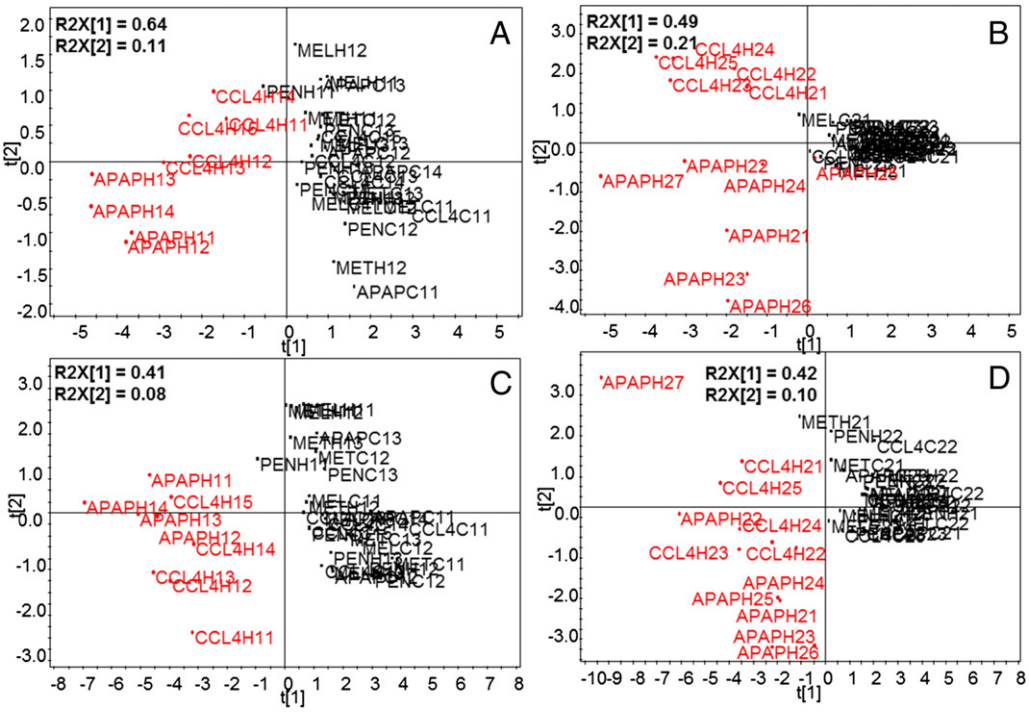


Fig. 2. The scores plot from the Simca PLS training set modeling using 6 metabolites at 6 h (A), 7 metabolites at 24 h (B), and 20 metabolites at both 6 h (C) and 24 h (D). The metabolites were chosen based on the VIP value from the Simca PLS modeling (Fig. 1C & D). Animal labeling is the same as Fig. 1.

be ideal for the prediction of liver injury [41]. The metabolic profiling has been successfully used to predict drug induced kidney injury in a preclinical study [42]. Hence, our efforts to discover a metabolic biomarker panel for prediction of liver injury will be based on the common pathways underlying hepatotoxicity: i) bile acid metabolism; ii) oxidative stress, iii) energy pathways related to mitochondrial impairment and iv) other hepatic cell regeneration pathways [41]. Forty-one blood metabolites, which were previously detected as commonly altered by APAP [22] and CCl_4 [23], were semi-quantified. The normalized levels of these metabolites are reported in Supplemental Table S1 for the 24 h samples and Supplemental Table S2 for the 6 h samples.

3.3. SIMCA Hepatotoxic Modeling Results

Fig. 1 shows the scores plot at 6 h (A) and 24 h (B) from the SIMCA PLS modeling of the sets using all 41 metabolites. Individual metabolite contributions expressed as VIP values are shown in Fig. 1C & D for 6 h and 24 h, respectively. Two well distinguished clusters can be observed in Fig. 1A & B. The animals with negative values on component 1 (t[1]) are those dosed with overt-hepatotoxicants, while those having positive values on t[1] are either control animals or animals dosed with non-hepatotoxicants. The predicted values from the 41 metabolite SIMCA models at 6 h and 24 h are listed in Table S3. The classification accuracy for the 6 h modeling set was 94%, while the accuracy for 24 h model was 89%. In the external test set (high dose) for 41 metabolite modeling, one rat treated with DAN was classified as being exposed to a hepatotoxicant and two DAN-treated rats were ambiguously (highlighted in orange in Table S3) classified at both 6 h and 24 h; while one rat treated with FEL was predicted as hepatotoxic and two rats treated with FEL were ambiguously classified at 24 h (Table S3).

