**Glycodeoxycholic Acid Levels as Prognostic Biomarker in Acetaminophen-Induced Acute Liver Failure Patients**


*Department of Pharmacology, Toxicology and Therapeutics, †Department of Biostatistics, ‡Department of Internal Medicine, University of Kansas Medical Center, Kansas City, Kansas 66160, §Department of Medical Toxicology, Banner Good Samaritan Medical Center, Phoenix, Arizona 85006, Department of Medicine, and Center for Toxicology and Pharmacology Education and Research, University of Arizona College of Medicine, Phoenix, Arizona 85006 and †Division of Digestive and Liver Diseases, Department of Internal Medicine, University of Texas Southwestern Medical Center, Dallas, Texas 75390

1To whom correspondence should be addressed at Department of Pharmacology, Toxicology and Therapeutics, University of Kansas Medical Center, 3901 Rainbow Blvd, MS 1018, Kansas City, Kansas 66160. Fax: (913) 588-7501. E-mail: hjaeschke@kumc.edu.

The authors certify that all research involving human subjects was done under full compliance with all government policies and the Helsinki Declaration.

**ABSTRACT**

Acetaminophen (APAP)-induced acute liver failure (ALF) remains a major clinical problem. Although a majority of patients recovers after severe liver injury, a subpopulation of patients proceeds to ALF. Bile acids are generated in the liver and accumulate in blood during liver injury, and as such, have been proposed as biomarkers for liver injury and dysfunction. The goal of this study was to determine whether individual bile acid levels could determine outcome in patients with APAP-induced ALF (AALF). Serum bile acid levels were measured in AALF patients using mass spectrometry. Bile acid levels were elevated 5–80-fold above control values in injured patients on day 1 after the overdose and decreased over the course of hospital stay. Interestingly, glycodeoxycholic acid (GDCA) was significantly increased in non-surviving AALF patients compared with survivors. GDCA values obtained at peak alanine aminotransferase (ALT) and from day 1 of admission indicated GDCA could predict survival in these patients by receiver-operating characteristic analysis (AUC = 0.70 for day 1, AUC = 0.68 for peak ALT). Of note, AALF patients also had significantly higher levels of serum bile acids than patients with active cholestatic liver injury. These data suggest measurements of GDCA in this patient cohort modestly predicted outcome and may serve as a prognostic biomarker. Furthermore, accumulation of bile acids in serum or plasma may be a result of liver cell dysfunction and not cholestasis, suggesting elevation of circulating bile acid levels may be a consequence and not a cause of liver injury.

**Key words:** acetaminophen; bile acid; serum biomarker; cholestasis; acute liver failure; hepatotoxicity
Acetaminophen (APAP)-induced acute liver failure (AALF) is a major cause of morbidity in Western countries (Lee, 2012). Accurate prediction of the course and outcome of injury in AALF is an important clinical challenge. Previous work from our laboratory and others has confirmed serum biomarkers such as HMGB1, cytokeratin 18, mitochondrial DNA, nuclear DNA fragments, and glutamate dehydrogenase can serve as both mechanistic (Antoine et al., 2012; McGill et al., 2012, 2014a) and prognostic biomarkers (Antoine et al., 2012, 2013; McGill et al., 2014b; Schomaker et al., 2013) that can accurately predict the course of injury. Advanced metrics such as the King’s College Criteria (Rutherford et al., 2012) and the model for end-stage liver disease (MELD) score (Schmidt and Larsen, 2007) are currently used to assess potential outcome; however, novel biomarkers have been proposed that might enhance the accuracy and speed of the determination of the course of injury (Antoine et al., 2012, 2013; McGill et al., 2014a; McGill and Jaeschke, 2014; Rutherford et al., 2012; Weerasinghe et al., 2014).

A number of mechanisms behind APAP-induced liver injury in rodents have been elucidated. Activation of APAP to the electrophile N-acetyl-p-benzoquinone imine by cytochrome P450s results in protein binding, mitochondrial dysfunction, and oxidative stress (Jaeschke et al., 2012) which results in RIP1- and RIP3-dependent programmed necrosis (Ramachandran et al., 2013, Zhang et al., 2014). Due to cellular rupture during necrosis, numerous cellular products are released that can be detected in serum of mice with active liver injury (Antoine et al., 2012; McGill et al., 2012). Some of the products termed damage associated molecular patterns have been proposed as novel serum biomarkers that can provide information about the mechanisms of injury in human patients. For APAP-induced liver injury, a number of the same events that occur in mice have been confirmed in man, including release of HMGB1, mitochondrial DNA, and glutamate dehydrogenase (Antoine et al., 2012; McGill et al., 2012, 2014b). In addition to the release of cellular products by the liver, AALF results in multigorgan dysfunction and the accumulation of byproducts of this dysfunction. Many of these byproducts, such as elevations in serum creatinine, are components of essential prognostic scores in current use, such as the MELD score (Schmidt and Larsen, 2007).

