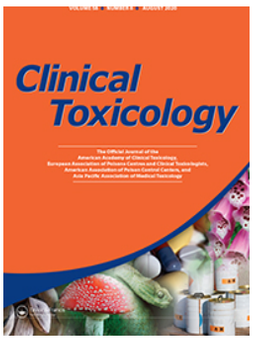

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Michael Ganetsky, Anders H. Berg, Joshua J. Solano & Steven D. Salhanick

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BASIC RESEARCH

Metabolomic analysis of acetaminophen induced subclinical liver injury

Michael Ganetsky[a], Anders H. Berg[b], Joshua J. Solano[c] and Steven D. Salhanick[d]

[a]Department of Emergency Medicine, Division of Medical Toxicology, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA, USA; [b]Department of Pathology and Laboratory Medicine, Cedars-Sinai Medical Center, Los Angeles, CA, USA; [c]Department of Integrated Medical Science, Charles E. Schmidt College of Medicine, Florida Atlantic University, Boca Raton, FL, USA; [d]Staff Toxicologist, Children's Hospital, Boston, MA, USA

ABSTRACT

Introduction: This study examines the metabolomic profile in humans following acetaminophen (APAP) induced subclinical hepatoxicity in the presence and absence of propylene glycol (PG), a cytochrome P450 2E1 inhibitor.

Methods: Plasma samples were collected during a previously performed randomized, cross-over trial where 21 subjects received APAP, four grams daily for two weeks in one arm and APAP, four grams daily with 20 mL PG in a second arm. Plasma collected at baseline and at day nine of each arm(time of peak elevation of liver function tests) underwent metabolomic analysis.

Results: There were reduced phase two metabolites in subjects who displayed liver injury. There was also decreased sulfonation capacity in all subjects as well as in subjects displaying liver injury relative to subjects not displaying liver injury as evidenced by decreased sulfonation of hepatically derived steroids. There were decreased levels of acylcarnitines in subjects who displayed liver injury relative to subjects not displaying liver injury, indicating inhibition of mitochondrial fatty acid β-oxidation.

Conclusions: Daily APAP dosing led to saturation of metabolic pathways and inhibition of mitochondrial function in subjects displaying subclinical liver injury.

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KEYWORDS
Acetaminophen; hepatotoxicity; propylene glycol; metabolomics

Introduction

Acetaminophen (APAP) toxicity is a leading cause of acute liver injury and responsible for a significant proportion of liver transplants that follow drug-induced liver failure in the United States [1,2]. Hepatotoxicity can develop from a large, acute ingestion, but also from repeated supratherapeutic ingestions [3]. Several studies have demonstrated a subclinical, self-limited rise in alanine aminotransferase (ALT) and aspartate aminotransferase (AST) following ingestion of the previously recommended therapeutic limit of four grams per day for several days [4,5].

The majority of ingested APAP is conjugated with glucuronide and sulfate while approximately 5–15% of a dose is metabolized *via* the cytochrome P-450 (CYP) system, primarily *via* CYP2E1 [6–9]. This reductive metabolism results in the production of a radical metabolite, n-acetyl-p-benzoquinone imine (NAPQI) [10]. Most investigators and clinicians believe that the hepatocellular damage is initiated by NAPQI arylating intracellular proteins once glutathione stores are depleted [11,12]. Data obtained from studies in rodents and *in vitro* reveals that CYP2E1 activity is primarily responsible for the development of hepatotoxicity [13].

We previously reported that after taking an equivalent 15 mg/kg APAP dose in solid versus liquid preparation, healthy adult volunteers produced significantly less CYP derived minor metabolites following ingestion of the liquid preparation [14]. We theorized that this effect was due to the presence of propylene glycol (PG), a common excipient found in the liquid preparation. In murine and *in vitro* models of APAP toxicity, PG reduces hepatic injury *via* inhibition of CYP2E1 [15,16]. Further, children are less susceptible to APAP toxicity [17]. We have speculated that this may be due to excipients, which inhibit reductive metabolism in the pediatric preparations that they are more likely to ingest.

Prior studies indicate that humans taking four grams of acetaminophen daily will develop a mild, typically self-limited rise in ALT and/or AST [4,5]. We subsequently performed a study to determine if the addition of PG alone administered with a daily APAP dose of four grams over two weeks could reduce the incidence of this subclinical liver injury in healthy human volunteers by inhibiting the production of toxic minor metabolites. Our results suggested an intrinsic susceptibility to APAP hepatocellular injury that was CYP2E1 mediated but not protected by PG at the degree of CYP2E1 inhibition achieved [18].

