

## Systems Biology Investigation to Discover Metabolic Biomarkers of Acetaminophen-Induced Hepatic Injury Using Integrated Transcriptomics and Metabolomics

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### Abstract

**Background:** Drug-induced hepatotoxicity is one of the major reasons for drug recall and hence it is of major concern to the FDA and consumers. Overdose of acetaminophen (APAP) can cause acute hepatic injury. The current clinical biomarkers of liver injury are insufficient in predicting the extent of injury; thus novel biomarkers are needed to integrate with the current biomarkers for better risk assessment during drug development and clinical use.

**Methods:** Sprague-Dawley rats were orally gavaged with a single dose of 0.5% methylcellulose (control), 100 mg APAP/kg body weight or 1250 mg APAP/kg body weight. Urine, terminal blood samples and tissues were collected at 6, 24, 72, and 168 h for clinical chemistry and histopathology analyses. Based on the clinical chemistry data and histopathology, liver injury occurred in treated animals during the first 24 h, while recovery occurred during 72 to 168 h. A systems biology investigation of APAP-induced hepatic injury was conducted to elucidate novel metabolic biomarkers using an integrated transcriptomic and metabolomic approach. Both open metabolic profiling and broad metabolic profiling were utilized to examine metabolic changes in blood and open profiling was used to evaluate changes in the urinary metabolite profiles.

**Results:** In total, 270 metabolites were evaluated in blood and/or urine. Metabolites involved in energy, urea and bile acid pathways were found to have strong correlations to hepatic necrosis scores and elevated alanine aminotransferase levels. The pathways associated with these metabolites were altered by the first 72 h but had generally recovered by 168 h. Changes in hepatic gene expression of the bile acid pathway supported the interpretation from the metabolomics data.

**Conclusion:** The combination of the transcriptomics and metabolic profiling technologies discovered novel injury biomarkers (arginine, 2-oxoarginine, medium chain dicarboxylic acids,  $\alpha$ -ketoglutarate and bile acids), which are involved in energy, bile acid, and arginine metabolism pathway.

**Keywords:** Acetaminophen; Liver injury biomarker; Metabolic profiling; Transcriptomics; Hepatotoxicity

**Abbreviations:** ALP: Alkaline Phosphatase; ALT: Alanine Aminotransferase; ANOVA: Analysis of Variance; APAP: Acetaminophen; APAP-Glu: Acetaminophen Glucuronide; APAP-NAC: *N*-Acetyl-L-cysteine acetaminophen; APAP-Sul: Acetaminophen-Sulfate; AST: Aspartate Aminotransferase; BEH: Bridged Ethyl Hybrid; BUN: Blood Urea Nitrogen; GC/MS: Gas Chromatography/Mass Spectrometry; GGT: Gamma-Glutamyl Transferase; GSH: Glutathione; GSSG: Glutathione Disulfide; H1: High dose group 6 h after dosing; H2: High dose group 24 h after dosing; HD: High dose; 1250 mg APAP/kg body weight; LC/MS: Liquid Chromatography/Mass Spectrometry; LC/MS/MS: Liquid Chromatography/Mass Spectrometry/Mass Spectrometry; LD: Low dose; 100 mg APAP/kg body weight; MC: Methylcellulose; vehicle control; MRM: Multiple Reaction Monitoring; NAPQI: *N*-acetyl-*p*-benzoquinone; NMR: Nuclear Magnetic Resonance Spectroscopy; NOS: Nitric-Oxide Synthase; PCA: Principal Component Analysis; PLS-DA: Partial Least Squares-Discriminant Analysis; QC: Quality Control; SAME: *S*-Adenosyl-methionine; TCA: Tricarboxylic Acid; TQ-MS: Triple Quadrupole-Mass Spectrometry; UPLC/MS: Ultra Performance Liquid Chromatography/Mass Spectrometry; UPLC/QTOF-MS: Ultra Performance Liquid Chromatography/Quadrupole Time-Of-Flight Mass Spectrometry; VLDL: Very Low Density Lipoprotein

### Introduction

Drug-induced hepatotoxicity is one of the major reasons for drug failure and recall and hence it is of major concern to the FDA and consumers. An overdose of acetaminophen (APAP), a widely used over-the-counter analgesic and antipyretic drug, can lead to acute liver failure associated with hepatic centrilobular necrosis [1]. The acetaminophen-induced hepatic injury causes increases in alanine aminotransferase (ALT) but most often the liver recovers before liver failure occurs. Currently, serum ALT [2], aspartate aminotransferase, and bilirubin levels constitute the major biomarkers used to diagnose

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liver injury in the clinical setting. ALT is an organ damage biomarker for hepatocyte injury, however, other factors can also influence its blood levels [3,4]. Therefore, ALT is not specific for liver injury [5-8]. Unfortunately, ALT and AST are often considered as liver functional biomarkers; these enzymes are not actually related to liver function and therefore cannot be used to assess liver function or predict liver recovery. Bilirubin is a functional marker of the liver but generally does not increase until severe liver injury occurs. Therefore there remains a need to integrate novel biomarkers to further improve risk assessment in nonclinical and clinical studies during drug development.

The omics technologies, which comprise transcriptomics, proteomics and metabolomics, are promising platforms to obtain a more comprehensive understanding of the biochemical and cellular events that are associated with the damage that occurs during the toxicity phase and subsequent recovery. Recently, transcriptomic and proteomic technologies [9-13] have been successfully utilized to reveal that APAP can cause noteworthy changes in gene expression and protein modifications that alter function. These genes and proteins play important roles in pathways related to oxidative stress, mitochondrial function and mitochondrial structure as well as signaling pathways and hepatic necrosis. The metabolomics profiles, which primarily represent downstream changes in transcription and proteomic changes, have been adopted to investigate the mechanism of APAP-induced toxicity and to discover novel biomarkers of hepatotoxicity [12-17]. In previous studies, NMR spectroscopy and ultra performance liquid chromatography/mass spectrometry (UPLC/MS)-based metabolomics were employed to study urinary metabolic perturbations induced by APAP administration [15] and the drug's metabolites profile [14]. These results showed the depletion of antioxidants (e.g., ferulic acid), trigonelline, S-adenosyl-methionine (SAME), and energy-related metabolites caused by APAP administration. In other studies [12,13], NMR-based metabolomics and transcriptomics have been employed to investigate metabolic changes in intact liver tissue, tissue extracts and plasma from mice dosed with APAP. Results indicated that APAP can disrupt energy metabolism including glycolysis and lipid metabolism. Recently, Chen et al. [16] used LC/MS-based metabolomics and a knockout-gene mouse model and found that APAP administration disrupts fatty acid  $\beta$ -oxidation. The above mentioned studies focused on understanding mechanisms or discovering potential biomarkers related to APAP-induced hepatotoxicity.

The present study employed an integrated transcriptomics and multi-analytical platforms-based metabolomics approach in order to comprehensively understand the metabolome temporal changes induced by APAP administration. Four analytical platforms, including NMR, LC/MS open profiling, LC/MS/MS broad metabolite analysis and GC/MS, were utilized to examine metabolic changes in blood and urine from rats dosed with APAP. The multiple metabolomics profiling technologies in biofluids provided comprehensive insights into the metabolome changes and their corresponding pathways are altered by APAP, while the tissue transcriptomics data supported the pathway analysis. Novel liver injury metabolite biomarkers were also discovered through the correlation analysis between metabolites and hepatic necrosis scores and blood ALT levels. These injury and recovery biomarkers were involved in energy metabolism ( $\alpha$ -ketoglutarate and medium chain dicarboxylic acids), urea cycle metabolism and bile acid metabolism [17].

