Circulating Acetaminophen Metabolites Are Toxicokinetic Biomarkers of Acute Liver Injury

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Acetaminophen (paracetamol-APAP) is the most common cause of drug-induced liver injury in the Western world. Reactive metabolite production by cytochrome P450 enzymes (CYP-metabolites) causes hepatotoxicity. We explored the toxicokinetics of human circulating APAP metabolites following overdose. Plasma from patients treated with acetylcysteine (NAC) for a single APAP overdose was analyzed from discovery ($n = 116$) and validation ($n = 150$) patient cohorts. In the discovery cohort, patients who developed acute liver injury (ALI) had higher CYP-metabolites than those without ALI. Receiver operator curve (ROC) analysis demonstrated that at hospital presentation CYP-metabolites were more sensitive/specific for ALI than alanine aminotransferase (ALT) activity and APAP concentration (optimal CYP-metabolite receiver operating characteristic area under the curve (ROC-AUC): 0.91 (95% confidence interval (CI) 0.83–0.98); ALT ROC-AUC: 0.67 (0.50–0.84); APAP ROC-AUC: 0.50 (0.33–0.67)). This enhanced sensitivity/specificity was replicated in the validation cohort. Circulating CYP-metabolites stratify patients by risk of liver injury prior to starting NAC. With development, APAP metabolites have potential utility in stratified trials and for refinement of clinical decision-making.

Study Highlights

WHAT IS THE CURRENT KNOWLEDGE ON THE TOPIC?

☑ Acetaminophen overdose is common. Decisions regarding the need for treatment are frequently based on measurement of the blood acetaminophen concentration. However, acetaminophen must be metabolized to cause liver injury. Acetaminophen metabolites are present in the circulation after therapeutic dosing and overdose.

WHAT QUESTION DID THIS STUDY ADDRESS?

☑ Are circulating acetaminophen metabolites elevated with acute liver injury and can they predict injury better than acetaminophen parent drug concentration?

WHAT THIS STUDY ADDS TO OUR KNOWLEDGE

☑ Patients who developed acute liver injury had higher acetaminophen metabolites derived from the cytochrome P450 pathway that mediates toxicity. Hospital presentation metabolites were more sensitive and specific for liver injury compared with the parent drug.

HOW THIS MIGHT CHANGE CLINICAL PHARMACOLOGY OR TRANSLATIONAL SCIENCE

☑ Acetaminophen metabolites can predict liver injury and have potential utility in stratified trials and for refinement of clinical decision-making.

Acetaminophen (paracetamol, APAP) overdose is a common reason for attending the hospital and the leading cause of acute liver failure in the Western world.[1] In the United States, over 400,000 Emergency Department visits relating to APAP overdose were recorded between 2006 and 2010.[2] Annually in the UK, APAP overdose results in ~100,000 Emergency Department presentations and 50,000 hospital admissions,[3] and is the direct cause of death in around 150 people.[4]

The mechanism of acute liver injury (ALI) after APAP overdose is well defined and can be translated from rodents to humans using mechanistic biomarkers.[5] APAP is predominantly metabolized into nontoxic glucuronide (APAP-Glu) and sulfate (APAP-Sul) conjugates. A small fraction is metabolized by cytochrome P450 (CYP) enzymes into the reactive metabolite *N*-acetyl-p-benzoquinone imine (NAPQI). When NAPQI is formed it reacts with the cysteine sulfhydryl group on glutathione (GSH). Most APAP-GSH is subsequently converted into APAP-cysteine (APAP-Cys) and APAP-mercapturate (APAP-Mer) conjugates.[6] APAP metabolites are detectable in plasma from healthy volunteers after therapeutic doses and in patients after APAP overdose.[7–10] They rapidly increase after ingestion of a therapeutic dose, with APAP-Glu having a higher concentration than the

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parent drug from 1–2 hours after ingestion.[6] Urinary metabolites of APAP can identify subjects with liver injury in the context of therapeutic dosing.[11]

In overdose, glutathione can become depleted and NAPQI can then bind to sulfhydryl groups in cellular proteins.[6] This may lead to oxidative stress, mitochondrial injury, hepatocyte necrosis, and acute liver failure. The protein binding of NAPQI results in APAP protein adducts that can be quantified by measurement of APAP-Cys that is released from the protein fraction of serum or plasma following protease enzyme treatment.[12] This is a distinct pool of APAP-Cys to the *in vivo* glutathione-derived metabolite that is present in the nonprotein fraction of the circulation. Glutathione-derived APAP-Cys is removed by dialysis in studies designed to quantify circulating APAP protein adducts.[13–15] APAP protein adducts are released from necrotic hepatocytes, although this remains controversial.[16] The focus of the present study was the metabolism of APAP as opposed to quantification of cell death. Therefore, we measured APAP-Cys in the nonadduct fraction of plasma.

The current antidote, acetylcysteine (NAC), replenishes cellular GSH and is effective at preventing liver injury if administered soon after overdose.[17,18] NAC could also directly bind to NAPQI, although this is not a significant pathway in rodents.[19] The decision to start treatment with NAC is commonly based on the dose ingested and a timed blood APAP measurement, which is interpreted using a binary treat/no treat nomogram with the threshold for treatment at a level of low risk. Current clinical practice, therefore, treats a number of patients who would not come to harm if they did not receive NAC.[20] Despite this conservative approach there are still patients who develop acute liver injury (ALI). Targeted therapies that reduce cell death and aid tissue regeneration are in development.[21,22] To facilitate stratified clinical trials there is an unmet need for new biomarkers of liver injury. These need to be accurate at early timepoints, when current markers lack sensitivity and specificity.[23]