Bile acids are critical mediators of metabolism and dietary fat intake that are synthesized in the liver (Chiang, 2013). Although there are numerous bile acid species present in mammals, the most abundant in mice and man are the primary bile acids, cholic acid (CA) and chenodeoxycholic acid (CDCA), and their amino acid conjugate species (Trottier et al., 2011, 2012). In addition, gut bacteria carry out mono-dehydroxylation reactions that result in accumulation of deoxycholic acid (DCA) from CA. Although a majority of bile acids have a similar structure, liquid chromatography coupled to mass spectrometry (LC-MS) has provided a validated method to analyze individual bile acids in serum and liver (Alnouti et al., 2008; Zhang et al., 2012) or bile (Alnouti et al., 2008). Under conditions of cholestasis (Trottier et al., 2011; Woolbright et al., 2014b; Zhang et al., 2012), or under conditions of massive liver injury (Péan et al., 2013) bile acids accumulate in the serum due to perturbations in enterohepatic flow. In mice and rats given toxic doses of APAP, total and specific serum bile acid levels increase (Luo et al., 2014; Yamazaki et al., 2013). Although “total” serum bile acid levels typically increase to similar degrees, altered concentrations of “specific, individual” bile acids present in serum after liver injury could serve as predictors of the course of drug-induced liver injury (Luo et al., 2014; Yamazaki et al., 2013).

The goal of this study was to measure individual bile acid levels in the serum of ALF patients and to determine whether any differences were present that would allow for the prediction of progression of injury. Herein, we confirm that not only are total and individual bile acid levels substantially elevated in APAP-induced ALF patients, but there is preferential accumulation of glycodeoxycholic acid (CDCA) in non-surviving patients. Furthermore, we demonstrate that total and individual serum bile acid levels accumulate to a greater degree in ALF patients than in patients with cholestatic liver injury (CLI), indicating bile acid accumulation is not a function of cholestasis, but rather liver dysfunction.

### MATERIALS AND METHODS

**APAP-induced acute liver injury patients.** Patient samples and data for all studies were acquired under informed consent and approved by Institutional Review Boards (IRBs) and adhere to the 1975 Declaration of Helsinki. To assess the time courses of circulating bile acid concentrations after APAP overdose, plasma samples were obtained from APAP overdose patients at the University of Kansas Hospital in Kansas City, Kansas and the Banner Good Samaritan Medical Center in Phoenix, Arizona (Table 1). The diagnosis of APAP overdose was made by a physician based on standard clinical criteria (reported history of APAP overdose, detectable serum APAP, and/or aminotransferase level of ≥1500 IU/l). Patients were excluded from the study if they had evidence of liver injury from some other causes, eg, viral hepatitis. Blood samples were collected in heparinized tubes at the time of study admission and every 24 h thereafter until patient death or discharge. The whole blood was centrifuged (1000 g, 10 min) to obtain plasma and aliquots were stored at −80°C for later analysis. Samples were also collected from

<table>
<thead>
<tr>
<th>TABLE 1. Volunteers and Acetaminophen Overdose Patients</th>
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<tr>
<td></td>
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<td></td>
</tr>
<tr>
<td>Age (median, range)</td>
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<tr>
<td>Sex (% female)</td>
</tr>
<tr>
<td>Peak ALT (U/l) (mean ± SE)</td>
</tr>
<tr>
<td>Peak bilirubin (mg/dl) (mean ± SE)</td>
</tr>
<tr>
<td>Peak PTa (s) (mean ± SE)</td>
</tr>
</tbody>
</table>

Volunteers, Vol, acetaminophen-induced acute liver injury, AALI, acetaminophen-induced acute liver failure, AALF, S, survivors; NS, non-survivors; liver transaminases, LT.

*When available. Alanine aminotransferase, ALT; prothrombin time, PT; bilirubin, Bili.