The natural drops observed in the VIP plots (Fig. 1C & D) were used to select a subset of 6 metabolites at 6 h, 7 metabolites at 24 h, and 20 metabolites at both 6 h and 24 h that were further used to build new SIMCA and Matlab PLS models with a reduced number of metabolites. Fig. 2 shows the PLS-DA scores plot using 6 metabolites at 6 h (A), 7 metabolites at 24 h (B), and 20 metabolites at 6 h (C) and 24 h (D). The 6 metabolites in the 6 h model included the following metabolites: PC(36:4), palmitoylcarnitine, lysoPC(20:1), indoxyl sulfate, lysoPC(20:2) and lysoPC(18:2). The 7 metabolites at 24 h were as follows: cholic acid, chenodeoxycholic acid isomer, chenodeoxycholic acid, lysoPC(18:1), lysoPC(17:0), lysoPC(18:0) and homocysteine. Alternative models using the top 20 metabolites at both 6 and 24 h, (shown in Fig. 1C & D) were also explored. A total of 9 metabolites were common to both 6 h and 24 h models (Fig. S2). The common metabolites were: lysoPC(17:0), lysoPC(18:1), lysoPC(20:0), lysoPC(20:1), lysoPC(20:2), lysoPC(22:5), oleoylcarnitine, taurocholic acid and oxidized glutathione (GSSG). Similar to our models using all 41 metabolites, the PLS-DA scores plots (Fig. 2) indicate the presence of two distinct clusters; the animals with negative values on t[1] were those dosed with overt-hepatotoxicants while those on the positive axis of t[1] were from the control and those treated with high dose non-hepatotoxicants. Three animals were identified as outliers in Fig. 2: one rat treated with PEN (Fig. 2A & C) and one rat treated with MET (Fig. 2D) are located within the hepatotoxic cluster, while one rat treated with APAP (Fig. 2B) is located within the non-hepatotoxic cluster. The classification accuracy for the 6 h training set model using 20 metabolites was 100%, while the 24 h model misclassified two rats treated with APAP and CCl_4 resulting in 95% accuracy. Three rats from the external test set (high dose) based on 20 metabolites were ambiguously classified including one treated with DAN at 6 h and two rats treated with FEL at 24 h (Table S3). One of the FEL treated rat was predicted as being exposed to hepatotoxicant at 24 h. The accuracy for the 6 metabolite SIMCA model at 6 h was 94%. Two rats treated with a high dose of either DAN or FEL were ambiguously classified at 6 h. The accuracy for the 7 metabolite for 24 h SIMCA model was 89%. One rat treated with FEL was positively predicted as experiences hepatotoxicity while one rat treated with a high dose of DAN and two rats treated with FEL were ambiguously classified at 24 h (Table S3).

3.4. Matlab PLS Modeling and Risk Estimation Analysis

Alternatively, Matlab PLS models based on the algorithm shown in Scheme 2 were developed to predict the liver toxicity of FEL and DAN