## Materials and Methods

### Chemicals

Optima LC/MS grade acetonitrile and water were purchased from

Fisher (Pittsburgh, PA). Formic acid, leucine-enkephalin, imidazole, pentadecafluorooctanoic acid, L-tryptophan and all the MS standards were obtained from Sigma-Aldrich (St. Louis, MO). NMR solvents, 1,1-difluoro-1-trimethylsilyl methyl phosphonic acid (DFTMP) was obtained from Bridge Organics, (Vicksburg, MI) and 2,2-dimethyl-2-silapentane-5-sulfonic acid (DSS-d6) in deuterium oxide ( $D_2O$ ) was obtained from Chenomx (Edmonton, Alberta, Canada). Stable isotope labeled palmitoylcarnitine and myristoylcarnitine were purchased from Cambridge Isotope Laboratories (Andover, MA). The human serum standard reference material (SRM 909b) was obtained from the National Institute of Standards and Technology (NIST) (Gaithersburg, MD).

### Animal care and treatment

Fifty-two 10-13 week old male Sprague-Dawley rats (250-350 g) were obtained from the FDA National Center for Toxicological Research (NCTR) breeding colony. Animal rooms were maintained at 19-23°C, 40-70% relative humidity, with a 12 h dark/12 h light cycle. Animals accessed feed and water *ad libitum*. Experiments were conducted in accordance to the National Institutes of Health (NIH) guidelines and reviewed and approved by the Testing Facility Institutional Animal Care and Use Committee (IACUC). Group of 16 rats were orally gavaged with a single dose of 0.5% methylcellulose (MC, vehicle control), group of 10 rats were dosed with 100 mg APAP/kg body weight and group of 26 rats were dosed with 1250 mg APAP/kg body weight. The dose levels were selected based on the results from a range-finding study. In the range finding study, 1000 mg/kg caused minimal clinical chemistry changes and mild to moderate histopathology changes (e.g., glycogen depletion and single cell necrosis in the centrilobular regions of the liver). At 1500 mg/kg, 2 of 4 animals died after dosing and prior to necropsy. Therefore, the high dose of 1250 mg/kg was chosen to induce mild to moderate adverse effects. The low dose of 100 mg/kg was chosen because this dose is similar to the maximum human dose of APAP (1000 mg/day) when scaled on a body surface area basis (590 mg/m<sup>2</sup> for rat vs. 570 mg/m<sup>2</sup> for a 65 kg human). APAP (100 mg/kg) was expected to produce no adverse effects.

Urine samples were collected in polypropylene conical tubes embedded in a frozen ice/salt mixture to rapidly freeze the urine and avoid bacterial growth. Urine was collected from 0-6 h, 6-24 h, 48-72 h and 144-168 h post-dosing. Four rats from the control group were sacrificed at 6 h, 24 h, 72 h and 168 h, while 5 animals from the low dose group were sacrificed at 6 h and 24 h. Five animals from the high dose group were sacrificed at 6 h and 7 animals were sacrificed at 24 h, 72 h and 168 h. Terminal blood was collected from the caudal vena cava into serum separator tubes and tubes with EDTA for clinical chemistry analysis and metabolomics analysis, respectively. The blood samples were centrifuged (10°C, 2000×g, 10 min) and the serum (for open profiling by LC/MS and NMR at the National Center for Toxicological Research) and plasma (for broad profiling by LC/MS/MS and GC/MS at Metanomics Health) were removed and frozen at -80°C until analysis. Analytes evaluated using classical clinical chemistry approaches included creatinine, blood urea nitrogen (BUN), ALT, AST, alkaline phosphatase (ALP), GGT, triglyceride, cholesterol, glucose, sodium, potassium, calcium, phosphorus, albumin, total protein, and total bilirubin. At the time of sacrifice sections of liver were placed in RNA later solution (QIAGEN, Valencia, CA, USA) for gene expression analysis. Additional sections of liver were fixed in 10% neutral buffered formalin, routinely processed and embedded in paraffin, sectioned at 5  $\mu$ m, stained with hematoxylin and eosin, and examined by light microscopy. Lesions were scored on a 4-point scale (minimal, mild,

moderate, and marked) by a board-certified Veterinary Pathologist.

### Quality control in metabolomics

A quality control (QC) sample comprised of 40 or 26 common chemicals for LC/MS and NMR open profiling, respectively, was evaluated. Pooled serum or pooled urine and the QC sample were run every 10 serum or urine sample runs by UPLC/QToF-MS and UPLC/TQ-MS to monitor the analytical equipment variability. The QC and pooled urine samples were run alternately every five samples in the NMR analyses. For serum, the QC sample, pooled serum sample and NIST serum standard (SRM-909b) were analyzed alternately every four samples in NMR analyses.

### Open metabolic profiling by UPLC/QToF-MS

For serum sample preparation, 100  $\mu$ L serum was mixed with 300  $\mu$ L methanol and incubated at  $-20^{\circ}\text{C}$  for 20 min and then centrifuged at 13,000 rpm for 12 min at  $4^{\circ}\text{C}$  to precipitate proteins. The supernatant (300  $\mu$ L) was then transferred into clean tubes and evaporated to dryness using a SpeedVac concentrator (Thermo Scientific, Waltham, MA). The samples were reconstituted in 200  $\mu$ L 95:5 water/acetonitrile, vortexed for 2 min and kept at  $4^{\circ}\text{C}$  for 20 min. The resulting solution was then centrifuged at 13,000 rpm for 12 min at  $4^{\circ}\text{C}$ . The supernatant was transferred to autosampler vials for LC/MS analysis.

A 3  $\mu$ L aliquot of serum supernatant after methanol precipitation or 5  $\mu$ L of dilute urine (1:10 volume with water) 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 (for serum) or C18 column (for urine) with a dimension of 2.1 mm $\times$ 10 cm and 1.7  $\mu$ m particle size. The total running time for each serum and urine sample analysis was 21 and 13 min, respectively. The column was held at  $40^{\circ}\text{C}$ . The UPLC mobile phase consisted of 0.1% formic acid in water (solution A) and 0.1% formic acid in acetonitrile (solution B). While maintaining a constant flow rate of 0.4 mL/min, the serum metabolites were eluted using linear gradients of 2–80% solution B from 0 to 15 min and 80–98% solution B from 15 to 17 min. The final gradient composition was held constant for 2 min followed by a return to 2% solution B at 19.1 min. The gradient for urine analysis was described previously [14,15]. In brief, the metabolites were eluted at a constant flow rate of 0.4 mL/min using the following gradients of 0–30% B from 0 to 6 min, 30–50% B from 6 to 9 min, and 50–95% B from 9 to 11 min. The final gradient composition was held constant for 1 min followed by a return to 100% A at 12.1 min.

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 [18,19]. The capillary voltage was 3.2 kV for positive ionization mode and 2.4 kV for negative ionization mode, while the cone voltage was 40 V in both modes. MSE 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 and 12 min for urine 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 [18,19]. The aligned data from MarkerLynx analysis for QToF-MS data was filtered using

the pooled QC samples based on the followed criteria: i) ions with % RSD less than 30% in the pooled QC samples were included; ii) ions present in  $\geq 70\%$  of QC samples were included. The resulting dataset was analyzed by unsupervised PCA and supervised partial least squares discriminant analysis (PLS-DA).