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healthy volunteers at the University of Kansas Medical Center. Volunteers were recruited as control patients over a number of days and at different time points during the course of the study. Informed written consent was obtained from each patient or next of kin. Time from admission to hospital to first drawn blood sample was generally less than 1 day for this patient population.

AALF patients. To compare patients who did and did not survive, serum samples from AALF patients were obtained through the Acute Liver Failure Study Group network (Table 1). Serum samples were balanced for the number of survivors and non-survivors. The diagnosis of APAP overdose was made by on-site investigators using standard criteria, as described above. Patients were excluded from the study if they had evidence of liver injury from some other causes, eg, viral hepatitis. In other respects, typical AALF criteria were applied: elevated INR (> 1.5), hepatic encephalopathy, and liver failure within 26 weeks of illness onset without evidence of other liver diseases. Samples were centrifuged on-site to obtain serum and stored at ≤ 80°C. Only samples from patients with evidence of tissue necrosis (peak in-study alanine aminotransferase [ALT] > 1000 U/l) were used for our measurements. Informed written consent was obtained from next of kin. Time from admission to hospital to first drawn blood sample has been previously reported (McGill et al., 2014b).

CLI patients. For the cholestatic patients, patients admitted to the University of Kansas Hospital were enrolled in an IRB approved protocol. Informed written consent was obtained from each patient. The inclusion criteria for the study included subjects undergoing planned endoscopic retrograde cholangiopancreatography (ERCP) for medical diagnosis and potential treatment of cholestasis. Patients with viral hepatitis, or any form of well-defined intrahepatic cholestasis, or autoimmune hepatitis, were excluded from this group. This group was pared down via etiology, and only patients with extrahepatic cholestasis or presumed extrahepatic cholestasis prior to ERCP were included in this study. Etiologies included gallstones, biliary stricture, obstructive jaundice, and pancreatitis. Diseases with autoimmune hepatitis features such as primary biliary cirrhosis or primary sclerosing cholangitis were excluded. Patients were split into two groups: the uninjured group (UI) were patients defined as having ALT < 40 U/l and alkaline phosphatase (ALP) < 110 U/l and the CLI group which were defined as patients with ALT > 40 U/l and ALP > 110 U/l and clinically diagnosed cholestasis as evidenced by ERCP (Table 2).

Clinical data. Prothrombin time, serum ALT, serum ALP, and serum bilirubin were measured in clinical laboratories at the participating hospitals using standard clinical methods.

**TABLE 2. Cholestatic Patients**

<table>
<thead>
<tr>
<th></th>
<th>UI</th>
<th>CLI</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>11</td>
<td>23</td>
</tr>
<tr>
<td>Age (median, range)</td>
<td>64, 39–83</td>
<td>57, 33–79</td>
</tr>
<tr>
<td>Sex (% female)</td>
<td>45</td>
<td>43</td>
</tr>
<tr>
<td>ALT (U/l) (mean ± SE)</td>
<td>20 ± 2</td>
<td>227 ± 40</td>
</tr>
<tr>
<td>ALP (U/l) (mean ± SE)</td>
<td>61 ± 9</td>
<td>465 ± 87</td>
</tr>
<tr>
<td>Bili (mg/dl) (mean ± SE)</td>
<td>0.8 ± 0.1</td>
<td>5.6 ± 1.5</td>
</tr>
</tbody>
</table>

Alanine aminotransferase, ALT; alkaline phosphatase, ALP; bilirubin, Bili; uninjured patients, UI; cholestatic liver injury, CLI.