There is little data regarding the cause or significance of this observed rise in AST/ALT. Also, little data on other metabolic effects of chronic, frequent APAP dosing exists. Metabolomic analysis could provide insight into the cellular mechanisms responsible for the AST/ALT rise. The metabolome is the quantitative set of all the low-molecular weight molecules present in a particular physiological state. Metabolomic analysis attempts to quantify all the

CONTACT Steven D. Salhanick steven.salhanick@childrens.harvard.edu Staff Toxicologist, Children's Hospital, 300 Longwood ave, Boston, MA 02115, USA

metabolites of a cellular system typically using liquid chromatography and mass spectrometry followed by advanced data analysis to identify signal [19]. Previous metabolomic analysis of healthy volunteers with daily acetaminophen dosing has shown that NAPQI detoxification products can be identified prior to the rise in ALT and can distinguish subjects who will develop this rise [5].

Consequently, the goal of this follow up study was to investigate the metabolomic profiles of subjects who developed the subclinical transaminitis from APAP alone and in the presence of PG, an established CYP2E1 inhibitor, to determine cause of the observed rise in AST/ALT and elucidate any other effects of chronic APAP administration.

Methods

This study was a metabolomic analysis of plasma samples obtained from a previously published randomized, cross-over trial investigating the effect of PG on AST/ALT elevation following chronic APAP administration [18].

Previous study protocol

We had conducted a randomized, crossover trial with two separate study arms. Inclusion criteria for subjects were as follows: subjects were 20–40 years of age, had no current prescription or over the counter medication use, were not pregnant, had no prior significant medical history, and alcohol use was limited to two or fewer drinks no more than four times per week. Subjects were randomized to start with either APAP alone or APAP and PG. APAP was orally dosed at one gram, four times per day for 14 days. In the APAP + PG arm, the subjects concurrently took five milliliters (mL) of 99% pharmaceutical grade PG dissolved in five ounces of water with the APAP dose. Dosing of APAP was identical in both arms. Subjects were seen in our institution's General Clinical Research Center (GCRC) daily where blood was drawn for AST/ALT and to be interviewed by a nurse regarding any adverse effects or issues with compliance with the protocol. The subjects underwent a washout period of at least 14 days in between study arms. Subjects were allowed to continue taking APAP as long as AST or ALT did not reach three times the upper limit of normal. The protocol was approved by the institutional Committee on Clinical Investigation (IRB).

The primary outcome was the percent of subjects developing ALT or AST greater than two times baseline (responders) between the two arms. Baseline ALT was determined to be the mean of the ALT levels on the first three days of each study arm.

Metabolomic analysis

After completion of the initial trial, we subsequently performed metabolomic analysis on selected plasma samples stored from the initial study. Samples selected for metabolomic analysis included a baseline, which was day one of the APAP only group prior to APAP administration. Baseline samples were compared to samples from day nine (the day of mean peak AST/ALT) which were divided into the following groups: APAP only, APAP + PG, Responders (subjects with elevated AST/ALT) and Non-responders. Baseline samples were compared to each group and day nine groups were compared to each other.

Metabolomic analysis was performed by Metabolon (Research Triangle Park, NC). Samples were stored at −80 °C. Proteins were precipitated with methanol under vigorous shaking for 2 min (Glen Mills GenoGrinder 2000) followed by centrifugation. The resulting extract was divided into five fractions: two for analysis by two separate reverse phase (RP)/ultra-performance liquid chromatography (UPLC)- tandem mass spectrometry (MS/MS) methods with positive ion mode electrospray ionization (ESI), one for analysis by RP/UPLC-MS/MS with negative ion mode ESI, one for analysis by Hydrophilic interaction chromatography (HILIC)/UPLC-MS/MS with negative ion mode ESI, and one sample was reserved for backup. Samples were placed briefly on a TurboVap® (Zymark) to remove the organic solvent. The sample extracts were stored overnight under nitrogen before preparation for analysis. All methods utilized a Waters ACQUITY UPLC and a Thermo Scientific Q-Exactive high resolution/accurate mass spectrometer interfaced with a heated electrospray ionization (HESI-II) source and Orbitrap mass analyzer operated at 35,000 mass resolution. The sample extract was dried then reconstituted in solvents compatible to each of the four methods. One aliquot was analyzed using acidic positive ion conditions, chromatographically optimized for more hydrophilic compounds. In this method, the extract was gradient eluted from a C18 column (Waters UPLC BEH C18–2.1 × 100 mm, 1.7 μm) using water and methanol, containing 0.05% perfluoropentanoic acid (PFPA) and 0.1% formic acid (FA). Another aliquot was also analyzed using acidic positive ion conditions, however it was chromatographically optimized for more hydrophobic compounds. In this method, the extract was gradient eluted from the same afore mentioned C18 column using methanol, acetonitrile, water, 0.05% PFPA and 0.01% FA and was operated at an overall higher organic content. Another aliquot was analyzed using basic negative ion optimized conditions using a separate dedicated C18 column. The basic extracts were gradient eluted from the column using methanol and water, however with 6.5 mM Ammonium Bicarbonate at pH 8. The fourth aliquot was analyzed *via* negative ionization following elution from a HILIC column (Waters UPLC BEH Amide 2.1 × 150 mm, 1.7 μm) using a gradient consisting of water and acetonitrile with 10 mM Ammonium Formate, pH 10.8. The MS analysis alternated between MS and data-dependent MSn scans using dynamic exclusion. The scan range varied slighted between methods but covered 70–1000 *m/z*.