Although the efficacy of NAC has been established for over 35 years, the optimal dosing regimen is still undetermined. The Scottish and Newcastle Antiemetic Pretreatment for Paracetamol Poisoning study (SNAP) compared the conventional intravenous NAC regimen with an identical NAC dose given in a modified (shorter) regimen.[24] Patients who had ingested a single acute overdose were randomized to one of four treatment arms: modified NAC regimen pretreated with the intravenous antiemetic ondansetron (ondansetron-modified) or pretreated with placebo (saline) (placebo-modified); or the conventional NAC regimen with or without ondansetron (ondansetron-conventional and placebo-conventional). The primary finding of the SNAP study was that the modified regimen resulted in substantially reduced vomiting, anaphylactoid reactions, and treatment interruptions. Although that study was not powered for efficacy, there was no significant difference in liver injury between modified and conventional regimens. However, unexpectedly, significantly more ondansetron-treated patients developed an elevation in serum alanine aminotransferase (ALT) activity compared to placebo. Given that APAP overdose and NAC therapy are commonly accompanied by nausea and vomiting, it is important to understand whether ondansetron worsens liver toxicity as even small increases in ALT could result in extra NAC treatment and avoidable increases in length of hospital stay.

The primary objective of this study was to define the relationship between circulating APAP metabolites and ALI. The secondary objective was to explore the effect of ondansetron on APAP metabolism to provide a mechanistic explanation for the increase in liver injury with this commonly used antiemetic.

RESULTS

The relationship between APAP metabolites and ALI (defined as an increased serum ALT activity of 50% or more) was investigated using serial samples collected in the SNAP trial (the discovery cohort). There was subsequent validation in samples taken at first presentation to two hospitals as part of the Markers and Paracetamol Poisoning (MAPP) study (the validation cohort). An overview of APAP metabolism is presented in **Figure 1**, with the metabolites measured in this study indicated. Patient screening and recruitment to the original SNAP trial, and the current discovery cohort, is presented in **Figure 2**. The characteristics of those patients with blood samples available for this study were similar across SNAP treatment groups aside from the higher incidence of liver injury in ondansetron-treated patients **(Supplementary Table 1)**, which mirrors the whole SNAP trial cohort.

Patients with and without ALI in the SNAP "discovery" cohort and the MAPP "validation" cohort are compared in **Tables 1 and 2**, respectively. In the time window of this study all patients in the discovery cohort received the same total dose of NAC, given either by the conventional or modified protocol. In both cohorts the increase in ALT was modest in those patients with ALI, with a median peak serum ALT activity of 154 U/L (65–909) and a median peak International Normalized Ratio (INR) of 1.4 (1.3–1.6) in the discovery cohort and 252 U/L (22–1256) and 1.2 (1.1–1.6) in the validation cohort. This increase in INR may reflect APAP inhibition of vitamin K-dependent activation of clotting factors rather than liver synthetic dysfunction.[25] There was no change in kidney function with ALI in either cohort as reported by change in serum creatinine concentration.

APAP metabolite kinetics

APAP parent drug concentration measured by liquid chromatography, tandem mass spectrometry (LC-MS/MS) correlated significantly with the value from the clinical laboratory APAP assay. The Pearson r value (95% confidence interval (CI)) was 0.88 (0.84–0.92), $P < 0.0001$ with a correlation coefficient (R^2) of 0.78 **(Supplementary Figure 1A)**. In the discovery cohort, the plasma APAP and metabolite concentrations at pretreatment and at 12 h and 20.25 h after the start of NAC treatment are presented in **Supplementary Figure 1B**. APAP-Glu was the metabolite with the highest concentration followed by APAP-Sul, APAP-Cys, APAP-Mer, and APAP-GSH. All metabolites decreased after the start of treatment, and only APAP-Glu was higher in concentration than APAP parent drug.