and a subset of the non-DILI compounds forming the external test set. The performance metrics of these models are shown in Table 3. For three of these datasets (6 h with 6 and 20 metabolites; and 24 h with 7 metabolites), one latent variable (LV) was sufficient to generate models with accuracy for the training and the hold-out test sets exceeding 97.4%. It was impossible to estimate the accuracy of the external test as it contained chemicals displaying idiosyncratic behavior (i.e. cannot be classified as either hepatotoxic or non-hepatotoxic). One out of 4 rats treated with a high-dose of DAN at 6 h was classified as experiencing hepatic injury by both 6 and 20 metabolites models; while no rats treated with FEL at 6 h were classified as such. At 24 h, one out of five rats treated with a high-dose of FEL was classified as hepatotoxic; while no rats treated with DAN were classified as hepatotoxic. Thus, it can be concluded that both FEL and DAN displayed toxicity patterns that were time-dependent and had low incident rates. To further estimate the ability of these models to predict hepatotoxic potential, an external test set (low dose) comprised of animals treated with low doses of DILI, non-DILI and iDILI compounds were examined. For the low doses at 6 h, one out of 5 rats dosed with CCl_4 or FEL was classified as hepatotoxic using 6 and 20 metabolites Matlab PLS models, respectively. For the low doses at 24 h, 2 out of 5 samples from rats treated with APAP and 1 out of 5 samples from rats treated with MET were classified as experiencing hepatotoxicity using the 6 metabolites model; while 1 out of 5 samples from MEL or FEL were classified as experiencing hepatotoxicity using the 20 metabolites model. These predictions indicate that low doses of both APAP and CCl_4 have the potential to induce metabolites changes that are predictive of liver injury before overt clinical chemistry signs are observed. However, this effect can be observed earlier (at 6 h) in the case of CCl_4, while up to 24 h might be needed to observe metabolic changes in animals treated with low doses of APAP. An interesting finding in the external test was that some samples from high and low doses of MET were predicted as being exposed to a hepatotoxicant. Although MET is classified as a non-hepatotoxicant in this study, MET has been reported to cause liver injury in patients [36]. The Matlab PLS external test set (low doses) showed that the model predictions were below or near the 0.5 cutoff and it has not determined that 0.5 is the optimal cutoff for a hepatotoxic prediction in the Matlab PLS models.

To further understand the limitations of the temporal metabolic patterns used in the PLS models, a cross time examination was performed. The 7 metabolites model at 24 h was used to predict all animals with high doses at 6 h and the prediction accuracy (DAN and FEL excluded) was 78.9% (Table S3 cross time examination). High dose PEN at 6 h had 3 false positives. We reported that PEN had multiple bile acids released at 6 h [23] that may be due to efficacy of PEN on the gut microflora when taken orally. Very few APAP and CCl_4 samples were predicted as toxic using the 7 metabolites 24 h pattern on 6 h samples and this may be due to that the pattern at 24 h represents a later toxic response that is not present early in toxicity. The prediction accuracy was 96.2% when the 6 metabolites panel at 6 h was used to predict all animals with high doses at 24 h. The false predictions included the 5 rats treated with APAP that did not show signs of toxicity and two rats treated with CCl_4 clearly indicating that the 6 h pattern at 24 h was not as sensitive as the 24 h pattern at 24 h. We then used the 20 metabolites 6 h model to predict 24 h samples and 20 metabolites 24 h model to predict 6 h samples. The results of the 20 metabolites 6 and 24 h models cross examination are shown in Table S3. The prediction accuracy at 6 h was 84.2% using 20 metabolites modeling of 24 h, while the prediction accuracy at 24 h was 84.5% using 20 metabolites modeling of 6 h. The false-positive prediction (rats treated with PEN or MEL) and lack of predicting APAP as hepatotoxic at 6 h using the 24 h modeling were similar to the 7 metabolites model cross time examination. This clearly demonstrates that the pattern at 24 h represents a later toxic response that is not present early in toxicity (6 h). The results of the 20 metabolites 6 h model at 24 h were very similar to the 6 metabolites 6 h model at 24 h with one less APAP predicted as hepatotoxic. Thus, the results show that the 6 h pattern is only seen in some of the later (24 h), more toxic APAP and CCl_4 samples but not all hepatotoxicant samples; the early metabolic changes can persist through an extended time and both 6 and 20 metabolites 6 h models did predict 24 h non-hepatotoxicant or control samples except for the one MET sample that has consistently predicted as toxic.

The hepatotoxicity risk of each compound at high doses can be estimated by analyzing the probability density functions of the predicted quantitative values from the hold-out and external test sets (Fig. 3). The width and the height of the probability density curve can serve as

Table 3
Matlab PLS model predictions based on the algorithm from Scheme 2.

	6 h				24 h			
	Training	Hold-out test	Ext. test high dose	Ext. test low dose	Training	Hold-out test	Ext. test high dose	Ext test low dose
	6 metabolites (1LV)				7 metabolites (1LV)			
PEN	3−	3−	2−	5−	3−	3−	2−	5−
MEL	3−	3−	2−	5−	3−	3−	2−	5−
APAP	4+	4+		5−	6+/1−	6+/1−		3−/2+
CCl_4	5+	5+		4−/1+	5+	5+		5−
MET	3−	3−	2−	5−	3−	3−	1+	4−/1+
FEL			5−	5−			4−/1+	5−
DAN			3−/1+	5−			4−	4−
Control	18−	18−	15−		17−	17−	15−	
Accuracy (%)	100	100			97.4	97.4		
	20 metabolites (1LV)				20 metabolites (2LV)			
PEN	3−	3−	2−	5−	3−	3−	2−	5−
MEL	3−	3−	2−	5−	3−	3−	2−	4−/1+
APAP	4+	4+		5−	7+	7+		5−
CCl_4	5+	5+		5−	5+	5+		5−
MET	3−	3−	2−	5−	3−	3−	1+	5−
FEL			5−	4−/1+			4−/1+	4−/1+
DAN			3−/1+	5−			4−	4−
Control	18−	18−	15−		17−	17−	15−	
Accuracy	100	100			100	100		