Compounds were identified by comparison to >3000 library entries of purified standards or recurrent unknown entities. Biochemical identifications are based on three criteria: retention index within a narrow RI window of the proposed identification, accurate mass match to the library ± 10 ppm, and the MS/MS forward and reverse scores between the experimental data and authentic standards. The MS/MS

scores are based on a comparison of the ions present in the experimental spectrum to the ions present in the library spectrum.

Peak values measured for metabolites were quantified using area-under-the-curve. For studies spanning multiple days, a data normalization step was performed to correct variation resulting from instrument inter-day tuning differences. Each compound was corrected by registering the medians to equal one (1.00) and normalizing each data point proportionately. Data are presented as proportional increases or decreases from corrected median [20].

Repeated measures ANOVA contrasts were used to identify biochemicals that differed significantly between experimental groups. An estimate of the false discovery rate (q-value) is calculated to take into account the multiple comparisons that normally occur in metabolomic-based studies. Given the multiple comparisons inherent in analysis of metabolites, between-group relative differences are assessed using both Student's t-tests (p-value) and false discovery rate analysis (q-value). Pathways were assigned for each metabolite, also allowing examination of overrepresented pathways. Principal component analysis, hierarchical cluster analysis and random forest analysis were performed. Results were considered significant at $p \leq .05$.

Results

Relevant results from initial study

Twenty-first subjects completed the protocol. One subject was excluded from analysis due to non-compliance with the protocol, specifically the subject used multiple prescription medications and underwent general anesthesia during the study period. The mean age of subjects was 29 years (range 21–37). Eleven subjects were randomized to start in the APAP + PG group first. There was no difference for the primary outcome of percent responders in each crossover treatment group, defined as peak ALT rising to two times baseline ALT. There were 6/20 (29%) responders in the APAP only arm compared to 8/20 (40%) responders in the APAP + PG arm (chi-square $p = .59$). However, subjects who were responders in the APAP arm, tended to be responders in the APAP + PG arm; there were five subjects who were responders in both arms. Detailed additional results have been published separately [18].

Metabolomic analysis results

Metabolomic analysis was performed on 3 groups: baseline, APAP, and APAP + PG, and then further subdivided on responders and non-responders from both APAP and APAP + PG groups combined.

There are significant metabolomic differences between baseline and day 9 in all groups, primarily due to APAP metabolites

Random Forest analysis differentiating baseline from day nine APAP and day nine APAP + PG resulted in a predictive accuracy of 92.75% and 97.25% respectively, indicating profound differences in overall biochemical profiles. This is primarily due to exposure and metabolism of APAP and PG. The differences included the presence of APAP and metabolites, as well as PG confirming exposure. Presence of glucuronide- and sulfate-conjugated acetaminophen metabolites including 2-hydroxyacetaminophen sulfate, 2-methoxyacetaminophen sulfate, 4-acetamidophenylglucuronide and 2-methoxyacetaminophen glucuronide were noted in APAP and APAP + PG treated groups in relation to baseline control samples. (Figure 1) Additionally, NAPQI detoxification products such as 3-(cystein-S-yl) acetaminophen and 3-(N-acetyl-L-cystein-S-yl) acetaminophen was observed in APAP and APAP + PG treated groups, suggesting active detoxification of NAPQI. However, minimal differences in NAPQI detoxification products (3-(cystein-S-yl) acetaminophen and 3-(N-acetyl-L-cystein-S-yl) acetaminophen) were observed when

