A Metabolomic Analysis Of Thiol Response For Standard And Modified N-Acetyl Cysteine Treatment Regimens In Patients With Acetaminophen Overdose

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ARTICLE

A metabolomic analysis of thiol response for standard and modified *N*-acetyl cysteine treatment regimens in patients with acetaminophen overdose

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Abstract

N-acetylcysteine (NAC) is an antidote to prevent acetaminophen (paracetamol-APAP)-induced acute liver injury (ALI). The 3-bag licensed 20.25 h standard regimen, and a 12 h modified regimen, are used to treat APAP overdose. This study evaluated the redox thiol response and APAP metabolites, in patients with a single APAP overdose treated with either the 20.25 h standard or 12 h modified regimen. We used liquid chromatography tandem mass spectrometry to quantify clinically important oxidative stress biomarkers and APAP metabolites in plasma samples from 45 patients who participated in a randomized controlled trial (SNAP trial). We investigated the time course response of plasma metabolites at predose, 12 h, and 20.25 h post-start of NAC infusion. The results showed that the 12 h modified regimen resulted in a significant elevation of plasma NAC and cysteine concentrations at 12 h post-infusion. We found no significant alteration in the metabolism of APAP, mitochondrial, amino acids, and other thiol biomarkers with the two regimens. We examined APAP and purine metabolism in overdose patients who developed ALI. We showed the major APAP-metabolites and xanthine were significantly higher in patients with ALI. These biomarkers correlated well with alanine aminotransferase activity at admission. Receiver operating characteristic analysis showed that at admission, plasma APAP-metabolites and xanthine concentrations were predictive for ALI. In conclusion, a significantly higher redox thiol response with the modified NAC regimen at 12 h postdose suggests this regimen may produce greater antioxidant efficacy. At baseline, plasma APAP and purine metabolites may be useful biomarkers for early prediction of APAP-induced ALI.

Study Highlights

WHAT IS THE CURRENT KNOWLEDGE ON THE TOPIC?

N-acetylcysteine (NAC) is an effective antidote used to prevent acetaminophen (APAP)-induced acute liver injury (ALI). The 12 h modified NAC regimen has a

James W. Dear and Mei Li Ng contributed equally to this work.

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lower rate of adverse effects than the 20.25 h standard NAC regimen. However, the effect of NAC regimen on redox thiol and APAP metabolism have not been studied.

WHAT QUESTION DID THIS STUDY ADDRESS?

What are the effects of modified and standard NAC regimens on circulating thiol biomarkers and APAP metabolites?

WHAT DOES THIS STUDY ADD TO OUR KNOWLEDGE?

Patients who received 12 h modified NAC treatment have significantly higher circulating cysteine concentration at 12 h postinfusion than those who received 20.25 h standard NAC regimen. At baseline, plasma APAP and purine metabolites were significantly higher in patients who developed ALI.

HOW MIGHT THIS CHANGE CLINICAL PHARMACOLOGY OR TRANSLATIONAL SCIENCE?

This study suggests the 12 h modified NAC regimen provides greater antioxidant protection to APAP overdose patients in this time frame allowing further NAC therapy to be targeted earlier to patients at risk. At baseline, plasma APAP and purine metabolites are predictive biomarkers for APAP-induced ALI.

INTRODUCTION

Acetaminophen (APAP) toxicity increases the risk of acute liver injury (ALI) due to excess accumulation of its reactive metabolite, *N*-acetyl-p-benzoquinone imine (NAPQI). In APAP overdose, hepatic glutathione (GSH) is depleted leading to oxidative stress, mitochondrial dysfunction, and cell injury.[1] *N*-acetylcysteine (NAC) is an effective antioxidant against NAPQI-induced liver injury.[2] Its antioxidant effect is mainly attributed to NAC bioconversion to cysteine, a precursor for hepatic GSH biosynthesis.[3] The UK 20.25 h standard (now increased in duration to 21 h) and 12 h modified i.v. NAC protocols are two common regimens used to treat overdose patients in the United Kingdom.[4] A previous randomized controlled trial has demonstrated that the modified regimen (Scottish and Newcastle antiemetic pretreatment for paracetamol poisoning study [SNAP] regimen) produces a lower rate of adverse reactions associated with NAC, such as retching, vomiting, and anaphylactoid reactions,[4] while having similar treatment efficacy at preventing APAP-induced ALI.[5] Reduction of adverse reactions with the modified regimen is achieved by slowing the initial loading dose and thereby reducing the mean peak NAC concentration.[6] NAC is a potent antioxidant that increases thiol biomarkers of oxidative stress.[7] The aim of this study was to compare the effect of standard and modified NAC regimens on circulating antioxidants and thiol biomarkers in patients who had taken an acute APAP overdose. This exploratory study also examined the endogenous metabolism of APAP that may lead to the discovery of novel mechanisms underpinning APAP-induced ALI. We used liquid chromatography tandem mass spectrometry (LC-MS/MS) metabolomics to quantify plasma metabolite concentrations at three timepoints, in a subset of patients from the SNAP study.[8] We investigated whether there was any significant difference in plasma metabolite concentration between the standard and modified NAC regimens. Finally, we examined plasma APAP metabolite concentrations at three timepoints and identified additional APAP metabolites that are predictive for ALI. Overall, this study suggests a greater antioxidant effect in the 12 h modified NAC regimen, and identifies exploratory biomarkers for ALI risk assessment.