### MxP™ broad profiling of plasma by LC/MS/MS and GC/MS

MxP™ broad profiling based on GC/MS and LC/MS/MS techniques was used as described previously [20,21]. In brief, polar and non-polar metabolites were separated for both GC/MS and LC/MS/MS analysis by adding water and a mixture of ethanol and dichloromethane (1:2, v:v) to the plasma after protein precipitation. For GC/MS analysis, the non-polar fraction was treated with methanol under acidic conditions to yield the fatty acid methyl esters derived from both free fatty acids and hydrolyzed complex lipids. The non-polar and polar fractions were further derivatized with O-methyl-hydroxylamine hydrochloride and pyridine to convert oxo-groups to O-methyl-oximes and subsequently with a silylating agent before analysis [22]. For LC/MS/MS analysis, both fractions were reconstituted in water for the polar fraction or in THF:MeOH:Water (5:4:1, v:v:v) for the non-polar fraction. The sample to solvent ratio was 1:8. HPLC was performed by gradient elution using methanol/water/formic acid on reversed phase separation columns. Mass spectrometric detection technology was applied which allows target and high sensitivity MRM (multiple reaction monitoring) profiling in parallel to a full screen analysis (patent WO2003073464). For GC/MS and LC/MS/MS profiling, data were normalized to the median of reference samples which were derived from a pool formed from aliquots of all samples to account for inter- and intra-instrumental variation.

### Open metabolic profiling by NMR

For serum sample preparation, 20  $\mu$ L 4,4-dimethyl-4-silapentane-1-sulfonic acid (DSS) in  $\text{D}_2\text{O}$ , 5  $\mu$ L 55 mM difluoro trimethylsilanyl phosphonic acid (DFTMP) in sodium phosphate (NaPi) buffer, and 75  $\mu$ L NaPi buffer (pH=7.4) were added to 100  $\mu$ L serum. Urine samples were prepared by combining 400  $\mu$ L of urine, 185  $\mu$ L of sodium phosphate buffer (pH=7.4), 60  $\mu$ L DSS in  $\text{D}_2\text{O}$  and 55 mM DFTMP in NaPi buffer. Proton ( $^1\text{H}$ ) NMR spectra were acquired on a Bruker Avance spectrometer operating at 600.133 MHz for proton and equipped with a triple resonance cryoprobe. Water suppression was achieved through application of the Bruker “noesyprsat” pulse sequence, which irradiates the water resonance during a delay time ( $d_1=2.5$  sec) and a mixing time ( $d_8=100$  msec). For each sample, 32 scans were collected into 65,536 data points. A spectral width of 9615.39 Hz was utilized with an acquisition time of 3.41 seconds.

NMR spectra were processed using ACD/Labs 1D NMR Manager (Toronto, Canada) as described previously [14,15]. The raw FIDs were zero filled to 131,072 points, multiplied by a 0.3 Hz exponential function and Fourier transformed. The transformed spectra were then phased using the “simple method” and baseline corrected using the “Sp Averaging” method with a box half width of 61 points and a noise factor of 3. All spectra were auto referenced to the DSS peak at 0.0 ppm. Metabolite identification within an individual spectrum was accomplished using the Chenomx NMR Suite (Chenomx, Edmonton, Alberta, Canada), which has a database of >250 compounds. Spectra were grouped and normalized by total intensity. Additionally, the spectra were normalized by total urine volume collected to give total excreted.

## Targeted bile acids and acylcarnitines analysis in serum using a UPLC/TQ-MS

A Waters Xevo TQ-MS (Waters, Milford, MA) operated in positive mode was utilized for absolute quantification of bile acids and acylcarnitines in serum using stable isotope standards. MRMs for bile acids and acylcarnitines were optimized by direct infusion of standards.

## Gene expression analysis of liver tissue

Liver samples collected at 6 h, 24 h, 72 h and 168 h post-dosing were subjected to microarray analysis using the Whole Rat Genome Microarray (4x44K) (Agilent Technologies, Santa Clara, CA). Microarray intensity data were uploaded to the ArrayTrack™ (<http://www.fda.gov/ArrayTrack>) database and normalized using 75<sup>th</sup>-percentile scaling without background subtraction.

## Statistics

In the blood chemistry, transcriptome and metabolome analyses, the values in the treated group at the high dose and time point were compared to the respective control group. The data were analyzed by an *F*-test to evaluate the homogeneity of variance. If the variance was homogeneous, a Student's *t*-test was applied. If the variance was heterogeneous, an Aspin-Welch's *t*-test was performed. In every statistical analysis, a value of *p*<0.05 was considered significant. For the metabolome and transcriptome data from control and high dose groups, one-way ANOVA versus time was completed using ArrayTrack™ (<http://www.fda.gov/ArrayTrack>). The control group and dosed groups at each time point were first normalized using the Reference Average Comparison method prior to ANOVA analysis. Hepatocyte necrosis were scored in that pathology reports as 1 for

minimal necrosis; 2 for mild necrosis; 3 for moderate; 4 for marked necrosis. In order to discover biomarkers that can potentially serve as new injury biomarkers for APAP-induced hepatotoxicity, all of the metabolites identified from the various instrumental platforms were subjected to Pearson's rank correlation to the hepatocyte necrosis scores and log<sub>2</sub>ALT.

## Results

A range-finding study was performed to determine a high dose (HD) that would produce liver injury, including hepatic necrosis, at 24 h but that did not cause abundant morbidity. A low dose (LD) was chosen that caused no overt changes. Table 1 reports the histopathology observations and average values for selected clinical chemistry parameters for control, 100 (LD) and 1250 mg (HD) APAP/kg treatment. Hepatocyte glycogen depletion was observed in all seven rats in the HD group 24 h after dosing. The severity ranged from minimal to moderate. Additionally, there were regenerative responses by 72 h with a minimal increase in hepatocellular mitotic figures in two of seven rats in the HD group. There was heterogeneity seen in the HD group at 24 h; three animals showed no evidence of hepatic necrosis, one animal exhibited minimal hepatic necrosis, one with mild injury and two with moderate injury. At 72 h in the HD group, three rats had minimal injury and one with moderate injury while the remaining three animals had no injury. In contrast, none of the animals in the control or LD groups exhibited hepatic necrosis or regenerative changes at any time point. Centrilobular fibrosis was present in some of the HD group (four out of seven rats at 72 h and two out of seven rats at 168 h). This finding was not noted for rats treated with low dose APAP or vehicle control at any time point. No vacuolization was observed in any of the animals in this study. As expected, ALT and

Time post single dosing	6 hrs			24 hrs		72 hrs		168 hrs		
Dose (mg/kg)	0	100	1250	0	100	1250	0	1250	0	1250
Number of animals	4	5	5	4	5	7	4	7	4	7
<b>Histopathology (Liver)</b>										
Necrosis, centrilobular	-	-	-	-	-	+ (1/7) ++ (1/7) +++ (2/7)	-	+ (3/7) +++ (1/7)	-	-
Fibrosis, centrilobular	-	-	-	-	-	-	-	+ (3/7) ++ (1/7)	-	+ (2/7)
Mitosis, centrilobular	-	-	-	-	-	-	-	+ (2/7)	-	-
Hepatocyte glycogen depletion	-	-	-	-	-	+ (3/7) ++ (2/7) ++++ (2/7)	-	-	-	-
<b>Blood chemistry</b>										
ALT (U/L)	32.5±4.0	36.4±4.0	36.8±1.0	67.5±3.0	55.4±5.0**	459.9±673.5	53.8±8.5	57.4±5.4	58.0±5.1	59.0±8.7
AST (U/L)	110.3±17.0	106.2±24.9	92.8±8.4	98.1±14.1	102.8±19.5	3007±5166	87.5±9.3	106.4±34.7	106.8±40.4	103.3±22.8
ALP (U/L)	195.3±42.6	211.4±77.7	184.3±24.2	353.0±89.2	309.6±47.5	365.4±63.2	285.8±30.1	305.4±63.7	319.0±44.3	306.4±64.3
GGT (U/L)	5.5±0.6	5.6±1.7	6.5±0.6*	7.3±1.3	6.4±1.1	6.4±1.5	6.8±1.0	7.7±2.1	6.3±1.3	7.0±1.2
TBILI (mg/dL)	0.40±0.40	0.28±0.15	0.20±0.08	0.28±0.05	0.32±0.08	0.38±0.16	0.20±0.11	0.28±0.17	0.25±0.10	0.18±0.07
BUN (mg/dL)	17.0±1.4	16.6±1.8	11.5±1.7**	10.0±0.8	12.8±3.1	12.3±4.7	11.8±1.7	16.6±7.0	16.0±1.8	11.4±4.0
CRE (mg/dL)	0.38±0.13	0.36±0.05	0.35±0.06	0.38±0.10	0.38±0.08	0.40±0.12	0.43±0.05	0.48±0.18	0.43±0.10	0.38±0.04
CHOL (mg/dL)	63.8±8.7	61.4±13.6	78.5±15.5	61.3±10.8	60.6±5.4	92.0±19.6*	77.3±11.4	80.6±11.1	69.5±6.6	67.7±10.6
TRI (mg/dL)	70.5±50.2	77.8±24.2	98.5±30.2	170.0±38.7	156.2±56.7	179.6±106.0	74.3±20.0	96.9±51.9	115.0±59.1	101.6±23.6
GLU (mg/dL)	251.5±79.5	186.6±15.9	165.8±21.8	171.5±15.1	175.2±18.4	164.3±26.5	193.0±39.5	170.3±4.8	186.0±26.7	170.4±13.2