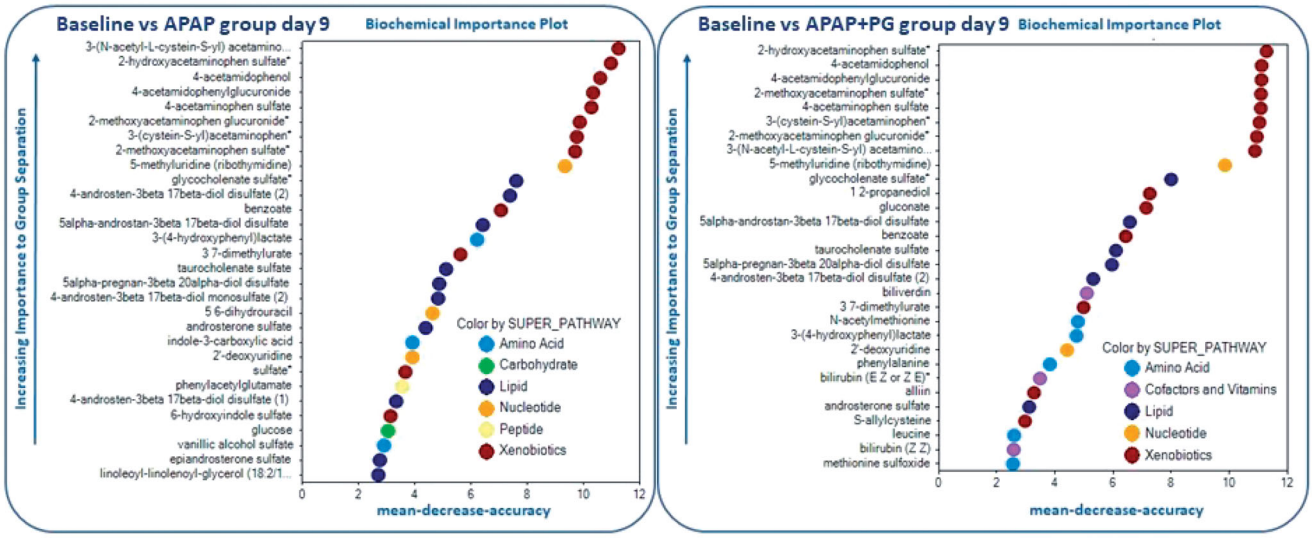


Figure 1. Random Forest classification of baseline group vs APAP group at day 9 (left) and baseline group vs APAP + PG group at day 9 (right). Differentiation mostly due to presence of APAP and metabolites. Predictive accuracy of 92.75% and 97.25% respectively.

comparing APAP and APAP + PG treated groups suggesting minimal inhibition of CYP 2E1 by PG.

Glucuronidation, sulfation and detoxification products were reduced in responders relative to non responders

There was a significant reduction in phase II metabolites (2-hydroxyacetaminophen sulfate, 4-acetamidophenylglucuronide, 4-acetaminophen sulfate) in the responders as compared to non-responders (Figure 2). However, there were mixed results comparing responders to non-responders for the CYP450 derived minor metabolites: 3-cystein-S-yl acetaminophen showed no difference but 3-N-acetyl-L-cystein-S-yl acetaminophen was reduced in the responders.

All groups (apap pg v apap and responders v non responders) showed decreased sulfonation capacity and responders had decreased sulfonation relative to non responders

There was a substantial decrease in 19 measured sulfated cellular metabolites (for example, glycocholenate sulfate, taurocholenate sulfate, and cysteine s-sulfate) in APAP and APAP + PG treated groups compared to baseline samples. There was also a decrease in sulfonated steroid metabolites such as pregnendiol disulfate and androsteroid monosulfate, suggesting competitive inhibition by APAP for liver steroid sulfonation pathways (Figure 3).

There was a decrease in fatty acid -oxidation in responders relative to non responders

The other affected metabolic pathway was a decrease in fatty acid β-oxidation in responders. A metabolic signature of reduced free fatty acid mobilization and β-oxidation was observed when comparing responders and baseline controls as evidenced by decreased levels of acylcarnitines. Several medium and long-chain carnitines, including decanoylcarnitine, hexanoylcarnitine, laurylcarnitine, octanoylcarnitine myristoylcarnitine, oleoylcarnitine and palmitoylcarnitine were considerably lower in responders compared to baseline. All 11 measured acylcarnitines were lower in the responders; 3 of 11 were significantly lower at $p \leq .05$ (Figure 4).

Discussion

APAP poisoning remains a significant clinical problem. Toxicity generally occurs following acute overdose or serial supratherapeutic doses. In the past several years a subclinical rise in transaminases has been observed in humans taking four grams per day [4]. There is controversy surrounding the clinical significance of this phenomenon. Some authors argue that it is clinically insignificant while others use it as evidence of toxicity occurring with low doses, supporting the contention that hepatotoxicity can occur with therapeutic dosing [21–23].

We performed the original study from which samples were obtained for this analysis to determine if PG, a CYP2E1 inhibitor, could suppress the phenomenon of subclinical rise in transaminases following daily therapeutic APAP dosing. This was done on the assumption that this rise in transaminases was due to a similar mechanism of toxicity as when a large single overdose or multiple supratherapeutic doses are taken. The generally cited mechanism is that there is production of a radical metabolite by CYP2E1 and other P450 isoforms which initiates liver injury. We did not find that PG decreased the incidence of subjects who developed elevated ALT levels ("responders"), despite some reduction in CYP2E1 derived metabolite levels. One of the reasons that this may have occurred was because a different mechanism of hepatocellular injury is at play. Our hope was to shed light on the mechanism by which subclinical rise in AST/ALT occurs in the setting of therapeutic APAP dosing. Further, one of several interesting findings from our initial study was that the same subjects were likely to be responders in both arms of the original study (APAP alone versus APAP with PG).