**RESULTS**

Plasma bile acid levels were first measured in a time course of patients hospitalized for acute APAP-induced acute liver injury (AALI) (Patient information in Table 1). Total plasma bile acid levels increased significantly versus both control volunteer patients and APAP overdose patients with normal liver transaminases (NLTs) (Figure 1A). Total plasma bile acid peaked around day 1, and levels fell slowly over the first 6 days, but remained elevated over control values, indicating persistent liver dysfunction in these patients, consistent with elevated bile acid analysis. Serum and plasma bile acids were measured using a method adapted from Alnouti et al. (2008) and Zhang et al. (2012). Serum and plasma samples were prepared using a methanol extraction procedure to facilitate the removal of serum proteins by centrifugation. This was done by mixing 20μl of serum with 80μl of methanol spiked with internal standard and briefly vortexing, then centrifuging at 14000 × g for 10 min. The supernatant was extracted and used for measurements. Bile acids in these extracts (5 μl) were then separated using a Waters (Waters, Milford, Massachusetts) Acquity ultra-performance liquid chromatograph (UPLC) equipped with a Waters Acquity BEH C18 column (1.7 μm, 130 Å, 2.1 mm × 100 mm) equipped with a Waters BEH C18 VanGuard pre-column (1.7 μm, 130 Å, 2.1 mm × 5 mm). The UPLC eluent was analyzed using a Waters Synapt HDMS quadrupole/ion-mobility/time-of-flight hybrid mass spectrometer equipped with an electrospray probe. Chromatography was performed at a flow rate of 0.3 ml/min using the following gradient program: mobile phase A (0.1% formic acid in water) was given at 0.3 ml/min 98% for 3.5 min (with the first 3 min directed to waste) prior to initiation of a linear gradient lasting 17.5 min that terminated at 98% mobile phase B (0.1% formic acid in acetonitrile). This was followed by a 2-min soak at 98% mobile phase B whereupon the mobile phase was returned to 98% over 2 min and equilibrated at these conditions for 5 min more. The mass spectrometer was operated in negative mode over a mass range of 100–1000 m/z with V-optics (normal dynamic range) enabled. The cone and capillary voltages were set to 35 V and 3.0 kV, respectively. The source and desolvation temperatures were set at 120°C and 350°C, respectively, while nitrogen was supplied as the cone (50 μl/min) and desolvation gases (700 μl/h). MS1 data were collected at an acquisition rate of one scan per second with Lock-spray correction (2 μg/μl leucine enkephalin, 20 μl/min, 5 s frequency, 10 scan average). Absolute quantification was accomplished by comparing against a standard curve of six different concentrations of each bile acid. Concentrations of bile acid standards were used to 100 ng/ml, 500 ng/ml, 2 μg/ml, 5 μg/ml, 10 μg/ml, and 25 μg/ml to create the standard curve. Bile acid concentrations falling below a 100 ng/ml threshold were listed as n.d. or not determinable.

Statistics. Normality was assessed using the Shapiro-Wilk test and all patient data were found to be non-normal. Differences between groups were tested for significance using the Mann-Whitney U test. Time course data were assessed using one-way ANOVA on ranks with Dunn’s post hoc test against control values. Receiver operating characteristic (ROC) curve analysis was performed in SigmaPlot 12.5 (Systat Software, San Jose, California). Logistic regression and calculation of odds ratios (ORs) were performed using SAS 9.4 (SAS Institute Inc., Cary, NC). In all cases, \( P < 0.05 \) was considered statistically significant.
focus on the administration of micromolar concentrations, as large majority of studies currently done on bile acids during liver injury, and are the only bile acids that accumulate represent 95%–99% of the bile acid pool in systemic circulation amidated conjugates of CA, CDCA, and DCA. These bile acids acid (GCA), GDCA, and taurodeoxycholic acid (TDCA), plus TBA were determined in each patient (Supplementary Table 1): litho-

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FIG. 1. Plasma bile acid levels were measured in patients with acetaminophen (APAP)-induced liver injury, control patients (Ctrl), and in patients with APAP overdose but normal liver transaminases (NLT). Total bile acid (TBA) values (A) were defined as the combined concentrations of TCA, GCA, GDCA, TCDCA, TCA, GCA, CA, CDCA, UDCA, LCA, CA, and TLCA. A time course was established post first blood draw for individual glycine conjugated bile acids with plasma ALT time course for the overdose patient group (B), and individual bile acid lev-

eral bile acids were next measured in a well-defined patient group with AALF, a separate population with advanced drug-induced liver failure (Patient information in Table 1) to determine whether individual bile acid concentrations could accurately predict patient outcome. Values were measured in samples from each patient at the day of study admission and in the samples collected nearest to the time of peak ALT to nor-

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cholestatic patients. These values mirrored relative ALT values for the two groups (Figure 3C), suggesting that the increase in serum bile acids may be related to the degree of liver injury, which directly leads to liver dysfunction.