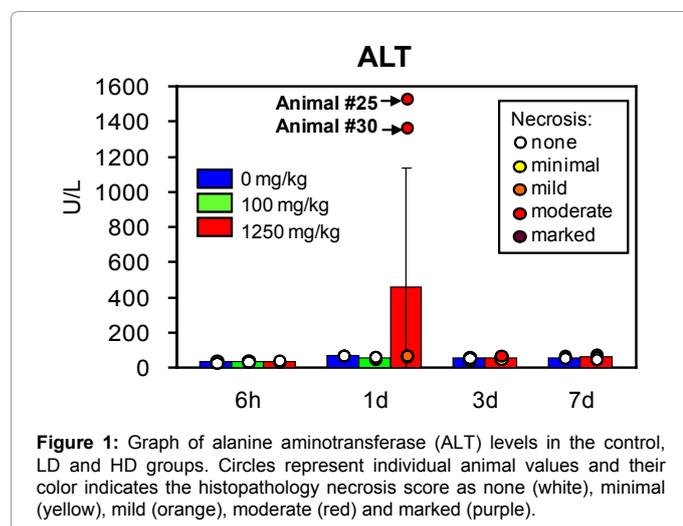
Table 1: Blood chemistry and histopathology.

Values for blood chemistry represented as mean ± SD. \* and \*\* represent *p*<0.05 and *p*<0.01, respectively. Histopathological lesions were qualified as: +, minimal; ++, mild; +++, moderate; +++++, marked

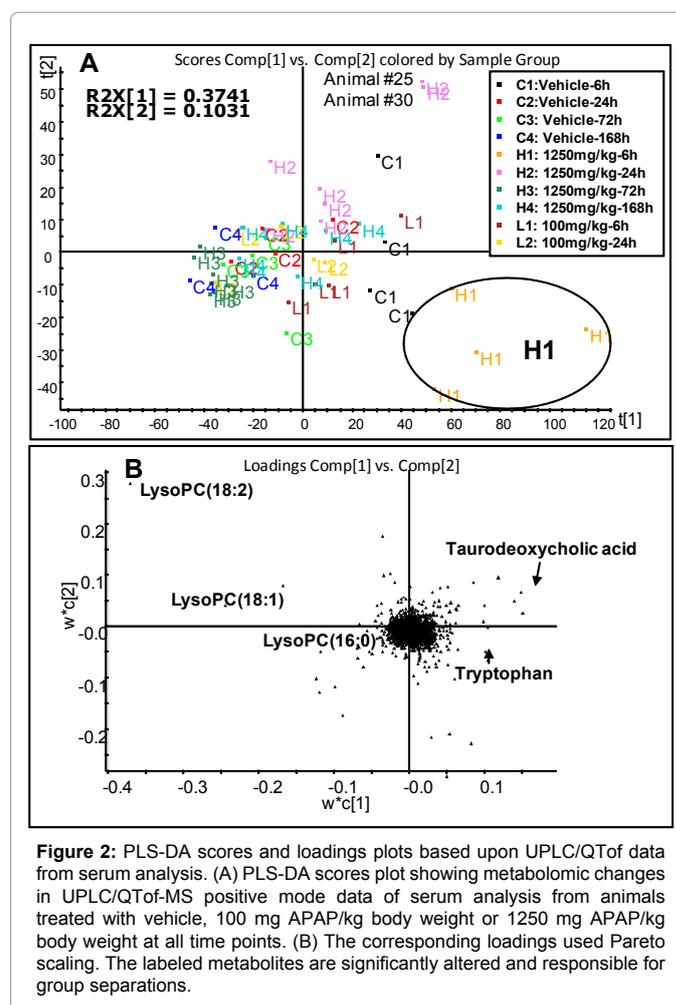
AST, which are markers of liver damage, were elevated (but not to a statistically significant extent) compared to control values at 24 h for the HD group. Glucose tended to decrease (though not significantly) at all time points, which is consistent with energy metabolism being disrupted in the HD group. Among the clinical chemistry data, only gamma-glutamyl transferase (GGT) and cholesterol were significantly increased at 6 h and 24 h post-dosing in the HD group, respectively. The lack of statistically significant changes in many of these parameters is due likely to the variability in response to the oral administration of APAP in the rat model as reported by other investigators [23]. As an example, figure 1 displays ALT levels in the control, LD and HD groups. Animals #25 and #30 at 24 h had high levels of ALT (>1000 units/liter) with moderate hepatic necrosis.

Four analytical platforms, including UPLC/QToF-MS, LC/MS/MS, GC/MS and NMR, were employed for the analysis of 52 blood samples, and UPLC/QToF-MS- and NMR-based metabolomics were conducted on 50 urine samples from the three treatment groups (control: (C), LD, and HD). Figure 2A shows the scores plot of the partial least squares discriminant analysis (PLS-DA) model generated from the UPLC/QToF-MS analysis of serum in the positive ionization mode. The HD group at 6 h after dosing (H1) is clearly separated from other treatment groups and time points and is consistent with initiation of events prior to clinical and histopathology changes. Five animals in the HD group at 24 h (H2) were clustered closely to the control group and two animals had minimal, mild or no hepatic necrosis with normal ALT levels. Since there was only one time point for each animal, it was not possible to determine whether the five HD animals from the 24 h collection had liver injury earlier and recovered at his time point or injury occurred later than 24 h. The remaining two animals (animal #25 and #30) are outliers from the H2 cluster, which showed moderate hepatic necrosis (scored as 3) and high levels of ALT. Figure 2B displays the loadings plot from PLS-DA indicating the weight of an ion feature, which is used to interpret the group separations in the scores plot. Some identified ions (labeled in Figure 2B), which are significantly responsible for the group separations are LysoPCs, amino acids and bile acids.

For urine open profiling, samples collected at 24 h, 72 h and 168 h were analyzed by QToF-MS and NMR. Urine samples at 6 h were excluded because of the abbreviated sample collection time interval versus those from 24 h and 72 h urine collection, which resulted in



**Figure 1:** Graph of alanine aminotransferase (ALT) levels in the control, LD and HD groups. Circles represent individual animal values and their color indicates the histopathology necrosis score as none (white), minimal (yellow), mild (orange), moderate (red) and marked (purple).