The most compelling result of this investigation was the decrease in circulating acylcarnitines suggestive of disturbance in mitochondrial fatty acid β-oxidation, potentially due to mitochondrial injury in responders as compared to baseline and non-responders (Figure 4). This difference in the metabolome does not explain why certain subjects were susceptible to the AST/ALT rise, but does demonstrate an association with mitochondrial dysfunction.

Mitochondrial dysfunction secondary to injury has been well described as crucial in the initiation of APAP induced liver injury. The likely mechanism for early mitochondrial injury is direct arylation of mitochondrial proteins leading to inhibition of respiration [24]. Drug-induced liver injury, including acetaminophen induced liver injury, is associated with disturbances in mitochondrial fatty acid β-oxidation [25]. Inhibition of fatty acid β-oxidation by APAP has been shown in animal models of APAP toxicity. In mice given toxic APAP doses, there is an early rise in acylcarnitine levels, followed by a delayed decrease below baseline levels, that correlates with ALT elevation [26]. In CYP2E1-null mice, there is reversible inhibition of β-oxidation but irreversible inhibition in wild type mice [27]. Inhibition may be due to direct mitochondrial damage or may be due to suppression of peroxise proliferator-activated receptor α (PPARα) regulated pathways. PPARα controls the expression of genes encoding peroxisomal and mitochondrial fatty acid β-oxidation enzymes; PPARα-humanized mice are protected from APAP toxicity felt to be from induction of a gene encoding mitochondrial uncoupling protein 2 (UCP2) which protects against reactive oxygen species generated during drug-induced hepatotoxicity [28].

However, we did not find a difference in circulating acylcarnitines between the APAP and APAP + PG groups. This is unexpected since PG, a known CYP2E1 inhibitor, should decrease NAPQI formation which is thought to be responsible for the mitochondrial injury. There are several potential reasons we did not see a difference. First and most likely, the PG dose may have been too low which is supported by similar concentrations of NAPQI detoxification products between both groups (Figure 2, top row). Second, there may be contribution to mitochondrial toxicity from CYP1A2 and