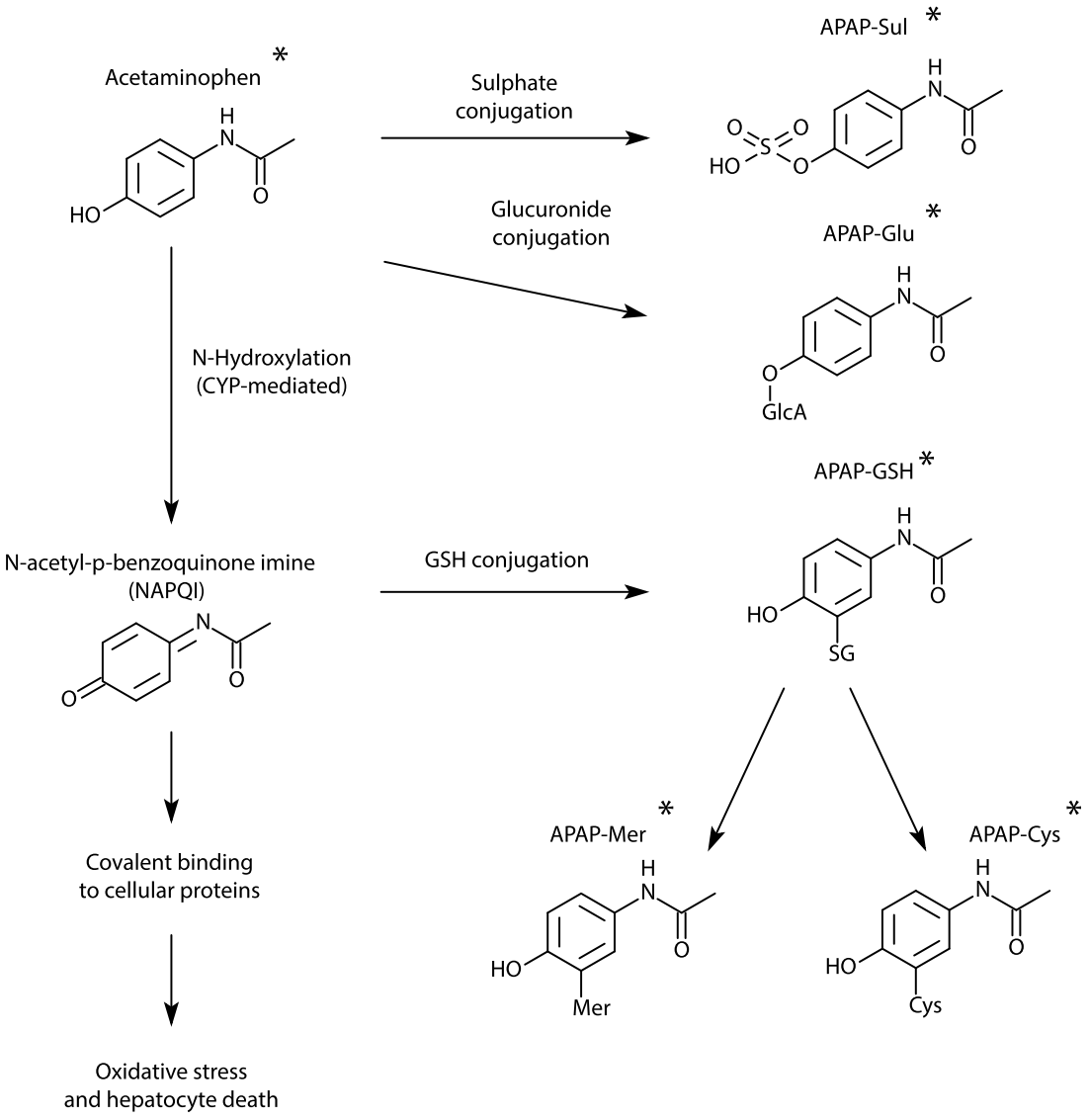

Figure 1 Pathways of acetaminophen (APAP) metabolism. APAP-sulphate (APAP-Sul); APAP-glucuronide (APAP-Glu); glutathione (GSH); APAP-glutathione (APAP-GSH); APAP-cysteine; (APAP-Cys); APAP-mercapturate (APAP-Mer). *Measured in this study.

Relationship between APAP metabolites and acute liver injury

Discovery cohort (SNAP). APAP half-life was longer in patients who developed liver injury compared to those with no injury: 3.11 h (2.38–4.38) vs. 2.36 h (2.02–2.68), $P = 0.004$ (**Figure 3a**). The concentrations of the APAP metabolites in patients without and with ALI are presented in **Supplementary Figure 2**. To compare the relative amount of metabolites formed by CYP activity compared to non-CYP conjugation, the $AUC_{(0\text{-}20.25h)}$ of CYP metabolites (APAP-Cys, APAP-Mer, APAP-GSH) was expressed as a fraction of the total $AUC_{(0\text{-}20.25h)}$ (CYP/total(%)). Patients who developed liver injury had a significantly higher $AUC_{(0\text{-}20.25h)}$ (CYP/total(%)) compared to those without liver injury (74 (58–746) vs. 47 (30–77), $P = 0.003$) (**Figure 3b**). $AUC_{(0\text{-}20.25h)}$ (CYP/total(%)) had a significant correlation with peak hospital stay ALT (**Figure 3c**).

APAP parent drug is used in clinical practice to stratify patients at hospital presentation. To explore the prognostic potential of metabolites formed by CYP activity the plasma concentration of the CYP metabolites (APAP-Cys, APAP-Mer, APAP-GSH) at pretreatment (0 h) were expressed as a fraction of the total metabolites (CYP/total (%)). Patients who developed liver injury had a significantly higher CYP/total (%) at pretreatment compared to those who did not develop liver injury, 2.21% (1.05–4.50) vs. 0.87% (0.58–1.43), $P = 0.0004$ (**Figure 3d**). The absolute concentration of APAP-Cys was significantly higher pretreatment with NAC in those patients with subsequent ALI (**Supplementary Figure 2**). Pretreatment CYP/total (%) remained higher in those patients who developed liver injury when the discovery cohort was censored by time from overdose to blood sampling (<8 h: ALI 3.12% (1.00–8.11) vs. no ALI 0.91% (0.59–1.40), $P = 0.006$; >8 h: ALI 2.16% (1.18–4.43) vs. no ALI 0.75% (0.50–1.70), $P = 0.05$).

The performance of each metabolite in the discovery cohort, alone and combined, was compared with regard to predicting