METHODS

Study design and patient cohort

The SNAP study was approved by the Medicines and Healthcare Products Regulatory Agency, the Scotland A Research Ethics Committee, UK (ref no 10/MRE00/20; EudraCT number 2009-017800-10, ClinicalTrials.gov Identifier NCT01050270), to examine the frequency of adverse reactions associated with the modified 12 h modified NAC regimen in APAP overdose.[8] This study was designed as 2 × 2 factorial, double-blind, randomized controlled trial. The trial procedure and patient recruitment protocol have been published in full.[4,8] The study was conducted in accordance with the Declaration of Helsinki and Good Clinical Practice guidelines. These patients were initially recruited between September 6, 2010, until December 31, 2012, from 3 sites in the United Kingdom, the Royal Infirmary in Edinburgh, and the Royal Victoria Infirmary, Newcastle, and subsequently at Aberdeen Royal Infirmary. Inclusion criteria were patients admitted to the hospital within 36 h of a single acute APAP overdose and

received NAC treatment according to standard UK guidelines. Exclusion criteria were patients who were unable to consent or not suitable for participation. Full written informed consent was obtained from each patient prior to participation in the trial, conformed with study protocol approved by the UK Medicines and Healthcare products Regulatory Agency and the Scotland A Research Ethics Committee, UK (ref. no. 10/MRE00/20).[8] All patients were randomized, with concealed allocation, into four parallel treatment groups: ondansetron pretreatment and the shorter NAC regimen (ondansetron-modified); ondansetron and the standard schedule (ondansetron-standard); placebo and the shorter acetylcysteine protocol (placebo-modified); and placebo and the standard regimen (placebo-standard). Treatment procedures were assigned to patients and their baseline and clinical characteristics were collected (Table 1). All adverse events presented during the treatment, any use and timing of rescue drug, clinical outcome, and survival data were documented by clinic staff in electronic case report form (CRF). Three blood samples were collected in EDTA tubes, from each randomized group at baseline (predose), 12 h, and 20.25 h after starting NAC regimen (with the predose blood samples were collected immediately before ondansetron pretreatment or placebo). Plasma samples were extracted and stored at −80°C until further analysis. This study analyzed samples taken in the setting of an acute medical emergency. A strength of this study was that the samples were collected soon after presentation to the hospital. This means that it was impossible to fast the patient. The timing of the patient's last meal was, unfortunately, not recorded within the SNAP trial. Our study was performed in a subset number ($N = 45$) of the SNAP cohort in whom were randomized to either 12 h modified NAC ($n = 26$) or 20.25 h standard NAC ($n = 19$) regimen (Figure S1).

The primary outcome of the study was progression to ALI after acute paracetamol overdose. ALI is defined as an increased serum alanine aminotransferase (ALT) of 50% or more (ALT rise ≥50%) at 20.25 h compared with the hospital admission value. As a result, we identified a total number of 4 patients who developed ALI, 3 patients received the 12 h modified NAC, and 1 patient received the 20.25 h standard NAC regimen.

Data collection and blood sample

Baseline demographics and clinical characteristics were collected at the time of recruitment and are presented in Table 1. Adverse reactions and treatment information related to the study were collected during the treatment phase via self-assessment and as questionnaires done by the patient and recorded on the CRF by trained clinical or research staff. Blood samples were collected by clinical staff before and after initiation of NAC treatment, at baseline, at 12 h, and at 20.25 h timepoints. Blood samples were analyzed for paracetamol concentration, full blood count, urea, creatinine, electrolytes, and liver function tests, as previously described.[8] The data reported here are from patients in whom rapid processing and freezing of samples was possible, in order that the metabolites would be stable. These were 45 of the trial patients recruited in Edinburgh.

Chemicals and reagents, sample preparation, and LC-MS/MS analysis

Chemicals and reagents, sample preparation, and LC-MS/MS analytical procedure were described in Supplementary Text.