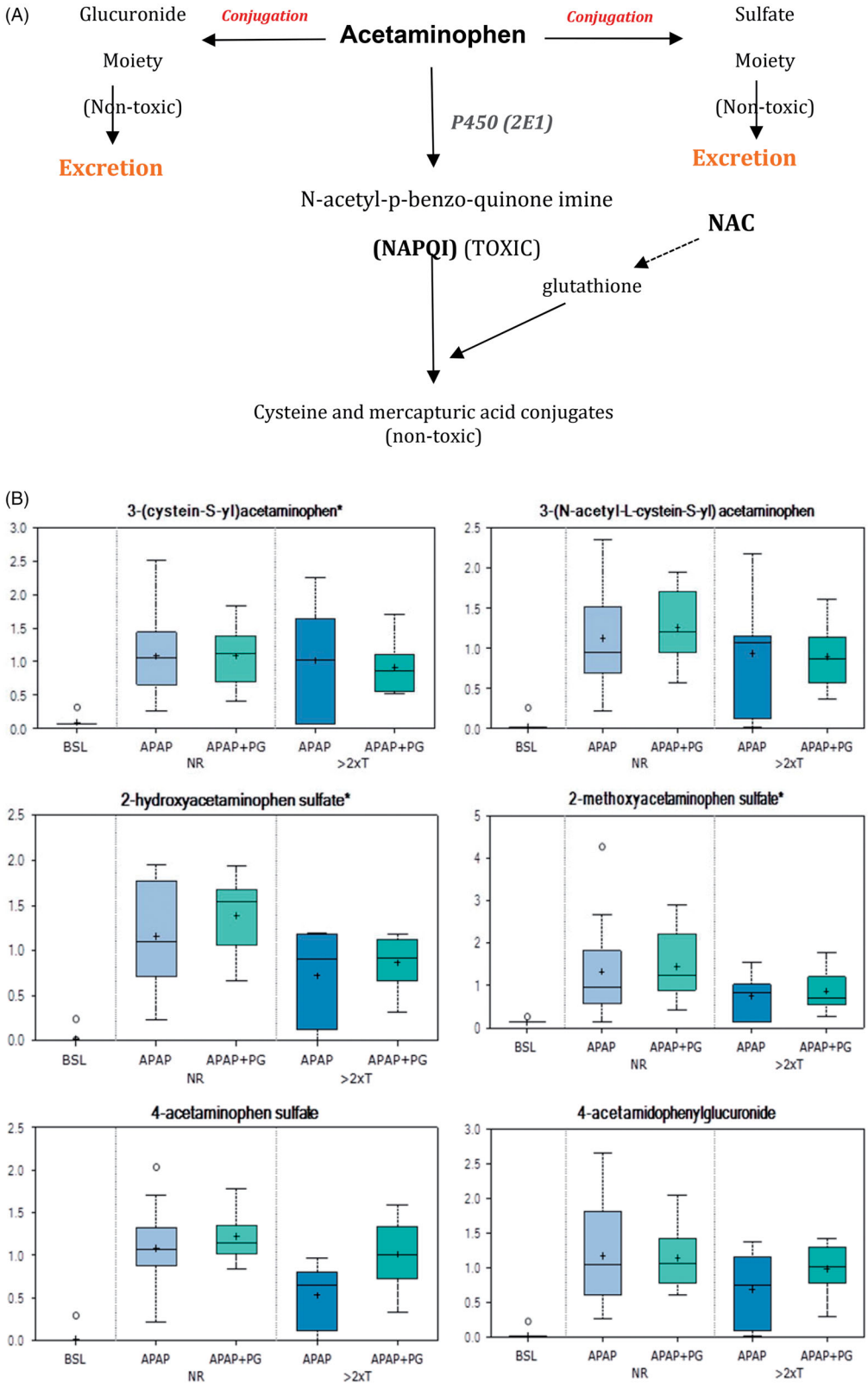


Figure 2. (A) Diagram of acetaminophen metabolism (B) Box plot of profile of selected acetaminophen metabolites. (BSL: baseline; NR: non-responder; >2xT: responders. Y-axis is scaled intensity) Top row are downstream of NAPQI, bottom 2 rows represent phase II conjugation metabolism. $p \leq .05$ considered significant.

CYP3A as these have also been implicated in NAPQI formation [7,8]. Finally, the mitochondrial injury may be independent of CYP2E1 activity.

Our findings indicating inhibition of mitochondrial β-oxidation of fatty acids in the setting of APAP-induced transaminitis is novel in humans and suggests that mechanisms of initiation of toxicity known to occur in animals and in cell culture are occurring in our subjects that displayed increases in ALT. Further, this finding suggests that rise in ALT noted with chronic dosing of four grams daily is due to