+ and − noted as hepatotoxic and non-hepatotoxic; HO = hold out.

an indicator of the range of biological responses. It is interesting to note that the probability density function of APAP at 24 h is characterized by a very wide curve, which is consistent with the observation that two animals had high degrees of necrosis, a few had minimal injury and a couple had no injury. The maximum peak of the distribution function may be related to average hepatotoxicity potential with a peak near 0 meaning no injury and a peak near 1.0 having overt injury. Animals treated with high doses at 24 h (Fig. 3C & D), showed that CCl_4 and APAP were the most toxic agents followed by FEL and DAN; MET, PEN and MEL were less toxic than other compounds. The results were consistent with the histopathology data that high dose CCl_4 caused more liver damage than did APAP, while other compounds did not cause hepatocyte necrosis (Table 1). Fig. 3A & B also show that CCl_4 and APAP are the most hepatotoxic compounds in both the 6 and 20 metabolites models. However, it is worth noting that a cutoff of 0.5 was used in the PLS model (≥ 0.5 as hepatotoxic, <0.5 as non-hepatotoxic), while all injured animals from the APAP and CCl_4 treatments had an average hold out predicted value of 0.936 at 24 h (when maximum injury occurred). Thus, it is not clear whether >0.5 is the optimal choice for a cut-off value of predicting whether hepatotoxicity occurred. The probability density functions of the idiosyncratic compounds may provide a better hepatotoxicity prediction in the clinic rather than a hard-cutoff value of 0.5 but more studies are needed before this can determined. Probability density function analysis was also employed to assess the hepatotoxic risk of each compound at low doses 6 h and 24 h post-dosing (Fig. S3). No Gaussian peak probability value was observed greater than 0.5 for any low dose hepatotoxic prediction in any of the Matlab PLS models.

Nine metabolites were common to both 6 h and 24 h (Fig. S2) models. These common metabolites are classified as energy-related metabolites (oleoylcarnitine, lipids (lysoPC(17:0), lysoPC(18:1), lysoPC(20:0), lysoPC(20:1) lysoPC(20:2), and lysoPC(22:5)), bile acids (taurocholic acid) and oxidative stress-related metabolites (oxidized glutathione, GSSG). The 9 metabolites are involved in common pathways underlying liver injury initiation and response to injury. Bile acids are synthesized in the liver, thus bile acid flux is a well-known liver function biomarker [43]. Blockade of bile flow from the liver to the intestine is one of the characteristics of liver diseases or liver injury. The rate of bile flow has been reported as an indicator for the recovery process post liver transplantation [43]. Acylcarnitines have been reported as potential biomarkers for APAP-induced hepatotoxicity, which could be caused by the disruption of fatty acid β-oxidation due to mitochondrial damage [44,45]. It has been reported that oxidative stress is involved in an animal model of idiosyncratic hepatotoxicity and antioxidant treatments have protective effects against hepatotoxicity in this model [46,47]. Glutathione has been reported to play an important role in detoxifying many reactive metabolites from hepatotoxicants. In humans, 1-lysoPC is produced from phosphatidylcholine by phospholipase A1 in the liver, brain and mitochondrion. LysoPC(17:0), lysoPC(18:1), lysoPC(20:0), lysoPC(20:1) lysoPC(20:2), and lysoPC(22:5) are included in the biomarker panel for both timepoints. Consistent with the findings in this study, altered lipid metabolism has been predicted based on the ten urinary microRNAs detected as potential biomarkers of hepatotoxicity-induced liver injury from the same APAP, CCl_4 and PEN studies [18]. The altered microRNAs, whose biological functions are related to the lipid metabolism, are significantly