**DISCUSSION**

**Bile Acids as Prognostic Serum Biomarkers in AALF**

A number of recent papers have focused on release of intracellular components to assess both translational mechanisms (McGill et al., 2012, 2014a) and potential prognostic biomarkers (Antoine et al., 2012, 2013; McGill et al., 2012, 2014b; Weerasinghe et al., 2014). More traditional metrics, such as the MELD score, typically include levels of circulating markers of liver function such as bilirubin levels (Schmidt and Larsen, 2007). As bile acid enterohepatic circulation is contingent upon active secretion by functional hepatocytes, we hypothesized that individual circulating bile acid levels might be predictive of future survival in APAP overdose patients. Of note, plasma levels of multiple individual bile acids and TBA levels rose significantly above control levels in patients with AALI, whereas patients without increases in transaminases had values similar to control patient levels of circulating bile acids. These findings suggest that the increase in serum bile acids may be a consequence of liver injury and dysfunction. The bile acid levels remained elevated over the first 6 days of the patient course, indicating serum bile acid levels may have prognostic value even in patients that have been hospitalized for longer periods. Leveraging the ALF Study Group sample population, we were able to show GDCA levels rose significantly higher in the non-surviving AALF patients than in
patients that survived APAP overdose, despite similar ALT values between groups. Although total serum bile acids were not different between groups at either peak ALT or day 1 of admission between the surviving and non-surviving AALF patients, GDCA values were increased significantly in the non-surviving patients. Although the mechanism behind why this specific bile acid is elevated in non-surviving patients was not addressed in this article, this difference in a single bile acid draws attention to the value of examining the entire bile acid spectrum, rather than simply measuring TBA levels. Furthermore, ROC analysis indicates that GDCA retrospectively predicted survival in this cohort of patients with AALF using either peak ALT values or day 1 of admission values, although the predictive value was modest on its own. Thus, while it is unlikely GDCA can outperform current prognostic metrics, measurements of GDCA can be done rapidly, so future work assessing the potential of GDCA to be used as part of a battery of tests, or as a component of a larger scoring system to assess progression to transplantation or death may be warranted.

Although female non-survivors still had significantly higher GDCA values than their surviving counterparts, this effect was lost in the male group. This was likely due to the much lower number of males in the study as the populations were 70%–80% female in both survivors and non-survivors. Although not statistically evaluated for every bile acid, this was largely conserved across the bile acid species that were tested with female patients being highly similar to results from both sexes together, and male patients being only slightly higher.

### Serum Bile Acid Levels as a Marker of General Liver Injury and Function

Previous research indicating that bile acids accumulate in the serum after APAP overdose in rats has also noted serum bile acid increases in multiple other models of liver injury (Luo et al., 2014; Yamazaki et al., 2013). Circulation of bile acids is dependent on active transport of bile acids from hepatocytes into the biliary tracts by canalicular transporters. This process is highly susceptible to perturbation, as even minor liver damage can cause increases in serum bile acids (Yamazaki et al., 2013), suggesting bile acids are a highly sensitive marker for liver injury and liver dysfunction. Total serum bile acid levels have been proposed as prognostic criteria either by themselves or as a battery before (Hoekestra et al., 2012; Shlomai et al., 2013); however, increased interest has developed recently in the analysis of

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**TABLE 3. Bile Acids and Outcome, Study Admission Samples**

<table>
<thead>
<tr>
<th>Bile Acid</th>
<th>AUC</th>
<th>P Value, AUC</th>
<th>Sens (95% CI) at 90% Spec</th>
<th>Spec (95% CI) at 90% Sens</th>
<th>OR (95% CI)</th>
<th>P Value, OR</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALT</td>
<td>0.581</td>
<td>&gt;0.05</td>
<td></td>
<td></td>
<td>1.000</td>
<td>&gt;0.05</td>
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<tr>
<td>Peak MELD</td>
<td>0.762</td>
<td>0.001</td>
<td>0.320 (0.150–0.535)</td>
<td>0.429 (0.245–0.628)</td>
<td>1.104</td>
<td>0.004</td>
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<tr>
<td>LCAa</td>
<td>ND</td>
<td>ND</td>
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<td>ND</td>
<td>ND</td>
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<tr>
<td>UDCA</td>
<td>0.486</td>
<td>&gt;0.05</td>
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<td></td>
<td>1.711</td>
<td>&gt;0.05</td>
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<tr>
<td>CDCAa</td>
<td>ND</td>
<td>ND</td>
<td></td>
<td></td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>DCA</td>
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<td>&gt;0.05</td>
<td></td>
<td></td>
<td>1.705</td>
<td>&gt;0.05</td>
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<tr>
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<td>&gt;0.05</td>
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<td></td>
<td>2.045</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>TCA</td>
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<td>&gt;0.05</td>
<td></td>
<td></td>
<td>1.011</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>GCDCA</td>
<td>0.532</td>
<td>&gt;0.05</td>
<td></td>
<td></td>
<td>1.000</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>GDCA</td>
<td>0.702</td>
<td>0.004</td>
<td>0.419 (0.246–0.609)</td>
<td>0.200 (0.077–0.386)</td>
<td>1.137</td>
<td>0.006</td>
</tr>
<tr>
<td>GCA</td>
<td>0.432</td>
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<td></td>
<td></td>
<td>0.996</td>
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<tr>
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</tr>
<tr>
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<td></td>
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<td>&gt;0.05</td>
</tr>
<tr>
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<td></td>
<td></td>
<td>1.002</td>
<td>&gt;0.05</td>
</tr>
</tbody>
</table>