**Figure 2:** PLS-DA scores and loadings plots based upon UPLC/QToF data from serum analysis. (A) PLS-DA scores plot showing metabolomic changes in UPLC/QToF-MS positive mode data of serum analysis from animals treated with vehicle, 100 mg APAP/kg body weight or 1250 mg APAP/kg body weight at all time points. (B) The corresponding loadings used Pareto scaling. The labeled metabolites are significantly altered and responsible for group separations.

major differences in the metabolite profiles not only related to APAP insults. PCA scores plots of QToF-MS and NMR data (Figures S1 and S2) from urine analysis showed that H2 was located furthest away from the LD and control groups, while other dosed groups at different time points were not completely separated from the controls. Metabolites related to energy metabolism, oxidative stress and other pathways were significantly altered in urine and were responsible for the group separations.

### Urinary levels of APAP drug metabolites

Table 2 reports the total urinary levels of APAP and APAP metabolites detected by NMR at 24, 72 and 168 h. APAP-sulfate (APAP-Sul) and APAP glucuronide (APAP-Glu) conjugates were the primary forms of APAP excreted at all time points and doses. The levels of the N-acetyl cysteine metabolite of APAP (APAP-NAC) were almost undetectable in the urine from rats dosed with 100 mg/kg at 24 hours, while APAP-NAC was 94.5 mM at 24 h and 5.2 mM at 72 hours in the high dose group.

### Metabolites and gene expression analysis

In total, 223 blood compounds and 47 urinary compounds were detected and semi-quantified; 16 were detected in both blood and urine (Table S1). Over 38% of the 270 detected compounds (including blood and urine) were fatty acids, lipids or fatty acid  $\beta$ -oxidation-related

Time post single dosing	24 hrs		72 hrs	168 hrs
Dose (mg/kg)	100	1250	1250	1250
APAP	0.51	130.43	3.92	ND
APAP-Glu	0.57	217.73	12.97	ND
APAP-NAC	0.39	94.47	5.16	ND
APAP-Sul	11.36	350.96	73.51	ND

ND = Not detected

**Table 2:** Total excreted APAP and APAP metabolites (mM) detected in urine by NMR analyses.

metabolites. The remaining compounds included 48 energy metabolism related metabolites (including those from the glycogenolysis/glycolysis, tricarboxylic acid (TCA) cycle and ketone metabolism pathways), and 36 amino acids and their metabolites. Twenty-seven of the metabolites were involved in the bile acid pathway. Since the majority of the significantly changed metabolites were fatty acid-related compounds, triple quadrupole (TQ)-MS was utilized for absolute quantification of the acylcarnitines and bile acids (Table S1) in blood in order to study the disturbance in the fatty acid  $\beta$ -oxidation pathway and in the bile acid metabolism pathway. TQ-MS data showed that bile acids were increased at 24 h in the HD group and acylcarnitines were increased at both 6 h and 24 h post-dosing in the HD groups (Table S1).

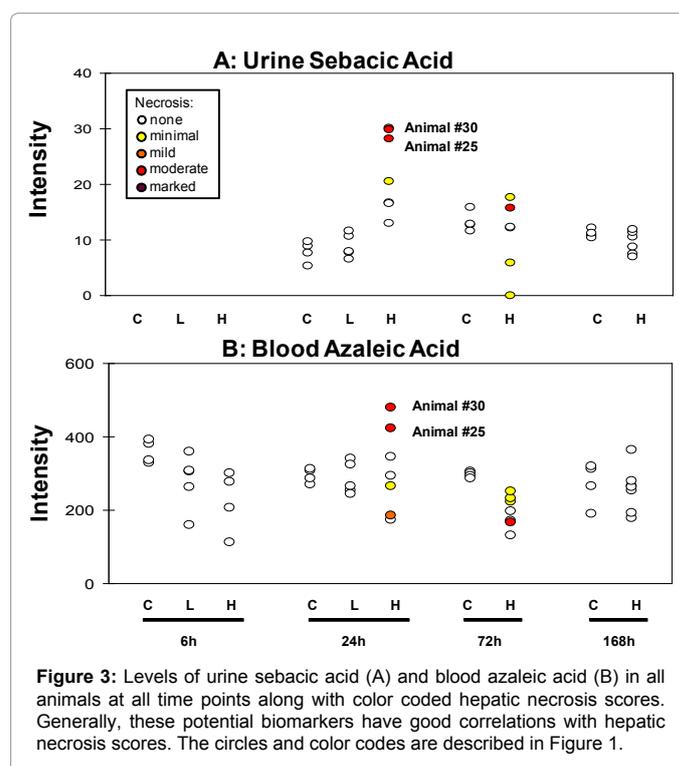
### Correlation of metabolites to hepatic necrosis and $\log_2$ ALT

In order to discover new injury biomarkers, 270 detected metabolites were correlated to hepatic necrosis scores and  $\log_2$ ALT using Pearson's rank correlation analysis. Table 3 lists the top 10 metabolites with strong correlations to  $\log_2$ ALT and hepatic necrosis for all animals at all time points and for HD animals at all time points to discover injury biomarkers. Discovery of injury biomarkers was primarily based on the correlations to  $\log_2$ ALT and to hepatic necrosis and evaluating whether the metabolites levels were altered significantly in animals #25 and #30 while remaining at normal levels for the rest of animals (Table 3). This process revealed that urea cycle metabolites (arginine and 2-oxoarginine), energy metabolites ( $\alpha$ -ketoglutarate, and medium chain dicarboxylic acids: decenedioic acid, sebatic acid, azelaic acid, and undecanedioic acid) and bile acids (taurocholic acid, taurodeoxycholic acid and sulfolithocholyglycine) were potential injury biomarkers. Generally, the levels of these potential biomarkers was significantly altered with moderate hepatic necrosis scores and increased ALT levels at 24 h, and returned to normal levels when the necrosis injury and ALT levels recovered at 72 and 168 h.

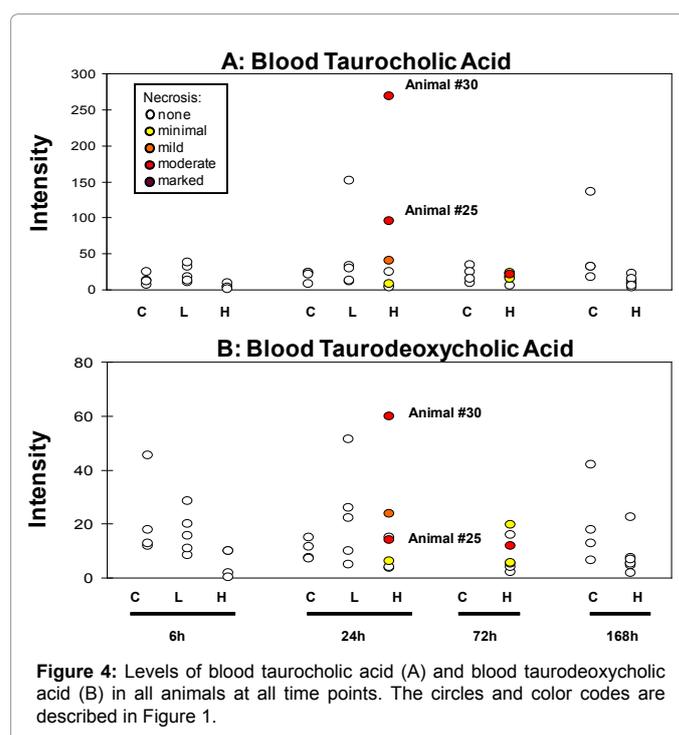
Figure 3 shows urinary levels of sebatic acid and blood levels of azelaic acid with each animal's corresponding hepatic necrosis score. The highest intensity levels of sebatic acid were observed at 24 h in animals #25 (~28 relative intensity level) and #30 (~29 relative intensity level) that were about 3.5 fold higher than observed in the control group at 24 h. The correlation coefficients of sebatic acid in all HD to hepatic necrosis and  $\log_2$ ALT were  $r=0.70$  and  $0.77$ , respectively. The correlation coefficients of azelaic acid at all high doses to hepatic necrosis and  $\log_2$ ALT were  $r=0.42$  and  $0.81$ , respectively. Generally, the levels of these medium chain dicarboxylic acids increased along with hepatic necrosis scores and ALT levels at 24 h and returned to control levels at 72 h and 168 h.