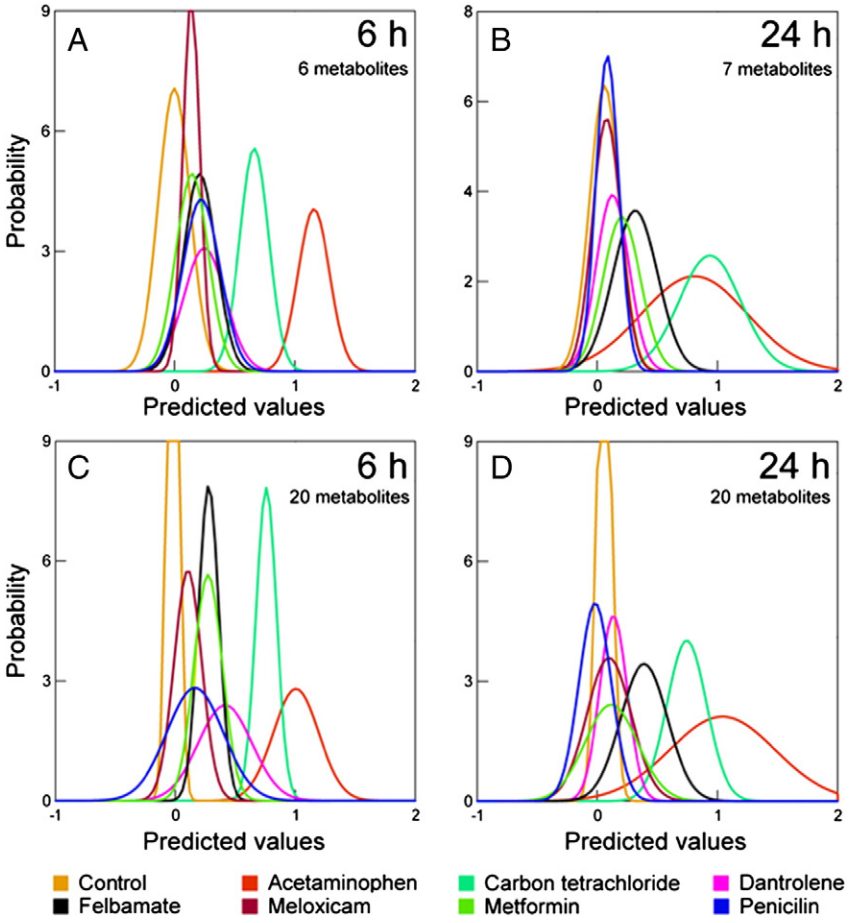


Fig. 3. Probability density functions of the average prediction values to assess the risk of each compound at 6 h using 6 metabolites (A) and 20 metabolites (B); and at 24 h using 7 metabolites (C) and 20 metabolites (D).

increased in APAP and CCl_4 treated animals but decreased in PEN dosed animals.

Fig. 4 shows the average blood levels of select metabolites including oleoylcarnitine, palmitoylcarnitine, lysoPC (20:1), and taurocholic acid for the treated groups of each compound and control at the 6 h and 24 h timepoints. Oleoylcarnitine (Fig. 4A) was significantly increased in rats treated with APAP and CCl_4 at 6 h and 24 h, and also in rats treated with MET at 6 h. Palmitoylcarnitine (Fig. 4B) was significantly increased in CCl_4-dosed rats and also appeared to be increased by APAP and FEL although the change was not significant at 24 h. At 6 h, palmitoylcarnitine was significantly increased in APAP, CCl_4, DAN and MEL treated rats. LysoPC(20:1) (Fig. 4C) appeared to be decreased by APAP and MEL and was significantly decreased by CCl_4, FEL and DAN at 24 h. At 6 h, lysoPC(20:1) was significantly decreased for APAP, CCl_4 and FEL treatments. Taurocholic acid (Fig. 4D) was significantly increased in CCl_4-dosed rats at 24 h and significantly decreased in MET-dosed rats at 6 h.

The 41 metabolites and 20 metabolites at 6 h and 24 h were analyzed to examine the pathway perturbations using MetaboAnalyst (http://www.metaboanalyst.ca/MetaboAnalyst/). Results (Fig. S5) showed that the 41 metabolites were primarily involved in the following pathways ($p < 0.05$): bile acid biosynthesis and glycerophospholipid, phenylalanine, and taurine metabolism were the major impacted pathways. In the 6 h 20 metabolites model, glycerophospholipid metabolism was significantly impacted ($p < 0.01$). The significantly impacted pathways by the 20 metabolites in the 24 h model were related to bile acid and glycerophospholipid metabolism.