*Not detectable in most samples. Not determined, ND; model for end-stage liver disease, MELD; odds ratio, OR; area under the curve, AUC; total bile acids, TBA; bile acids defined in text.*

**TABLE 4. Bile Acids and Outcome, Peak ALT Samples**

<table>
<thead>
<tr>
<th>Bile Acid</th>
<th>AUC</th>
<th>P Value, AUC</th>
<th>Sens (95% CI) at 90% Spec</th>
<th>Spec (95% CI) at 90% Sens</th>
<th>OR (95% CI)</th>
<th>P Value, OR</th>
</tr>
</thead>
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<tr>
<td>ALT</td>
<td>0.614</td>
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<td></td>
<td></td>
<td>1.000</td>
<td>&gt;0.05</td>
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<td>Peak MELD</td>
<td>0.762</td>
<td>0.001</td>
<td>0.320, 0.150–0.535</td>
<td>0.429, 0.245–0.628</td>
<td>1.104</td>
<td>0.004</td>
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<tr>
<td>LCAa</td>
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<td>ND</td>
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<td></td>
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<tr>
<td>UDCAb</td>
<td>0.527</td>
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<td></td>
<td>0.476</td>
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<td>ND</td>
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<td></td>
<td>1.621</td>
<td>&gt;0.05</td>
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<tr>
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</tr>
<tr>
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<td>&gt;0.05</td>
<td></td>
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<td>1.011</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>GCDCA</td>
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<td>&gt;0.05</td>
<td></td>
<td></td>
<td>1.001</td>
<td>&gt;0.05</td>
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<td>GDCA</td>
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<td>0.010</td>
<td>0.355, 0.192–0.546</td>
<td>0.065, 0.008–0.167</td>
<td>1.125</td>
<td>0.005</td>
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<td>0.993</td>
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<tr>
<td>TDCA</td>
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<td>1.037</td>
<td>&gt;0.05</td>
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<tr>
<td>TCDCA</td>
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<td></td>
<td>0.981</td>
<td>&gt;0.05</td>
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<tr>
<td>TBA</td>
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<td>&gt;0.05</td>
<td></td>
<td></td>
<td>1.001</td>
<td>&gt;0.05</td>
</tr>
</tbody>
</table>

*Not detectable in most samples.

*One outlier (>7 SD from mean) was excluded; this changed the sign of the logistic regression coefficient, but did not change the results of AUC or OR significance tests. Not determined, ND; model for end-stage liver disease, MELD; odds ratio, OR; area under the curve, AUC; total bile acids, TBA; bile acids defined in text.*
specific bile acids that compose a portion of this milieu as UPLC or HPLC/MS methods have been developed that allow for the rapid detection of multiple bile acids simultaneously in serum (Ainouti et al., 2008). As serum bile acid levels are also elevated and associated with disease progression in NASH patients (Bechmann et al., 2013), studies assessing individual bile acids in populations such as these may yield novel prognostic markers that can differentiate between benign and advanced forms of disease. More research is necessary on other etiologies of liver disease to determine whether individual bile acids such as GDCA or others directly correlate with disease progression and outcome.

Increased Serum Bile Acid Levels as a Source of Injury In vivo

An increase in circulating bile acid levels during liver injury is established in multiple models of injury in addition to APAP overdose (Bechmann et al., 2013; Luo et al., 2014; Peán et al., 2013; Yamazaki et al., 2013). In this study, we surprisingly found that serum bile acid levels were significantly increased up to 5-fold higher in AALF patients versus patients with CLI. This included significant increases in bile acids such as GDCA that are commonly thought to be a direct cause of CLI in man (González et al., 2011; Malhi et al., 2011; Woolbright and Jaeschke, 2012).