Figure 4 shows individual animals' blood levels of taurocholic acid, taurodeoxycholic acid and their associated hepatic necrosis scores. The highest intensity levels of taurocholic acid were observed at 24 h in animals #25 (~95 relative intensity level) and #30 (>250 relative intensity level). The intensity levels for these animals were

approximately 4 to 10 times of the levels observed in the control group at the 24 h time point. The correlation coefficients of taurocholic acid at all HD to hepatic necrosis and to  $\log_2$ ALT were  $r=0.60$  and  $0.86$ , respectively. The correlation coefficients of taurodeoxycholic acid at all HD to hepatic necrosis and  $\log_2$ ALT was  $r=0.60$  and  $0.64$ , respectively. The levels of these bile acids and others (Table S1) increased along with



**Figure 3:** Levels of urine sebatic acid (A) and blood azelaic acid (B) in all animals at all time points along with color coded hepatic necrosis scores. Generally, these potential biomarkers have good correlations with hepatic necrosis scores. The circles and color codes are described in Figure 1.



**Figure 4:** Levels of blood taurocholic acid (A) and blood taurodeoxycholic acid (B) in all animals at all time points. The circles and color codes are described in Figure 1.

hepatic necrosis scores and ALT levels at 24 h and returned to control levels at 72 h and 168 h.

Figure 5 shows the individual rat's blood levels of arginine and  $\alpha$ -ketoglutarate and their associated hepatic necrosis scores. The correlation coefficients of arginine to hepatic necrosis and to  $\log_2$ ALT at all high doses were  $r=-0.47$  and  $-0.66$ , respectively. The lowest levels of arginine were observed in animals #25 (0.04 relative intensity level) and #30 (0.04 relative intensity level) which is about a factor of 25 lower than was observed in the control group at 24 h. Overall, the levels of arginine decreased along with hepatic necrosis scores and ALT levels at 24 h and returned to control levels at 72 h and 168 h. The correlation coefficients of  $\alpha$ -ketoglutarate to hepatic necrosis and  $\log_2$ ALT at all high doses were  $r=0.72$  and  $0.96$ , respectively. The highest levels of  $\alpha$ -ketoglutarate was observed in animals #25 (3.2 relative intensity ratio levels to internal standard) and #30 (3.8 relative intensity ratio level to internal standard) at 24 h. At 72 h, the highest level of  $\alpha$ -ketoglutarate was observed in animal #39 that had moderate necrosis levels and normal ALT levels. Generally, the levels of  $\alpha$ -ketoglutarate increased along with hepatic necrosis scores and ALT levels at 24 h and returned to control levels at 72 h and 168 h.

### Bile acids synthesis and secretion pathways

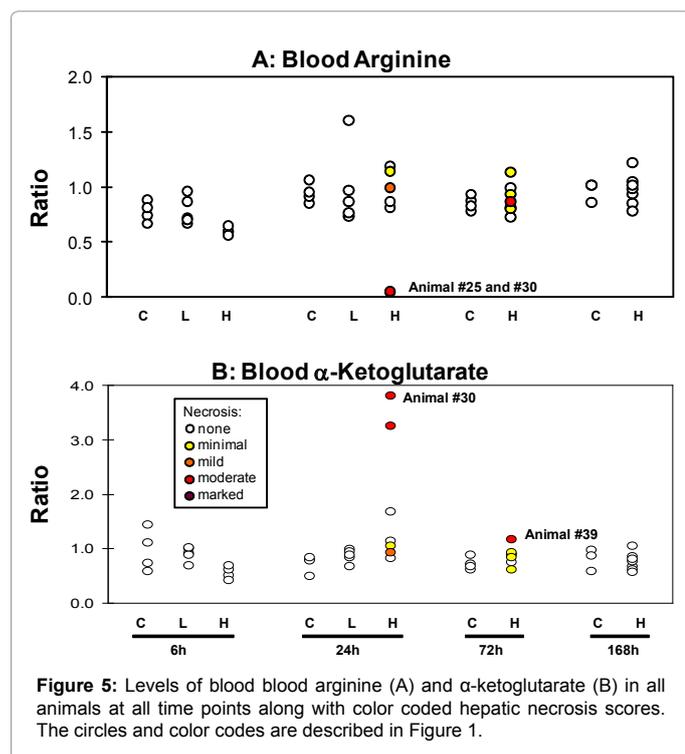
Since blood levels of bile acids had strong correlation to hepatic necrosis and  $\log_2$ ALT, perturbations in the bile acid pathway induced by APAP were further investigated with hepatic transcriptomics. Figure 6 shows the transcripts and bile acids involved in the bile acid metabolism pathway. Bile acids were measured with three different MS methods (TQ, LC/MS open profiling and LC/MS/MS broad profiling) with generally very good agreement in the overall bile acid changes at most time points. In general, the bile acids tended to be decreased at 6 h and were increased at 24 h in the HD group. Cholic acid was decreased at 6 h, significantly increased at 24 h and increased at 72 h for all three analytical techniques. Deoxycholic acid was decreased at 6 h,