3.5. Pearson's Correlation Results

Pearson's correlation was employed to evaluate the effects of food intake changes on the metabolite levels (Supplemental Table S4). All metabolites had correlations to dietary intake that were less than 0.5 ($|r| < 0.5$) except hippuric acid which had strong correlation ($r = 0.61$) at 24 h. At 6 h, lysoPC(18:1) and lysoPC(22:5) were significantly correlated to food intake ($p < 0.05$). At 24 h, lysoPC(18:1), lysoPC(20:2), lysoPC(22:5), oleoylcarnitine,palmitoylcarnitine, PC (38:6) and phenylalanine were significantly ($p < 0.05$) correlated to the food intake.

This preclinical study discovered biomarkers associated with drugs/toxicants that caused liver injury to predict hepatotoxicity of some drugs that are known to cause idiosyncratic liver injury in clinical studies even though there was no significant alteration in ALT, AST and ALP levels. This can be explained by the nature of the toxicity process in an organism. As indicated in Scheme 1 and as noted by Gregus [14], chemical-induced toxicity is a multiple-step process. First, the chemical compound is absorbed, distributed, metabolized and finally excreted (ADME) from the biosystem. The toxicant, which may be the parent compound or its metabolite(s), may interact with macro-biomolecules (DNA, RNA, proteins) as well as with endogenous metabolites, and result in varying degrees of harm to the cells. The result of the toxicant interaction with a target or targets is cellular dysfunction/destruction or neoantigen formation [14]. *N*-acetyl-*p*-benzoquinone imine, the reactive metabolite of APAP is known to bind to proteins [48,49] and this is believed to be the primary mechanism for hepatic necrosis. In much the same manner, the trichloromethyl radical generated from CCl_4 binds to microsomal triglyceride transfer protein and disrupts triglyceride exportation from the liver causing steatosis [50]. FEL has been shown to metabolize to the reactive metabolite, *3*-carbamoyl-*2*-phenylpropionaldehyde derivative, and this has been hypothesized to be involved with the iDILI induced by this drug [51]. DAN is metabolized to the electrophilic metabolite aminodantrolene and this has been suggested to play a role in iDILI [52]. This is consistent with the report that structural alerts for reactive metabolites were reported for 78–86% of the top 200 DILI causing drugs including FEL and DAN [53]. The total amount of injury depends on the amount of the reactive toxicants in the cell, what type of cellular disruption the toxicants induce, and other factors such as genetics, disease status and environment. Further, some drugs or their reactive metabolites can form immunogenic haptens and subsequently stimulate immune reactions. Therefore, human leukocyte antigen (HLA) polymorphisms have been explored as risk factors for iDILI. The conduct of genome-wide association studies in a genotyped population has identified strong association of HLA-B*57:01 allele with flucloxacillin toxicity [54]. In the current study, there is no report indicating HLA or genetic factors are a risk factor for FEL- or DAN-