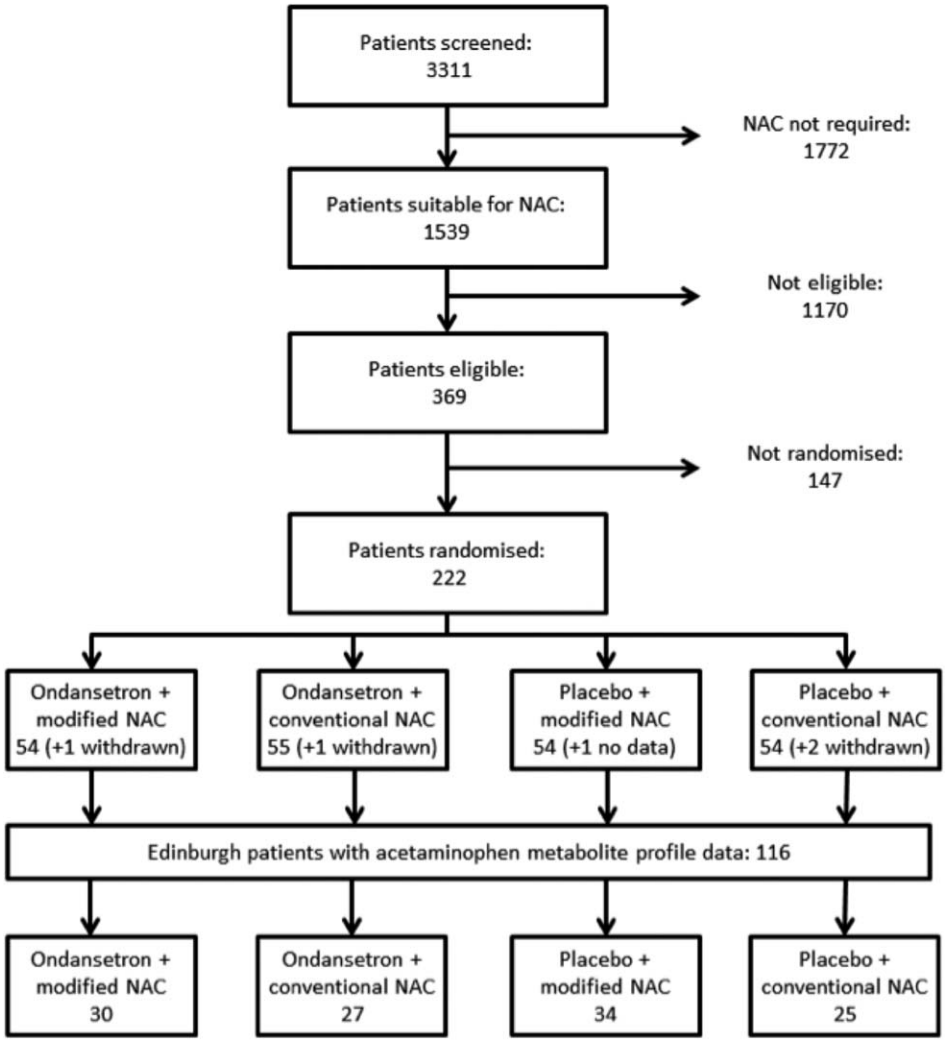

Figure 2 Study profile. The number of patients screened, suitable for NAC, eligible, and randomized into the original SNAP trial together with the number of patients (116) and their respective treatment arms in whom APAP metabolites were measured.

ALI at pretreatment using receiver operator characteristic analysis (ROC) (**Table 3**). The CYP metabolites had a superior predictive performance in comparison with the current markers (ALT and APAP parent drug had ROC-AUC of 0.67 (0.50–0.84) and 0.50 (0.33–0.67), respectively) **(Supplementary Figure 3a–d)**. In this discovery cohort the optimal metabolite combination was the ratio of APAP-Cys (CYP mediated) and APAP-Sul (non-CYP mediated), with an ROC-AUC of 0.91 (0.83–0.98). This metabolite combination at presentation had a significant correlation with peak ALT activity **(Supplementary Figure 3e)**.

Validation cohort (MAPP). The validation cohort consisted of 150 patients recruited from two geographically distinct hospitals, different from the site of recruitment for the discovery cohort. In blood samples collected at first presentation to hospital after single APAP overdose (before NAC was commenced), CYP/total (%) in those patients who developed liver injury was significantly higher compared to those who did not develop liver injury (0.95% (0.46–1.78) vs. 0.53% (0.34–0.84), $P = 0.02$) **(Supplementary Figure 3f)**. APAP-Cys and APAP-Mer were significantly higher in those patients with subsequent ALI **(Supplementary Figure 2)**.

Consistent with the results from the discovery cohort, CYP metabolites had superior predictive performance in comparison with the current standard markers (**Table 3**). In the validation cohort the sum of all the CYP metabolites had the largest ROC-AUC (0.83 (0.71–0.94)). As in the discovery cohort, APAP and ALT had no predictive value as assessed by ROC analysis (APAP ROC-AUC 0.57 (0.41–0.73); ALT ROC-AUC 0.51 (0.35–0.67)).

Effect of ondansetron on APAP metabolism

In the SNAP trial, patients pretreated with ondansetron had a higher incidence of liver injury that may reflect an effect on APAP metabolism. However, when liver injury patients were excluded, there was no difference in APAP half-life with ondansetron treatment compared to placebo, 2.48 h (2.07–2.97) vs. 2.23 h (1.97–2.56), $P = 0.10$. There was also no difference in $AUC_{(0\text{-}20.25h)}$ (CYP/total (%)) when ondansetron was compared to placebo (ondansetron: 54 (34–93) vs. placebo 43 (25–70), $P = 0.15$).

Table 1 Patient characteristics of the discovery cohort divided by absence or presence of acute liver injury according to the British National Formulary 2009[37]

	>50% ALT rise	No > 50% ALT rise	*P*-value
Number	14	102	
Median (IQR) age (years)	28 (21-32)	37 (26-48)	**0.03**
Median (IQR) weight (kg)	62 (56-71)	70 (59-80)	0.27
Number of females	11 (79%)	61 (55%)	0.17
Median (IQR) time from ingestion to treatment (h)	6.7 (7.8-10.7)	7.2 (8.0-10.0)	0.63
Number with ingestion to treatment <8hr	8 (57%)	65 (64%)	0.63
Median (IQR) ingested acetaminophen (mg/kg)	332 (190-393)	222 (165-313)	0.06
Number who ingested acetaminophen ≥16g	9 (64%)	44 (43%)	0.14
Median (IQR) admission alanine aminotransferase (U/L)	24 (18-82)	18 (13-26)	**0.04**
Median (IQR) peak alanine aminotransferase (U/L)	154 (65-909)	18 (14-27)	**< 0.0001**
Median (IQR) admission INR	1.0 (1.0-1.2)	1.0 (0.9-1.0)	**0.01**
Median (IQR) peak INR	1.4 (1.3-1.6)	1.1 (1.0-1.2)	**< 0.0001**
Median (IQR) admission billirubin (μmol/l)	12 (7-17)	7 (5-9)	**0.009**
Median (IQR) admission GGT (U/l)	19 (12-42)	25 (16-42)	0.25
Median (IQR) admission creatinine (μmol/l)	69 (59-81)	65 (59-74)	0.44
Median (IQR) peak creatinine (μmol/l)	70 (59-81)	67 (60-79)	0.68
Median (IQR) change in creatinine (%)	-5.4 (-22.8-3.1)	-6.0 (-12.6-1.7)	0.46
Alcohol ingested	1 (7%)	59 (58%)	**< 0.0001**
Other drugs ingested	9 (64%)	67 (66%)	0.92
Nutritional deficiency	2 (14%)	17 (17%)	0.82
Debilitating disease	0 (0%)	2 (2%)	0.60
Chronic alcohol use	0 (0%)	44 (43%)	**0.002**
Identified as high risk	2 (14%)	57 (56%)	**0.004**
Number who received ondansetron	11 (79%)	46 (45%)	**0.02**
Number who received modified NAC	7 (50%)	57 (56%)	0.68

P-value for difference between groups was determined by Mann-Whitney test or chi-square test.