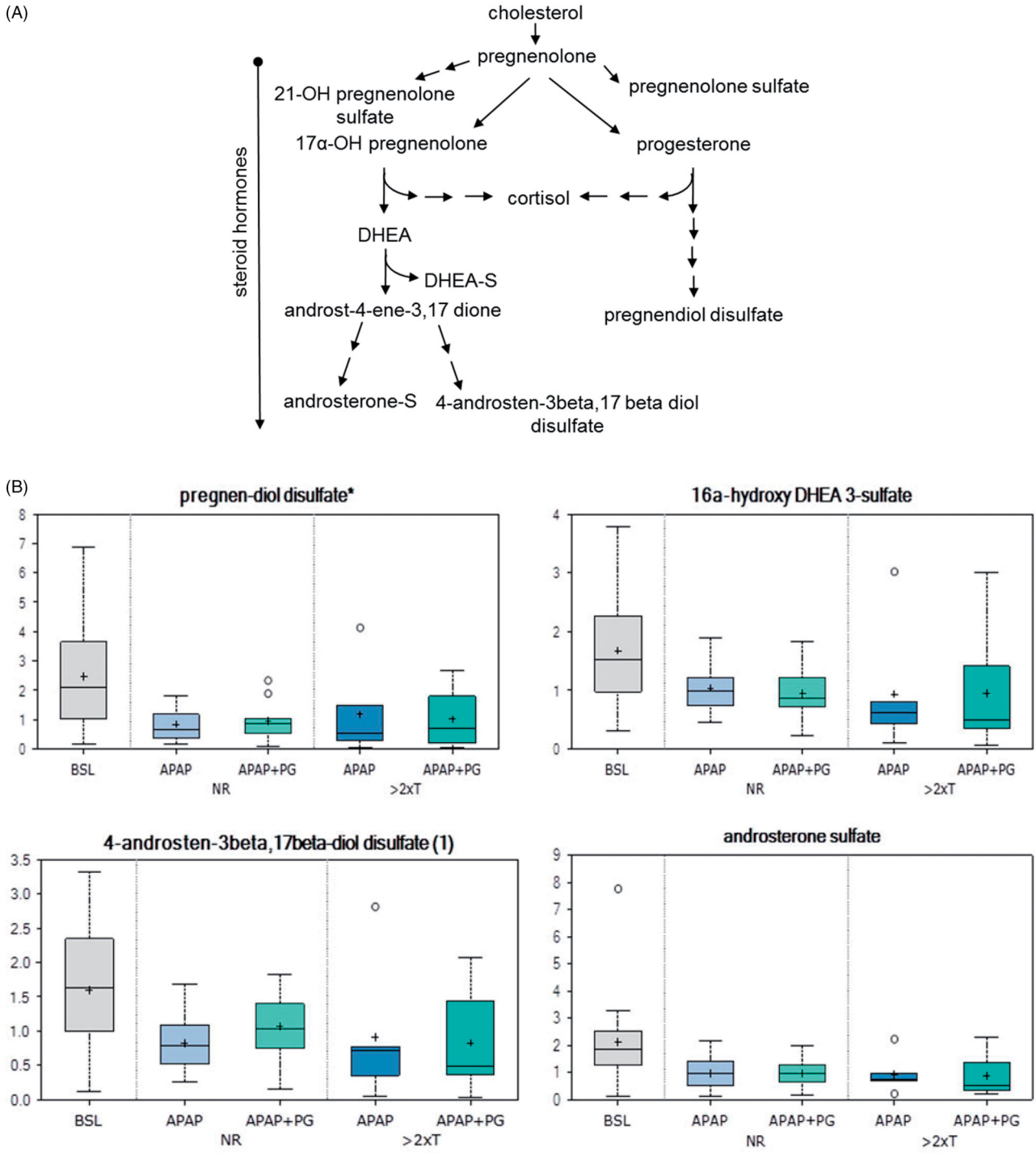
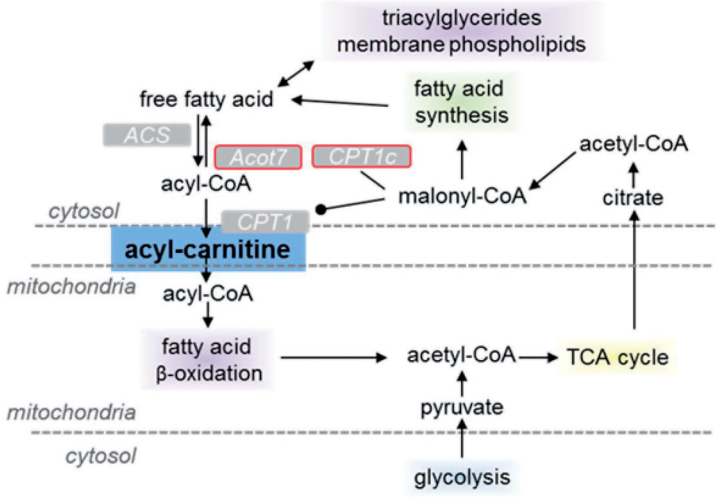
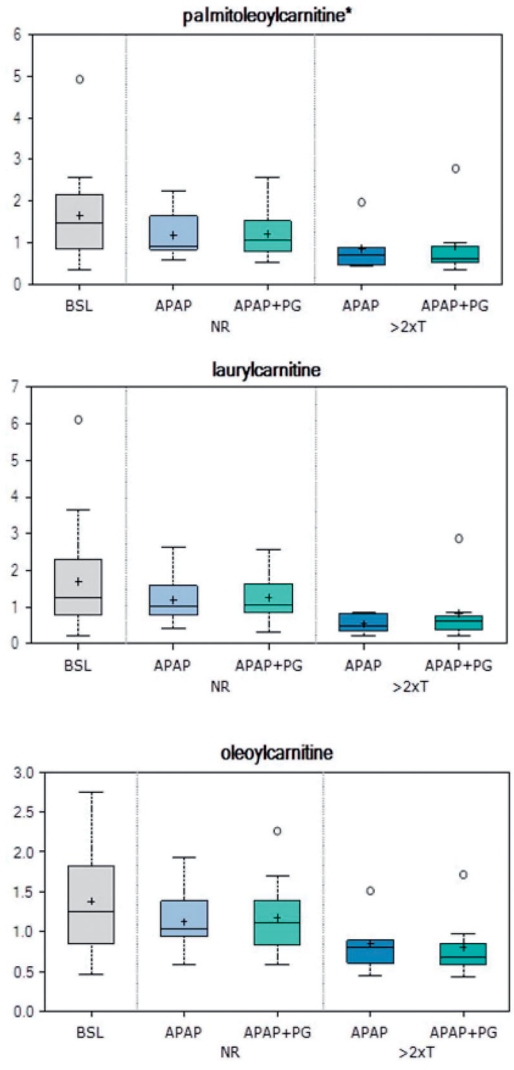


Figure 3. (A) Diagram of sulfated steroid metabolic pathway (B) Box plot of profile of selected sulfonation products demonstrating decreased sulfation capacity compared to baseline. (BSL: baseline; NR: non-responder; >2xT: responders. Y-axis is scaled intensity) Bottom 2 rows are steroid metabolites. $p \leq .05$ considered significant.

a similar mechanism as that of acute overdose. Lastly, our findings suggest that the phenomenon of subclinical rise in liver enzymes could be used as a human model for investigating the initiation mechanisms of APAP induced hepatotoxicity.