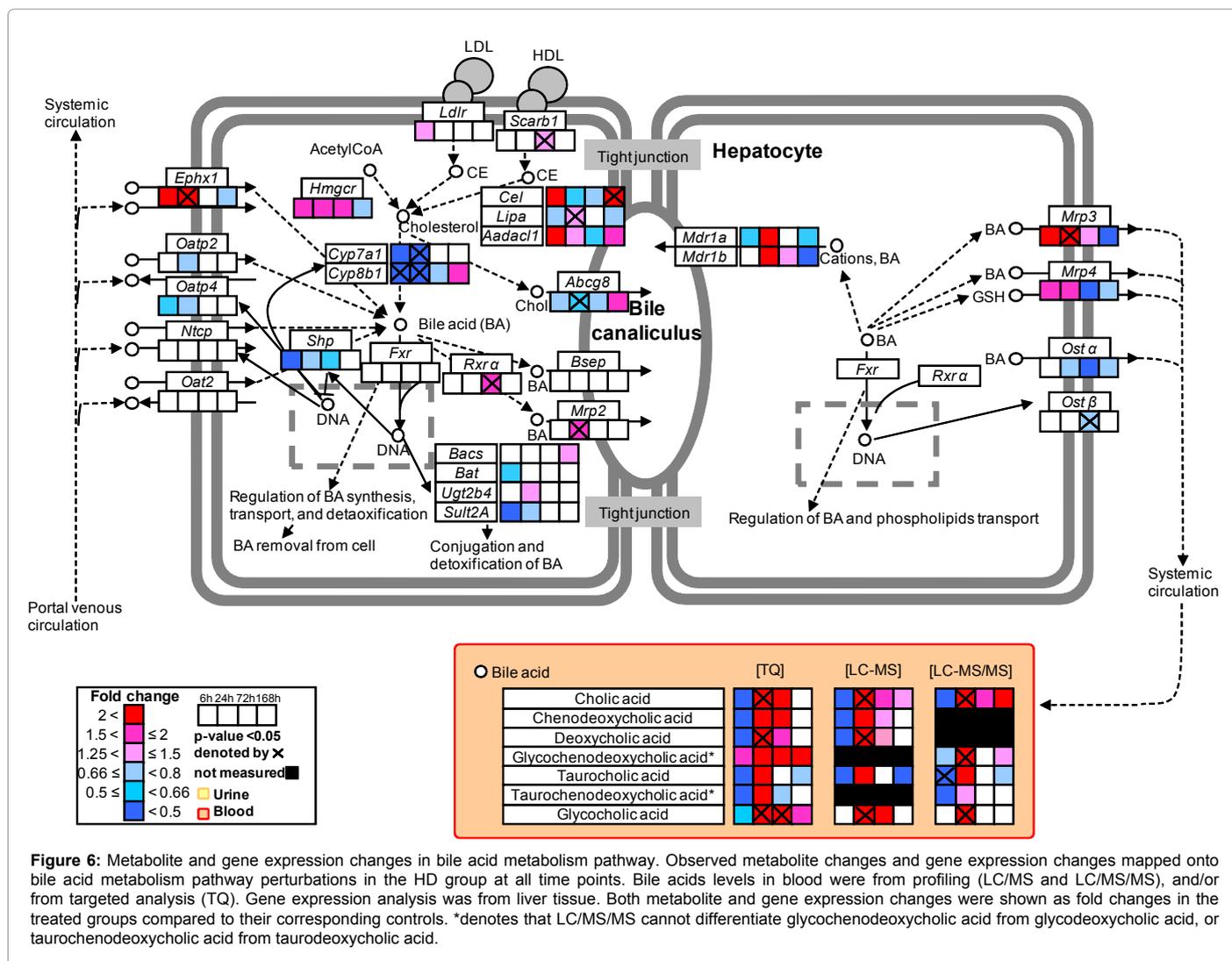
significantly increased at 24 h and increased at 72 h in both TQ and LC/MS analyses. The taurine conjugated bile acids were not significantly increased at any time point. Glycocholic acid was significantly increased at 24 h in all three analytical techniques but the TQ approach showed a slight decrease at 6 h and significant increase at 72 h while LC/MS only showed a non-significant increase at 72 h in serum and LC/MS/MS did not detect any changes at any other time points in plasma. The levels of glycochenodeoxycholic acid/glycodeoxycholic acid had the largest variations between TQ and LC/MS/MS and this may be the result of the LC/MS/MS method detecting multiple bile acids at the same retention time while LC/TQ was able to separate these bile acids. The expression of bile acid synthesis-related genes (Cyp7a1 and Cyp8b1) were down-regulated and bile acid transporter genes (Abcc2, Abcc3 and Abcc4, also called Mrp2, Mrp3 and Mrp4, respectively) were up-regulated at 6 and/or 24 h at HD although the changes in Mrp4 did not attain statistical significance, while a cholesterol transporter gene Abcg8 was down-regulated at 24 h (Table S2).

### Discussion

The integrated transcriptomics and metabolomics data demonstrated the time- and dose-dependent effects of APAP on genes in liver tissue and metabolites in blood and urine. Histological examination of the liver and classical clinical chemistry evaluation were performed to facilitate the discovery of liver injury biomarkers, and further to understand the metabolome alterations induced by APAP at the molecular level. Novel injury biomarkers were discovered based on the Pearson's rank correlation analysis between metabolite levels in blood and urine and hepatic necrosis and  $\log_2$ ALT in HD groups as well correlation analysis at all doses and all time points. Strong correlations discovered that energy metabolites (dicarboxylic acids and  $\alpha$ -ketoglutarate), bile acids and arginine metabolites could be good injury and recovery indicators (Table 3).

It has been reported that excess APAP can covalently bind to proteins which will lead to mitochondrial damage and global energy failure [10,24,25] through inhibition of fatty acid  $\beta$ -oxidation [12,16]. The energy metabolism pathway includes fatty acid  $\beta$ -oxidation and TCA cycle. Elevations in serum fatty acids (such as palmitic acid, table S1) and acylcarnitines (such as palmitoylcarnitine) indicated that fatty acid  $\beta$ -oxidation was inhibited by APAP before classical liver injury markers like hepatic necrosis and ALT levels increased. Blood levels of acylcarnitines increased by several fold during the injury initiation stage at 6 h; however, the levels returned to normal at 24 h and 3d during injury progression. As such, acylcarnitines could be early biomarkers to predict liver injury but not good indicators for injury progression. However, medium chain dicarboxylic acids, decenedioic acid and sebacic acid in urine, and azelaic acid and undecanedioic acid in blood, could be potential liver injury biomarkers since their levels had good correlations at 24 h ( $r>0.52$ ,  $p<0.05$ ) to  $\log_2$ ALT and hepatic necrosis (Figure 3). Generally, levels of these dicarboxylic acids were increased in the most afflicted animals (#25 and #30), while they were near normal levels for the rest. Increases in medium chain dicarboxylic acids could result from ketosis or from reduced fatty acid  $\beta$ -oxidation capability [26]. Further, the energy loss from fatty acid  $\beta$ -oxidation inhibition would stimulate a shift of energy metabolism to glycogenolysis/glycolysis to maintain energy homeostasis. Energy loss was supported by the histopathology and clinical chemistry data that showed glycogen depletion in the liver tissue of all seven animals and decreases in glucose in serum from the HD group at 24 h. These results are consistent with a shift of energy metabolism to glycolysis in order to compensate for the loss of ATP due to fatty acid  $\beta$ -oxidation





**Figure 6:** Metabolite and gene expression changes in bile acid metabolism pathway. Observed metabolite changes and gene expression changes mapped onto bile acid metabolism pathway perturbations in the HD group at all time points. Bile acids levels in blood were from profiling (LC/MS and LC/MS/MS), and/or from targeted analysis (TQ). Gene expression analysis was from liver tissue. Both metabolite and gene expression changes were shown as fold changes in the treated groups compared to their corresponding controls. \*denotes that LC/MS/MS cannot differentiate glycochenodeoxycholic acid from glycodeoxycholic acid, or taurochenodeoxycholic acid from taurodeoxycholic acid.

Metabolite	Platform	Biofluid	$\log_2$ ALT		Hepatic necrosis	
			All Doses animals	HD ALL	All Doses animals	HD ALL
Arginine	LC-MS/MS	Blood	-0.50	-0.66	-0.41	-0.47
2-Oxoarginine	LC_POS	Urine	-0.58	-0.59	-0.60	-0.54
Decenedioic acid	LC_NEG	Urine	0.64	0.62	0.59	0.51
Taurodeoxycholic acid	LC_NEG	Blood	0.30	0.64	0.27	0.60
Sulfolithocholylglycine	LC_NEG	Blood	0.65	0.72	0.54	0.57
Sebacic acid	LC_NEG	Urine	0.75	0.77	0.74	0.70
Azelaic acid	LC_POS	Blood	0.52	0.81	0.22	0.42
Undecanedioic acid	LC_POS	Blood	0.59	0.83	0.32	0.47
Taurocholic acid	LC-MS/MS	Blood	0.73	0.86	0.27	0.60
$\alpha$ -Ketoglutarate	GC-MS	Blood	0.88	0.96	0.70	0.72
$\log_2$ ALT	Clinic	Blood	1	1	0.72	0.73

**Table 3:** Metabolites with strong Pearson's correlations to  $\log_2$ ALT and hepatic necrosis scores. Pearson's rank correlations were based on the correlations between metabolites and  $\log_2$ ALT and hepatic necrosis scores at all dose levels (control, LD, HD) and metabolites at high dose (HD) from all time points to discover good injury biomarkers.

disruption. This was evidenced by increased levels of  $\alpha$ -ketoglutarate in animals #25 and #30 at 24 h and #39 at 72 h in blood in treated animals. Increases in urine levels of  $\alpha$ -ketoglutarate have been reported as a mitochondrial malfunction biomarker in transaldolase

deficiency patients [27]. Halmes et al. [28] also reported that enzyme activity of glutamate dehydrogenase, catalyzing the reversible oxidative deamination of glutamate to  $\alpha$ -ketoglutarate in the energy metabolism, is significantly decreased in mice 1 h post-dosing with

APAP. As such,  $\alpha$ -ketoglutarate levels can reflect energy fluctuation, which can be influenced by mitochondrial damage in hepatic cells as well as hepatocyte recovery. Indeed, blood  $\alpha$ -ketoglutarate had the strongest correlation to  $\log_2$ ALT and hepatic necrosis ( $r=0.88$  and  $0.70$ , respectively) based on Pearson's rank correlation analysis. Therefore, blood  $\alpha$ -ketoglutarate is related to energy metabolism and could be an injury biomarker but it probably would not be specific to the liver.