APAP-Cys/APAP-Sul was higher in the pretreatment blood sample from patients randomized to ondansetron compare to placebo. *Post hoc* analysis of the SNAP trial by logistic regression modeling demonstrated that when APAP-Cys/APAP-Sul was added to the stratified randomization process the incidence of ALI in the ondansetron treated patients was not different from placebo (**Table 4**).

Effect of modified NAC regimen on APAP metabolism

There was no difference in APAP half-life or $AUC_{(0\text{-}20.25h)}$ (CYP/total(%)) between SNAP trial conventional and modified NAC treatment (half-life: 2.19 h (1.97–2.54) vs. 2.44 h (2.08–2.84), $P = 0.08$. $AUC_{(0\text{-}20.25h)}$ (CYP/total (%)) 42 (32–89) vs. 53 (26–76), $P = 0.95$).

DISCUSSION

This study demonstrates that the cytochrome P450 enzyme-mediated mechanism of APAP toxicity described in rodent models translates to humans. The key novel findings were that a higher percentage of circulating metabolites formed by cytochrome P450 enzymes (CYP metabolites) were present in patients with liver injury and these metabolites were superior to both ALT and APAP with regard to early ALI risk stratification. The potential value of CYP metabolites to future clinical trials was demonstrated by their incorporation *post-hoc* into the SNAP trial. This showed that the reported increase in ALI with ondansetron was no different than placebo. This work has the potential to be built on and produce an important change in the management of APAP overdose—a very common medical emergency with suboptimal tools for patient stratification.

We measured five APAP metabolites (two non-CYP-mediated and three CYP-mediated) alongside APAP parent drug. APAP half-life was 2–2.5 h in patients who did not develop ALI and was prolonged to over 3 h in people with ALI. The prolongation of APAP half-life was smaller than reported in previous studies

Table 2 Patient characteristics of the validation cohort divided by absence or presence of acute liver injury (>50% increase in ALT)

	>50% ALT rise	No. >50% ALT rise	*P*-value
Number	19	131	
Median (IQR) age (years)	41 (19-65)	36 (22-48)	0.60
Number of females	12 (63%)	86 (72%)	0.83
Median (IQR) time from ingestion to sampling (h)	5.0 (4.0-8.0)	5.5 (4.0-13.25)	0.65
Number with ingestion to treatment <8hr	13 (57%)	94 (64%)	0.76
Median (IQR) ingested acetaminophen (gram)	13 (22-35)	15 (9-21)	**0.01**
Number who ingested acetaminophen ≥16g	11 (58%)	58 (44%)	0.27
Median (IQR) admission alanine aminotransferase (U/L)	18 (12-34)	18 (13-28)	0.86
Median (IQR) peak alanine aminotransferase (U/L)	252 (22-1256)	19 (14-28)	**< 0.0001**
Median (IQR) admission INR	1.0 (1.0-1.0)	1.1 (1.0-1.2)	**0.0004**
Median (IQR) peak INR	1.1 (1.0-1.1)	1.2 (1.1-1.6)	**< 0.0001**
Median (IQR) admission billirubin (μmol/l)	8 (6-15)	5 (5-9)	**0.04**
Median (IQR) admission GGT (U/l)	26 (15-40)	17 (13-47)	0.88
Median (IQR) admission creatinine (μmol/l)	59 (48-68)	57 (51-68)	0.99
Median (IQR) peak creatinine (μmol/l)	64 (55-78)	61 (55-70)	0.70
Median (IQR) change in creatinine since admission (%)	2.3 (-5.9-16.7)	-2.0 (-12.6-8.2)	0.14

P-value for difference between groups was determined by Mann-Whitney test or chi-square test.

(half-life up to 6.9 h), which is likely due to their patients having more severe ALI, as indicated by an ALT activity of ≥1,000 U/l.[6] The present study suggests that mild ALI is associated with a reduction in the capacity to metabolize APAP. This increase in half-life might reflect an intrinsic lower capacity to metabolize APAP that results in liver injury after overdose due to increased production of NAPQI. Alternatively, liver injury may cause a lower metabolic capacity. In this study there was no evidence of a difference in renal function between those patients with and without ALI, which otherwise could have affected metabolite clearance. APAP-Glu and APAP-Sul (formed through phase II non-CYP metabolism) were the highest concentration APAP metabolites in the circulation.[6,26,27] In previously published studies, about one-third of APAP was metabolized into APAP-Sul and two-thirds into APAP-Glu. The APAP-Sul pathway becomes saturated even at therapeutic doses[26,27] and the higher capacity of the APAP-Glu pathway is likely to explain the higher circulating concentration of APAP-Glu, which is in agreement with earlier reports.[6,7]