Another novel result from this study is the effect of APAP daily dosing on sulfation pathways. With APAP treatment, the sulfation pathway becomes saturated, while glucuronidation and oxidation increase, and a smaller amount of APAP is excreted unchanged. Sulfotransferases (SULT), a family of cytosolic enzymes, carry out sulfation of APAP and steroid hormones. SULTs transfer a sulfo group from 3′-phosphoadenosine-5′-phosphosulfate (PAPS) to APAP making it more polar and prone to elimination [29]. In the liver, SULT1A1, SULT2A1 and SULT1A3/4 are shown to catalyze APAP sulfation [30]. This general decline in sulfated metabolites with daily APAP treatment likely reflects saturated sulfation pathway due to augmented sulfation of particularly high levels of APAP in the liver. Importantly, a significant decrease in many sulfated steroids with chronic APAP treatment could be





Sub Pathway	Biochemical Name	Treatment Groups Baseline			
		NonResponders		Responders	
		APAP	APAP+PG	APAP	APAP+PG
Fatty Acid Metabolism (Acyl Carnitine)	octanoylcarnitine	0.80	0.76	0.45	0.48
	decanoylcarnitine	0.74	0.80	0.34	0.42
	cis-4-decenoyl carnitine	0.76	0.73	0.61	0.52
	laurylcarnitine	0.70	0.75	0.32	0.49
	myristoylcarnitine	0.75	0.82	0.54	0.57
	palmitoylcarnitine	0.82	0.93	0.67	0.63
	palmitoleoylcarnitine*	0.72	0.74	0.52	0.55
	stearoylcarnitine	0.87	0.93	0.80	0.72
	linoleoylcarnitine*	0.79	0.80	0.68	0.69
	oleoylcarnitine	0.81	0.85	0.61	0.58
	myristoleoylcarnitine*	0.70	0.68	0.37	0.47
Carnitine Metabolism	deoxycarnitine	0.80	0.81	0.74	0.72
	carnitine	0.93	0.93	0.86	0.83

Figure 4. Alteration in fatty acid oxidation in responders. (In table, shaded represents decrease, *=significant at $p \leq .05$. For box plots of selected metabolites, BSL: baseline; NR: non-responder; >2xT: responders. Y-axis in box plots is scaled intensity).

indicative of altered liver function, since the liver is one the major sites of steroid sulfation mediated by SULTs.

A previous metabolomic study found a similar decrease in sulfated steroid hormones in subjects taking acetaminophen [31]. This interference of acetaminophen with seemingly unrelated steroid metabolism has potentially as of yet undetermined clinical implications such as disruption of hormonal hemeostasis especially in pregnancy and impact on neurosteroids that have roles in nociception and inflammation. Interestingly, several epidemiologic studies have identified an association between maternal acetaminophen use during pregnancy and development of ADHD-like behaviors and autism spectrum symptoms [32–36]. The results of this study provide a plausible biological mechanism for this association.

Employing metabolomics has several limitations applicable to this study. The metabolome is very sensitive to changes in the subjects' environment, and we were unable to control for all possibilities such as diet, drugs of abuse, or alcohol intake. Additionally, the metabolome provides a phenotypic representation of cellular mechanisms. Our results demonstrate an alteration of a marker of mitochondrial dysfunction but does not further delineate the mechanistic details.

Our results raise many more questions for future research. Since β-oxidation inhibition likely plays an early role in initiation of APAP-hepatotoxicity, serum acylcarnitines may be useful as an early marker of APAP toxicity, or early prognostic tool for patients with APAP overdose. Also, given the saturation of sulfation in daily APAP dosing, there are likely uncharacterized drug-drug and drug-metabolic pathway interactions from daily APAP use.

Conclusion

Our previous study found that certain subjects are susceptible to subclinical transaminitis from APAP daily dosing, which was not protected by the addition of PG, a CYP2E1 inhibitor. In this follow up metabolomic analysis, these

subjects also demonstrated an exaggerated reduction of sulfated metabolite production suggesting that reduced ability to maintain sulfonation pathway may predispose humans to toxicity. Further, we found an inhibition of fatty acid β-oxidation, supporting the role of mitochondrial dysfunction as an initiator of APAP-induced liver injury. Lastly, saturation of sulfation mechanisms impact other pathways that rely on sulfation, such as steroid metabolism.

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Disclosure statement

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ORCID

Steven D. Salhanick http://orcid.org/0000-0002-1533-5159

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