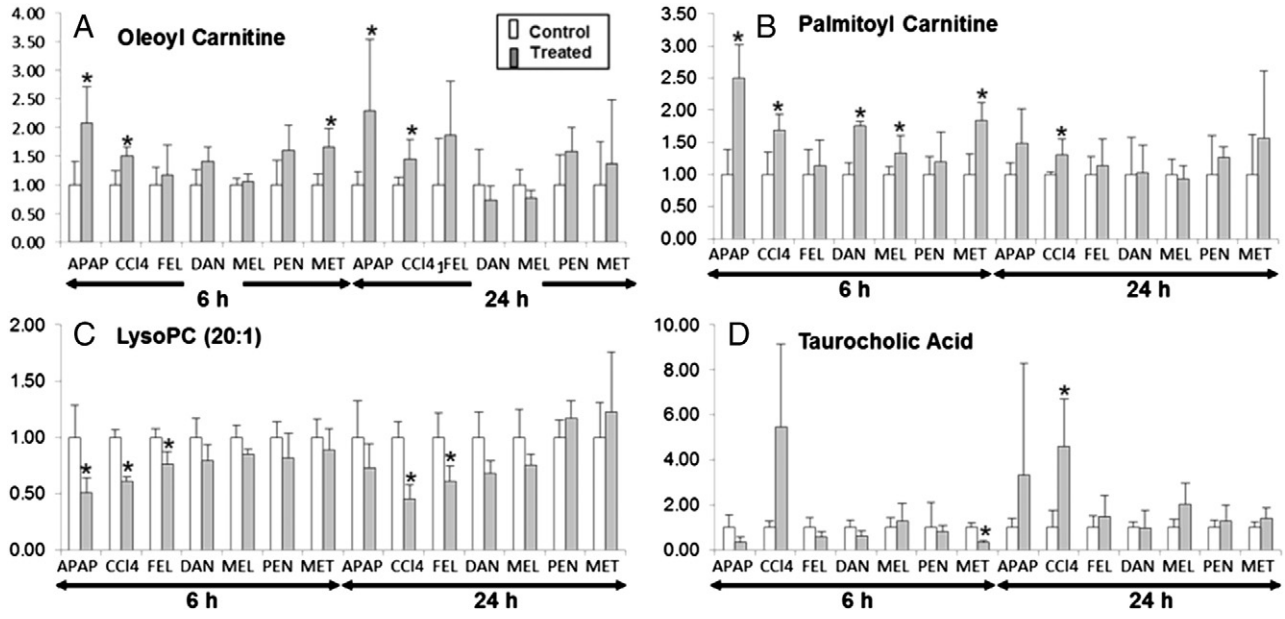


Fig. 4. Bar graphs of the normalized data of oleoylcarnitine (A), palmitoylcarnitine (B), lysoPC(20:1) (C), and taurocholic acid (D) in animals 6 h and 24 h post-dosing with vehicle, overt-hepatotoxicants (APAP and CCl_4), idiosyncratic hepatotoxicants (FEL and DAN), or non-hepatotoxicants (MEL, PEN and MET). * indicates $p < 0.05$ level compared the values from treatment with their corresponding control values.

induced toxicity. Therefore, the model may not be applicable to predict drugs interacting with HLA variants.

Once toxic injury occurs, the cell may try to avoid apoptosis or necrosis by protective or repair mechanisms. For example, toxicant-induced changes in mitochondria permeability are usually injuries that cause cell death but there are occasions that a cell can go through lysosomal autophagy limiting the injury and promoting survival [55]. These phenomena can be explained by self-protection of an organism, where reactive metabolites or parent compounds can be deactivated through conjugation to glutathione, which protects a cell from oxidative stress [56,57]. Glutathione can also bind to proteins in a process named as "glutathionylation", which potentially can protect them from additional damage due to toxicant binding to cysteine groups in the proteins [57,58]. The cell can also protect itself from DNA adducts by removing the modified DNA from the biosystem [59]. The system's response is to repair and protect damaged cells or to adapt to the insult then return to homeostasis. If the damage is beyond repair, the cell may go through apoptosis or necrosis and release cellular biomarkers like ALT [38]. If the toxic injury continues to persist and many cells in the liver are not able to recover, the liver may fail. The metabolic biomarkers discovered in this study at 6 h were not exactly the same as those discovered at 24 h, which might be caused by the temporal corresponding toxicity response status after drug/chemical exposure to a biosystem. Of note, no signs of liver damage were observed in animals treated with iDILI compounds which caused metabolome changes.

The biomarkers observed in these studies were changed upon exposure to DILI compounds (rats with elevated ALT from APAP and CCl_4 treatments) and were used to predict iDILI compounds (rats with normal ALT, DAN and FEL treatments). The metabolic biomarker panel involving multiple pathways has promise in predicting the response status of the biosystem to toxicants. Although the correlation results of metabolites with food consumption showed that changes in food intake were correlated to some metabolite levels, the combined metabolic biomarker panel can shed some light on DILI prediction. In this particular case, taurocholic acid ($r = -0.20$), deoxycholic acid ($r = 0.15$) and taurodeoxycholic acid ($r = -0.11$) were not affected by dietary intake over the seven studies, while the lysoPCs ($0.04 < r < 0.40$) and acylcarnitines ($-0.04 < r < -0.12$) showed some small correlations but they were in opposite directions. Palmitoylcarnitine and oleoylcarnitine were negatively correlated to diet, meaning when diet decreased during toxicity its levels increased. This is the opposite of what would be expected and has been reported [60] in acute calorie restriction in mice. The study in mice indicated that increased β-oxidation of fatty acids and lower levels of de novo fatty acid synthesis should lower the levels of palmitoylcarnitine and other medium chain acylcarnitines. These phenomena indicated that changes in acylcarnitines could be very likely due to toxicity instead of altered dietary intake. LysoPC(22:5) showed significant positive correlations to dietary intake and may be related to lower dietary intake during toxicity or to some lipid metabolism that reduced the levels that occur during toxicity. To further evaluate the potential of the metabolic biomarker panel, more drugs or chemicals, more animals and longer chronic dosing of idiosyncratic compounds studies should be tested in dietary controlled studies.