This raises the question whether systemic increases in bile acid levels can be a direct cause of liver injury, or whether they are just an indicator of liver dysfunction. The current study supports the conclusion that increases in serum bile acid levels during liver injury do not directly cause cell death. The role of bile acids in liver injury has been most studied with regard to their role in CLI. Serum or plasma concentrations of hydrophobic toxic bile acids have been suggested to be a major mediator of injury during cholestasis (Jang et al., 2012), NASH (Bechmann et al., 2013), and regeneration during massive liver injury via partial hepatectomy (Péan et al., 2013). Although the predominant hypothesis in the field of CLI is that the accumulation of bile acids in serum and liver, directly results in cell death (Guicciardi et al., 2013), recent work has cast some doubt on this hypothesis (Trottier et al., 2011; Woolbright et al., 2013; Zhang et al., 2012). Concentrations of approximately 40 μM GDCA are known to cause apoptosis of rat hepatocytes in vitro (Spivey et al., 1993). This led to the hypothesis that accumulation of low levels of bile salts such as GDCA results in apoptosis that drives CLI (reviewed in Guicciardi et al., 2013). Data in the current study dispute this idea. Concentrations of all bile acids in human cholestatic patients were significantly below 40 μM in this study, indicating it is unlikely hepatocytes are exposed to such high concentrations of toxic bile acids at the basolateral surface during cholestasis (Figure 3). In fact, AALF patients had serum GDCA concentrations of almost 25 μM, over 4 times the concentrations of approximately 6 μM in cholestatic patients. Despite these significant elevations in toxic bile acids, AALI is known to be an almost entirely necrotic pathology in human patients (Antoine et al., 2012; McGill et al., 2012) and human hepatocytes (Xie et al., 2014), and the mechanism of APAP-induced liver injury is not generally thought to involve increases in bile acid levels. In fact, in rodents, bile acids serve a protective role as depletion of bile acids by cholesteryamine enhances injury, whereas feeding CA enhances regeneration by sustaining glutathione levels (Bhushan et al., 2013), which is known to be protective (Ni et al., 2012). Moreover, human hepatocytes are apparently resistant to bile acid-induced apoptosis, as doses used to cause injury in vitro to primary human hepatocytes are typically 10-fold higher than those used to cause injury in rodent hepatocytes (Galle et al., 1990; Gonzalez et al., 2011; Spivey et al., 1993). Acutely, it requires in vitro administration of concentrations of toxic bile acids similar to biliary concentrations before human or mouse hepatocytes undergo necrosis, with little evidence for apoptotic cell death (Woolbright et al., 2014a). Thus, it seems unlikely that serum concentrations of bile acids contribute “as a direct cause of CLI,” or in any other disease with a mild increase in serum bile acid levels. Rather, if intrahepatic bile acid accumulation is the major cause of injury during cholestasis, the source of the bile acid accumulation must be due to either rupture of the biliary tracts where bile acids are present in millimolar quantities, or direct inhibition of bile acid export from hepatocytes for toxic concentrations to reach these levels (Jennitz et al., 2010; Padda et al., 2011; Woolbright et al., 2014a). As such, increases in serum bile acid levels in man during CLI (as well as other forms of liver injury) are likely an effect of liver injury and dysfunction, but not a cause, and do not directly result in cell death. Although increased serum bile acid levels may affect pathology in some models, serum bile acid concentrations alone are likely insufficient to directly cause injury in vivo.

Summary and Conclusions

Our data indicate that serum or plasma concentrations of individual bile acids hold some potential as biomarkers of liver injury and dysfunction, and moreover, GDCA may be a viable prognostic indicator of the course of APAP toxicity, especially if combined with other prognostic indicators. Furthermore, these data suggest a re-evaluation of serum bile acids as a source of pathology, as their role during liver injury may be far more complex. In conclusion, further studies may be warranted on the use of GDCA as a prognostic biomarker for...
AALF outcome clinically either alone or as part of a suite of tests.

**SUPPLEMENTARY DATA**

Supplementary data are available online at http://toxsci.oxfordjournals.org.

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**REFERENCES**