Current practice worldwide is to measure plasma or serum APAP as a central part of risk stratification after overdose. However, APAP *per se* is relatively nontoxic without CYP-mediated metabolism.[28,29] The CYP generated reactive metabolite, NAPQI, mediates ALI following APAP overdose.[30] Therefore, biomarkers that report activity of CYP-mediated APAP metabolism may, theoretically, refine patient care pathways. *A priori*, it could be hypothesized that APAP-GSH, APAP-Cys, and/or APAP-Mer would be either higher in those with liver injury because of increased CYP metabolism or lower because of reduced glutathione bioinactivation of NAPQI. This study demonstrates that patients with ALI have a relatively higher circulating fraction of CYP metabolites compared to phase II metabolites. Importantly, from a clinical perspective, prior to NAC treatment the fraction of CYP-mediated metabolites was higher in people who subsequently developed ALI. Although all patients included in this study received NAC treatment following measurement of their plasma APAP concentration, the absolute value of APAP had no predictive value for the development of subsequent ALI. We chose not to interpret APAP with regard to time from overdose—such as by creating multiple nomogram lines[31]—to facilitate head-to-head comparison with metabolites measured in the same sample. By contrast with APAP, the CYP metabolite APAP-Cys was able to predict the onset of ALI with an ROC-AUC of 0.75 in the discovery cohort and an ROC-AUC of 0.82 in the validation cohort. APAP-Cys is commonly used as a surrogate measure of circulating APAP-protein adducts. In this study the protein fraction was removed prior to mass spectrometry, which distinguishes it from the protocol used for adduct measurement. Therefore, the data presented in this article are likely to accurately reflect APAP-Cys derived from glutathione conjugation with NAPQI. When the ratio of APAP-Cys and APAP-Sul was calculated, prediction accuracy was further increased to an ROC-AUC of 0.91 in the discovery cohort. The optimal measure of CYP metabolism remains to be determined by future larger studies.

Multiple new biomarkers of hepatocyte injury,[5] inflammation,[32] tissue regeneration,[21] and kidney injury[33] have recently been identified. These markers have high sensitivity and specificity for reporting injury or assessing prognosis (depending on the

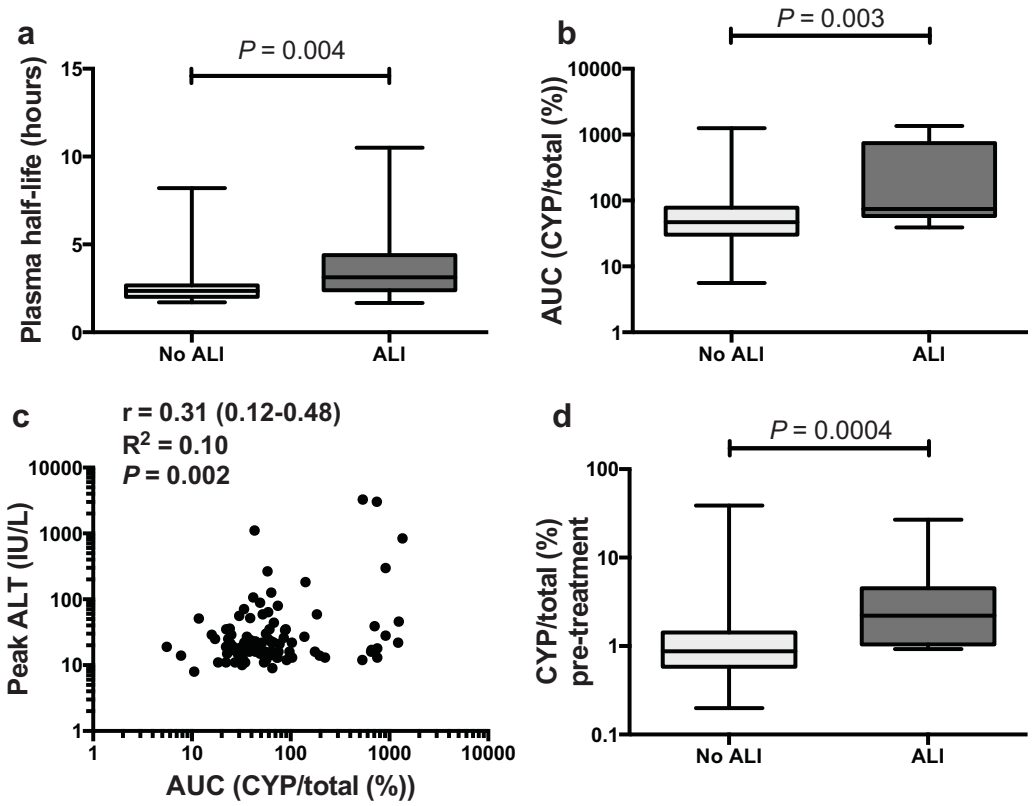

Figure 3 Discovery cohort. (**a**) APAP half-life in patients developing acute liver injury (ALI, as defined by >50% ALT increase) (n = 14) and those with no injury (no ALI) (n = 102). (**b**) Area under the curve (AUC) for the proportion of total metabolites formed by CYP enzyme activity (CYP/Total(%)) from time 0 to 20.25 h after starting NAC in liver injury (ALI) (n = 14) and nonliver injury (No ALI) patients (n = 102). (**c**) Correlation between the AUC for the proportion of total metabolites formed by CYP enzyme activity (CYP/Total(%)) from time 0 to 20.25 h after starting NAC and peak hospital stay serum alanine transaminase (ALT) activity (n = 116). (**c**) At pretreatment before NAC and ondansetron or placebo. Metabolites formed by CYP enzyme activity are expressed as a proportion of total circulating metabolites (CYP/total (%)) in liver injury (n = 14) and nonliver injury patients (n = 102). In (**a,b,d**): boxes show median ± IQR, whiskers represent range.

context of use). CYP metabolites complement these markers by offering potential refinement of risk stratification beyond the measurement of APAP parent drug. It has been proposed that the product of the APAP concentration and serum ALT activity can determine the risk of liver injury independent of time from drug ingestion.[34] A combination of CYP metabolites and one or more highly sensitive liver injury markers (such as miR-122) promises to more accurately identify patients at risk of liver injury despite NAC treatment. Using this new combination in routine clinical practice still requires development. However, in the context of clinical trials these markers may offer value in the near future. The potential for refinement of clinical trials was demonstrated when the SNAP trial results were reanalyzed with the pre-ondansetron treatment ratio of APAP-Cys/APAP-Sul included. In this refined analysis the apparent increase in ALI reported with ondansetron was not different from placebo. This is because the more sensitive CYP pathway biomarker demonstrated that, by chance, more patients with a toxic metabolite profile were randomized to ondansetron compared to placebo. Future clinical trials of novel therapeutic strategies could be enhanced by measurement of CYP metabolites that may identify patients who will develop ALI despite treatment with NAC. For both CYP metabolites and other novel biomarkers to be useful in clinical trials, a key roadblock to be overcome is the development and validation of point-of-care assays that can provide measurements within the timeframe for patient identification and trial recruitment.[35]