Statistical analysis

Numerical data are presented as mean ± SD, or median and interquartile range. Categorical data are presented as frequency and percentage (n [%]), except for receiver operating characteristic (ROC) data where 95% confidence intervals (CIs) are presented. The final quantitative metabolomics data were normalized by signal drift and batch corrected using a custom script. Metabolomics data were log-transformed to adjust for normality distribution. Pairwise statistic compared means of all metabolite concentrations between the two regimens was performed by nonparametric Mann–Whitney U test. Multiple groups comparison was performed by nonparametric Kruskal–Wallis test with Dunn-Bonferroni post hoc adjustment. All statistical analyses were performed by SPSS version 24.0 (SPSS Inc.).

RESULTS

Effect of NAC regimen on plasma cysteine and cystine concentrations

We analyzed the plasma concentration of various oxidative stress biomarkers in serum samples from patients treated with NAC following acute APAP overdose. The details of the redox effects of NAC administration, assessed at baseline (before NAC started but after APAP ingestion), 12 h, and 20.25 h postadministration, together with the concentrations of APAP, NAC and its metabolites (cysteine and cystine), amino acids, and other established oxidative stress biomarkers from purine, ergothioneine, acyl carnitine, and F2-isoprostane pathways are shown for the standard three-bag regimen (Table 2) and the modified NAC regimens (Table 3). We also compared the timepoint effect of each regimen on the biomarkers (Table S1).

TABLE 1 Baseline demographic and clinical characteristic

	N-acetylcysteine regimen		Ondansetron pretreatment					
	Modified ($n = 26$)	**Standard ($n = 19$)**	**Active ($n = 18$)**	**Placebo ($n = 27$)**	**Ondansetron-modified ($n = 11$)**	**Ondansetron-standard ($n = 7$)**	**Placebo-modified ($n = 15$)**	**Placebo-standard ($n = 12$)**
Demographics								
Edinburgh	26 (100%)	19 (100%)	18 (100%)	27 (100%)	11 (100%)	7 (100%)	15 (100%)	12 (100%)
Age, y	38 (30–53)	38 (34–50)	35 (30–52)	40 (34–53)	34 (29–51)	36 (32–45)	40 (32–56)	41 (36–48)
Weight, kg	64 (55–79)	64 (53–73)	60 (52–69)	67 (58–78)	57 (53–69)	60 (55–69)	68 (61–80)	66 (55–74)
Women	16 (62%)	12 (63%)	12 (67%)	16 (59%)	6 (55%)	6 (86%)	10 (67%)	6 (50%)
Male	10 (38%)	7 (37%)	6 (33%)	11 (41%)	5 (45%)	1 (14%)	5 (33%)	6 (50%)
Clinical characteristic								
Time from ingestion to treatment, <8 h	17 (65%)	12 (63%)	11 (61%)	18 (67%)	7 (64%)	4 (57%)	10 (67%)	8 (67%)
Ingested acetaminophen, mg/kg	206 (146–332)	267 (200–346)	219 (182–302)	233 (140–367)	185 (162–295)	223 (207–290)	222 (139–335)	284 (154–373)
Number who ingested acetaminophen ≥16 g	12 (46%)	9 (47%)	6 (33%)	13 (48%)	4 (36%)	2 (29%)	8 (53%)	5 (42%)
Alcohol ingested	9 (35%)	11 (58%)	9 (50%)	11 (41%)	5 (45%)	4 (57%)	4 (27%)	7 (58%)
Other drugs ingested	20 (77%)	13 (68%)	13 (72%)	18 (67%)	8 (73%)	5 (71%)	10 (67%)	8 (67%)
Benzodiazepines	3 (12%)	4 (21%)	0 (0%)	7 (26%)	0 (0%)	0 (0%)	3 (20%)	4 (33%)
Opioids	3 (12%)	4 (21%)	1 (6%)	6 (22%)	1 (9%)	0 (0%)	2 (13%)	4 (33%)
Non-opioid analgesics	10 (38%)	5 (26%)	6 (33%)	10 (37%)	4 (36%)	2 (29%)	6 (40%)	3 (25%)
SSRIs	2 (8%)	3 (16%)	1 (6%)	4 (15%)	0 (0%)	1 (14%)	2 (13%)	2 (17%)
NSAIDs	6 (23%)	4 (21%)	3 (17%)	7 (26%)	2 (18%)	1 (14%)	4 (27%)	3 (25%)
Tricyclic antidepressants	0 (0%)	1 (5%)	1 (6%)	0 (0%)	0 (0%)	1 (14%)	0 (0%)	0 (0%)
Cardiovascular drugs	0 (0%)	1 (5%)	0 (0%)	1 (4%)	0 (0%)	0 (0%)	0 (0%)	1 (8%)
Nutritional deficiency	5 (19%)	3 (16%)	4 (22%)	4 (15%)	1 (9%)	3 (43%)	4 (27%)	0 (0%)
Debilitating disease	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Chronic alcohol use	7 (27%)	9 (47%)	5 (28%)	11 (41%)	3 (27%)	2 (29%)	4 (27%)	7 (58%)
Identified as high risk[†]	11 (42%)	11 (58%)	8 (44%)	14 (52%)	4 (36%)	4 (57%)	7 (47%)	7 (58%)

Note: A total of 45 patients were randomized to four treatment groups. Data are presented as frequency *n* (%) or median (interquartile range).