4. Conclusions

In order to discover a metabolic biomarker panel, forty-one metabolites previously observed in APAP and CCl_4 studies were semi-quantified in the 7 compound studies. The SIMCA PLS modeling results showed 89% accuracy in the modeling set. This model was further used to predict rats that were treated with non- (MET, PEN, MEL) or idiosyncratic (DAN or FEL) hepatotoxicants at 6 and 24 h. Two animals (one dosed with DAN at 6 h and one dosed with FEL at 24 h) were predicted as being exposed to hepatotoxicants (Table S3). The Matlab PLS algorithm generated models with 100% accuracy for the training, and 97.4% accuracy for the hold-out test sets (Table 3). Both, SIMCA and Matlab models predicted identical rats treated with a high dose of DAN at 6 h or FEL at 24 h as being exposed to hepatotoxicants. This is a higher rate than the false positive prediction rate for control animals in the external test and hold-out training sets where there were no false negatives at 6 h or 24 h. Interestingly, one rat treated with high dose of MET at 24 h was predicted after hepatotoxicant exposure although MET was considered as non-hepatotoxic in the experimental design. This phenomena supported the clinical report that MET could cause hepatotoxicity alone [36]. The common metabolites observed in the models at 6 h and 24 h are involved in fatty acid β-oxidation, bile acid metabolism and glycerophospholipid metabolism, which have been noted to be linked to the underlying mechanism of hepatotoxicity. The panel showed its potential to distinguish idiosyncratic hepatotoxicants prior to liver damage. Based on a 0.5 cutoff, the Matlab PLS model predicted one DAN at 6 h and one FEL at 24 h animals as being exposed to hepatotoxicants. The metabolic profile is related to injury and response of the animals to the drug/toxicant administration. For example, the APAP rats at 24 h that did not show overt liver injury were able to repair their injury but still had similar changes in the metabolites as the animals with liver injury. This may also be the case for one dosed with FEL or DAN. It is very challenging to predict how many clinical patients or previous preclinical studies on FEL or DAN would be classified in this category. Cross time examination results showed that the 24 h-metabolite models predicted animals at 6 h with less accuracy than the 6 h models used in predicting the hepatotoxic effect on rats at 24 h. The metabolites found effective at 24 h were not highly predictive of the hepatotoxic effect at 6 h and therefore the metabolites in the 24 h model may represent later metabolic changes to toxicity than occurs early on. Likewise, the 6 h metabolites were not as sensitive to hepatotoxicity at 24 h as the 24 h metabolite models, since the 6 h model contained mostly early changes and was not covering as many toxicity pathways as the metabolite in the 24 h model were. More studies are needed to further test the sensitivity, specificity and limitations of the temporal biomarker panels, and to test the translation potential to other species before major conclusions can be made about the usefulness of the metabolic patterns, associated pathways and models. In terms of sensitivity and reproducibility, not only more acute and idiosyncratic drugs need to be tested, but several idiosyncratic drugs connected to HLA hypersensitivity need to be evaluated to determine whether they would have a different metabolic pattern. For specificity, more non-hepatotoxic drugs need to be tested as well as drugs that cause damage to other organs. More animal numbers and more species are needed for temporal pattern refinement and Gaussian probability fitting for the chemical idiosyncratic hepatotoxic risk predictions. Nonetheless, the mechanism-based biomarker panel could provide complementary prediction information in hepatic injury by themselves or in conjunction with current clinical biomarker (ALT, AST and ALP) and other emerging biomarkers such as proteomic, transcriptomic or miRNA biomarkers that may improve regulatory and clinical decision making.

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