Our data are from an early-phase discovery study that only included patients treated with NAC (because they had serial blood tests). Future work will include those patients deemed not to require treatment after overdose based on interpretation of the currently used biomarkers. In this study we interpreted the APAP metabolite data at first presentation without regard for time from ingestion. In the future, nomograms may be developed that are analogous to the APAP treatment lines. The increase in ALT used to define our primary outcome of ALI (50% rise) was modest. This was chosen by the SNAP trialists before this randomized clinical trial started and so was also used in this follow-up biomarker discovery study. The incidence of larger increases in ALT (such as >1,000 U/L) was too low in both our discovery and validation cohorts for robust analysis. Therefore, future studies will be needed to define the relationship between CYP metabolites and more severe injury.

In summary, circulating APAP metabolites formed by CYP enzymes are toxicokinetic biomarkers that stratify patients by their risk of subsequent ALI prior to starting NAC. With

Table 3 Predictive accuracy of current and new biomarkers compared to ROC-AUC = 0.5

	Discovery cohort N = 116					Validation cohort N = 150				
Metabolite/ Biomarker	ROC-AUC (95% CI)	P value	SENS (95% CI)	PPV (%)	NPV (%)	ROC-AUC (95% CI)	P value	SENS (95% CI)	PPV (%)	NPV (%)
APAP-CYS/ APAP-Sul	0.91 (0.83-0.98)	**< 0.0001**	0.71 (0.42-0.92)	50	96	0.76 (0.63-0.88)	**0.0003**	0.43 (0.35-0.52)	38	92
CYP%	0.78 (0.67-0.90)	**0.0006**	0.36 (0.13-0.65)	33	91	0.66 (0.51-0.82)	**0.02**	0.11 (0.06-0.17)	14	87
Sum CYP metabolites	0.75 (0.61-0.88)	**0.003**	0.48 (0.38-0.58)	40	93	0.83 (0.71-0.94)	**< 0.0001**	0.44 (0.36-0.53)	39	92
APAP-CYS	0.75 (0.61-0.88)	**0.003**	0.36 (0.13-0.65)	33	91	0.82 (0.71-0.94)	**< 0.0001**	0.44 (0.35-0.52)	39	92
INR	0.70 (0.54-0.86)	**0.03**	0.23 (0.05-0.54)	24	89	0.71 (0.57-0.85)	**0.005**	0.07 (0.03-0.13)	9	87
ALT	0.67 (0.50-0.84)	**0.04**	0.29 (0.08-0.58)	28	90	0.51 (0.35-0.67)	0.86	0.16 (0.03-0.40)	19	88
APAP-Sul	0.65 (0.48-0.82)	0.06	0.50 (0.23-0.77)	41	93	0.53 (0.38-0.67)	0.75	0.11 (0.06-0.17)	14	87
APAP-Glu	0.63 (0.44-0.82)	0.11	0.36 (0.13-0.65)	33	91	0.61 (0.47-0.76)	0.11	0.11 (0.06-0.17)	14	87
APAP-GSH	0.61 (0.46-0.76)	0.19	0.21 (0.05-0.51)	22	89	0.67 (0.61-0.74)	**0.004**	0.41 (0.21-0.64)	37	91
APAP-Mer	0.59 (0.40-0.77)	0.29	0.21 (0.05-0.51)	22	89	0.76 (0.62-0.90)	**0.0003**	0.26 (0.19-0.34)	27	89
APAP LC/MS	0.50 (0.33-0.67)	0.97	0.14 (0.02-0.43)	16	88	0.57 (0.41-0.73)	0.32	0.05 (0.02-0.11)	7	87
APAP hospital lab	0.55 (0.37-0.73)	0.78	0.00 (0.00-0.04)	0	87	0.58 (0.42-0.73)	0.29	0.09 (0.04-0.15)	12	87

The positive and negative predictive values (PPV and NPV, respectively). Table with ROC-AUC (area under the curve with 95% CI), sensitivity (at 90% specificity) with 95% CI, and statistical significance for different metabolites measured at pretreatment in the discovery and hospital presentation in the validation cohort. *P*-value represents significance level are also presented for each metabolite/biomarker.

development, there is the potential for enhanced patient identification for entry into clinical trials of novel treatment pathways and refined clinical decision-making.

METHODS

Patients

All patients were treated with NAC for a single acute APAP overdose. To determine the need for NAC treatment, plasma APAP concentration was measured by the Paracetamol Assay from Cambridge Life Sciences (Cambridgeshire, UK) in the clinical biochemistry laboratories at each center. The APAP concentration was interpreted using the contemporaneous UK APAP treatment nomogram.

Discovery cohort. Patients from the SNAP trial (EudraCT number 2009-017800-10) were recruited at the Royal Infirmary of Edinburgh (RIE), UK. Details of the full SNAP protocol are reported in Thanacoody *et al.*[36] In brief, patients were eligible for entry into the SNAP trial if they presented within 36 h of a single acute APAP overdose and required treatment with NAC, based on standard UK guidance for management. Full informed consent was obtained and the study was approved by the UK Medicines and Healthcare products Regulatory Agency and the Scotland A Research Ethics Committee, UK (ref. no. 10/MRE00/20).

Plasma EDTA blood samples were collected before ("pretreatment"), 12 h and 20.15 h after the start of conventional or modified NAC treatment (with intravenous ondansetron or placebo treatment immediately after the pretreatment blood draw). Plasma was separated and the samples were stored at −80°C until analysis. For all study participants, demographics and blood results were recorded.