Changes in multiple bile acids were observed and with high correlations to hepatic necrosis as well as to  $\log_2$ ALT. Bile acid secretion is a marker of liver function and restoration of flow has been reported as a crucial step in the recovery process post liver transplantation [29]. Bile acids play important roles in lipid digestion and absorption in the small intestine as well as in regulating cholesterol homeostasis [30]. Bile acids are complex metabolic regulators and signaling factors for governing cholesterol catabolism as well as their own auto-regulation [31,32]. Bile acids are produced from cholesterol by cytochrome P450-mediated oxidation in the liver. As such, it is important to understand the underlying bile acid pathway and its impact on the biosystem at the molecular level. In this study, 29 genes and 7 bile acids related to bile acid metabolism were analyzed as shown in figure 6. At 6 h, one gene and one bile acid were significantly altered compared to control levels. At 24 h, 7 genes and 3 bile acids were significantly altered in the HD dose group. At 72 h, two genes and one bile acid were altered, while one gene and no bile acids were significantly changed at 168 h. Although only 3 bile acids were significantly increased at 24 h, all 7 bile acids were increased at this time point. Decreases in the expression of bile acid synthesis-related genes (*Cyp7a1* and *Cyp8b1*) and cholesterol transporter gene (*Abcg8*) and increases in the expression of bile acid transporter genes (*Mrp3* and *Mrp4*) at 6 h and/or 24 h post-dosing were detected (Figure 6). The decreases in *Abcg8* and increases in *Mrp3* and *Mrp4* gene expressions combined with increases in blood bile acids (Table S1) at 24 h may suggest that a mild form of intrahepatic cholestasis (a condition where bile cannot flow from liver to the duodenum) occurred. Slight increases in GGT at 6 h (Table 1), an indication of cholestasis, provided further evidence that short-term cholestasis had occurred. Further, the attenuation of bile acid synthesis from cholesterol would stimulate pathways to generate very low density lipoprotein (VLDL). This hypothesis was supported by the up-regulation of sterol *O*-acyltransferase genes (*Soat1* and *Soat2*) involved in VLDL synthesis (Figure 6). Furthermore, increases in free fatty acids in serum and the expressions of the microsomal triglyceride transfer protein gene (*Mttp*) involved in VLDL synthesis indicated that VLDL production from free fatty acids (due to fatty acid  $\beta$ -oxidation inhibition) was also stimulated. Taken together, the results indicated that bile acids may play important roles in energy expenditure through regulating the mobility/storage of free fatty acids. The up-regulation of the expression of ATP-binding cassette genes *Mrp2* and *Mrp3* (also named as *Abcc2* and *Abcc3*) may also be an attempt by the hepatocytes to transport residual APAP metabolites into the bile and portal blood [33,34]. APAP-GSH, APAP-NAC and APAP-Glu conjugates are substrates of MRP2 and MRP3 [33,34]. These results are consistent with a xenobiotic detoxification metabolism wherein the expressions of *Mrp* and *Mdr* transporters are increased to facilitate the detoxification process [35-37]. Thus, these findings generally indicate an adaptive response to reduce accumulation of toxic bile acids in the liver and to modulate energy expenditure as well as to accelerate the excretion of drug metabolites from the hepatocytes into the serum or urine. Taurocholic acid and its secondary metabolites taurodeoxycholic acid, and sulfolithocholylglycine, had strong correlations to  $\log_2$ ALT and therefore could be potential liver injury biomarkers. When these bile

acids returned to normal levels at 168 h it may indicate that the liver was functioning and recovered from injury. Total serum bile acids levels in acetaminophen overdose patients have reported to be more sensitive of mild liver damage than transaminases [38]. These findings are consistent with a previous report that found serum bile acid levels could be more sensitive biomarkers of organic solvent-induced hepatotoxicity than traditional ALT, AST, GGT and ALP [39].

Blood arginine and urine 2-oxoarginine (involved in arginine metabolism) had strong negative correlations ( $r<-0.5$ ,  $p<0.05$ ) to  $\log_2$ ALT and hepatic necrosis. Similar to ALT, arginase I (an enzyme that catalyzes the conversion of arginine into ornithine and urea) can be released from hepatocytes undergoing apoptosis. Arginase I in blood is one of several recently proposed liver injury biomarkers to be quantified by the Predictive Safety Testing Consortium (PSTC) and the European Medicines Agency. After evaluating three potential biomarkers (alpha-glutathione-S-transferase, arginase I, and 4-hydroxyphenylpyruvate dioxygenase) in 34 acute liver toxicity rats, Bailey et al. reported that arginase I was the most sensitive biomarker to predict biliary injury, and more specific compared with ALT and the other two potential biomarkers [40]. Blood arginine levels can also be affected by nitric oxide synthases (NOSs), which convert arginine to citrulline and nitric oxide (NO; an important signaling molecule to control blood pressure, insulin secretion and nervous system development, etc.). NO can also react with oxygen radicals to form reactive nitrogen species peroxynitrite, which can damage macromolecules such as enzymes in the mitochondria via oxidation, hydroxylation and nitration [41,42]. NOS have been found to be involved with APAP toxicity [42]. For example, neuronal NOS (nNOS) was reported to be related to the deactivation of manganese superoxide dismutase in a study comparing neuronal NOS knockout and wild-type mice dosed with 300 mg APAP/kg [43]. Taken together, the reduction of blood arginine levels can reflect arginase I levels released from hepatocytes as well as NOS enzymes activity during liver injury. Therefore, blood arginine and its metabolite 2-oxoarginine in urine might be potential injury biomarkers in addition to the current clinical biomarkers.

## Conclusion

Transcriptomic and multi-analytical platform based metabolomics techniques were successfully applied to systematically investigate the metabolome changes in blood and urine induced by APAP administration. The changes in the metabolites and transcripts in the pathways associated with APAP-induced toxicity are time dependent and generally recover to control levels at 168 h. In this work, four analytical platforms were utilized for non-targeted profiling to examine endogenous metabolite changes in blood and urine during liver injury and recovery from such. Further, the liver transcriptomics data were combined to successfully interpret the underlying biological meanings of the metabolomic results from a systems biology perspective. Both metabolomics analysis in blood and urine, and transcriptomics analysis in liver tissue suggested that energy metabolism and bile acid pathways were disturbed during liver injury. Furthermore, liver injury biomarkers involved in energy metabolism pathway (medium chain dicarboxylic acids and  $\alpha$ -ketoglutarate) and bile acid pathway (taurocholic acid and taurodeoxycholic acid) and arginine metabolites (arginine and 2-oxoarginine) were also discovered. This was a limited study in which the toxic dose of acetaminophen caused moderate necrosis and ALT increases in two rats and therefore further studies and needed to evaluate these potential metabolic biomarkers of liver injury.

## Acknowledgements and Disclaimers

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