Validation cohort. Adult patients (16 and over in Scotland, 18 and over in England) were recruited to the MAPP study if they fulfilled the study inclusion and exclusion criteria. Full informed consent was obtained from every participant and ethical approval for this study was from the South East Scotland Research Ethics Committee and the East of Scotland Research Ethics Committee via the South East Scotland Human Bioresource. Research nurses at each site identified participants on admission to hospital. The inclusion criteria were: a history of APAP overdose that the treating clinician judged to warrant treatment with intravenous NAC as per the contemporaneous UK guidelines; the first blood sample collected within 24 h of last APAP ingestion and the patient had the capacity to consent. Patients were excluded if any of the following applied: patient detained under the Mental Health Act (UK); patient has known cognitive impairment; inability to provide informed consent for any reason or an unreliable history of overdose. Patients having taken a single acute APAP overdose were recruited at St. Thomas Hospital London, UK (n = 59) and Aberdeen Royal Infirmary, UK (n = 91).

Primary endpoint

The primary endpoint was ALI; predefined by SNAP as a rise in serum ALT activity of 50% or more at 20.25 h compared to the hospital admission value.[36]

Table 4 Effect of APAP-Cys/APAP-Sul on acute liver injury in patients treated with ondansetron compared to placebo in SNAP trial

Model	Ondansetron versus placebo Odds ratio (95% CI), *P* value for developing ALI
Full SNAP trial, adjusted[a] as in Lancet paper[21]	0.303 (0.108, 0.851), 0.024
Full SNAP trial, unadjusted[b]	0.332 (0.124, 0.886), 0.028
This study subset of SNAP, unadjusted	0.211 (0.055, 0.801), 0.022
This subset, adjusted for APAP-Cys/APAP-Sul	0.465 (0.097, 2.226), 0.338

[a]Adjusted by the variables in the minimization algorithm, and center. [b]Obtained with a model in which only treatment and regimen were included.

Chemicals and reagents

High-performance liquid chromatography (HPLC)-grade methanol and water were from Fisher Scientific (Loughborough, UK). Acetic acid and formic acid were from Sigma-Aldrich (Gillingham, UK). APAP was from Apollo (Denton, Manchester, UK). APAP-Mer, APAP-GSH, APAP-Sul, APAP-d4 (APAP-d4), and APAP-sulphate-d3 (APAP-SUL-d3) were from Santa Cruz Biotechnology (Heidelberg, Germany). APAP-Cys and APAP-Glu were from CGeneTech (Indianapolis, IN).

Sample preparation and analysis by LC-MS/MS

APAP and metabolites were extracted from plasma by liquid–liquid extraction with acidified methanol. Briefly, 10 μL plasma was enriched with 10 ng APAP-d4 (APAP-d4) and 10 ng APAP-SUL-d3 as internal standards and 0.8 mL methanol (w/0.2% acetic acid) was added, vortexed, and incubated for 20 min on ice. After centrifugation (3000*g*, 10 min, 10°C) to pellet protein in the sample, the supernatant was reduced to dryness under nitrogen at 40°C and reconstituted in mobile phase (200 μL water/methanol (65:35, v/v)) and centrifuged for a second time.

Analysis was carried out by LC-MS/MS. Liquid chromatographic separation was achieved using an Aria CTC autosampler and Allegros pump on an ACE Excel 2 SuperC18 column (150 × 3 mm; 2 μm) protected by a Kinetex KrudKatcher (Phenomenex, UK) at 20°C and detected on a TSQ Quantum Discovery triple quadrupole mass spectrometer (Thermo Fisher Scientific, UK) operated by selective reaction monitoring. The mobile phase consisted of 0.1% formic acid in water and 0.1% formic acid in methanol at a flow rate of 0.3 mL/min. Gradient elution was achieved with a total run time of 9 min from 35% to 5%. The mass spectrometer was operated in polarity switching electrospray mode (300°C, 3 kV). In positive mode, transitions monitored for were m/z 152 → 110.0, 93.1 at 20 and 13 V and m/z 156.1 → 114.1, 97.1 at 15 and 22 V for APAP and APAP-d4, respectively. For the positively ionized APAP metabolites, APAP-Cys, APAP-Mer, and APAP-GSH, m/z 271.1 → 182.0, 207.6 at 8 and 9 V, m/z 313.0 → 140.1, 208.1 at 28 and 16 V and m/z 457.2 → 140 at 33 V were monitored.

For the negatively ionized APAP metabolites APAP-Sul, APAP-Glu, and the internal standard APAP-SUL-d3 m/z 229.8 → 107.0, 150.1 at 36 and 15 V, m/z 326.0 → 113.0, 150.0 at 28 and 16 V and m/z 233.0 → 109.5, 181.4 at 30 and 5 V were monitored.

Statistical analysis

All data are presented as median and interquartile range (IQR), except for ROC data, where 95% CIs are quoted. Comparisons were made using the Mann–Whitney *U*-test. All LC/MS-MS data were transformed from mass to molar concentrations before analyses were performed. APAP plasma half-life was estimated using a nonlinear fit, assuming first-order kinetics. All calculations and ROC analysis were performed using GraphPad Prism software (GraphPad Software, La Jolla, CA). Logistic regression models were run using SAS v. 9.4 (Cary, NC).

Additional Supporting Information may be found in the online version of this article.

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CONFLICT OF INTEREST/DISCLOSURE

The authors have no conflicts of interest to disclose.

AUTHOR CONTRIBUTIONS

J.W.D., A.D.B.V., and D.J.W. wrote the article; J.W.D., M.E., A.G., D.J.A., D.J.W., N.B., and D.N.B. designed the research; A.D.B.V., R.A.K., J.H.S., N.Z.H., A.G., D.M.W., P.D., J.G.C., and J.I.C. performed the research; J.W.D., J.I.C., D.J.A., S.C.L., and N.B. analyzed the data.

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