Abbreviations: NSAIDs, nonsteroidal anti-inflammatory drugs; SSRIs, selective serotonin reuptake inhibitors.

[†]indicates groups at baseline with high risk for acetaminophen-induced hepatotoxicity.

TABLE 2 Concentration of metabolites in blood samples from 19 paracetamol overdose patients collected at predose, 12 h, and 20.25 h following standard NAC regimen

	Standard regimen ($n = 19$)			p value		
Metabolite	**Predose ($n = 19$)**	**12 h ($n = 19$)**	**20.25 h ($n = 19$)**	**Predose vs. 12 h**	**Predose vs. 20.25 h**	**12 h vs. 20.25 h**
APAP clinical	132.61 ± 72.89	5.75 ± 1.35	4.54 ± 1.12	<0.0001	<0.0001	0.044
APAP LC-MS/MS	146.86 ± 97.04	3.30 ± 2.46	1.49 ± 1.48	<0.0001***	<0.0001***	0.032*
APAP-sulfate	62.30 ± 26.45	18.18 ± 12.45	6.31 ± 4.31	<0.0001***	<0.0001***	0.002**
APAP-glucuronide	252.25 ± 126.23	28.15 ± 19.13	10.04 ± 7.08	<0.0001***	<0.0001***	0.007*
APAP-cysteine	1.19 ± 0.36	0.89 ± 0.13	0.77 ± 0.03	0.009*	<0.0001***	0.069
APAP-glutathione	0.41 ± 0.11	0.34 ± 0.18	0.25 ± 0.23	0.325	0.033*	0.151
APAP-NAC	1.09 ± 0.48	0.35 ± 0.19	0.15 ± 0.04	<0.0001***	<0.0001***	0.001**
N-acetylcysteine	0.00 ± 0.00	9.88 ± 2.83	9.13 ± 1.64	<0.0001***	<0.0001***	0.754
Cysteine (Cys)	42.20 ± 11.03	164.91 ± 116.50	131.95 ± 68.19	<0.0001***	<0.0001***	<0.0001***
Cystine (CySS)	27.55 ± 5.88	22.76 ± 1.32	24.33 ± 3.18	0.001**	0.049*	0.154
Cys/CySS ratio	1.55 ± 0.42	7.26 ± 5.33	5.44 ± 2.83	<0.0001***	<0.0001***	0.639
Methionine	7.79 ± 4.56	8.27 ± 4.57	11.10 ± 4.50	0.988	0.042	0.049
Betaine	6.72 ± 5.13	6.63 ± 3.43	7.65 ± 5.05	0.693	0.474	0.782
Choline	3.75 ± 2.10	4.04 ± 4.02	4.03 ± 2.04	0.422	0.630	0.175
Oxoproline	26.38 ± 20.15	25.16 ± 22.24	22.91 ± 17.76	0.651	0.759	0.965
Cystathione	6.38 ± 0.01	6.40 ± 0.04	6.41 ± 0.04	0.318	0.159	0.841
Taurine	30.71 ± 7.53	33.54 ± 11.80	39.38 ± 13.69	0.669	0.044	0.101
S-adenosyl-homocysteine	11.48 ± 2.21	12.33 ± 5.29	14.56 ± 8.34	0.812	0.606	0.480
Hypoxanthine	171.97 ± 104.00	146.86 ± 156.73	113.98 ± 144.49	0.117	0.111	0.833
Xanthine	1.82 ± 1.50	2.08 ± 3.14	2.91 ± 7.87	0.586	0.264	0.976
Uric acid	271.48 ± 76.45	211.12 ± 47.43	266.80 ± 102.30	0.014	0.404	0.190
Allantoin	0.26 ± 0.15	0.31 ± 0.22	0.24 ± 0.16	0.845	1.000	0.695
Histidine	80.96 ± 18.24	94.39 ± 21.34	94.07 ± 16.75	0.052	0.028*	0.919
Hercynine	38.47 ± 13.06	47.01 ± 15.95	39.59 ± 14.91	0.113	1.000	0.243
S-Methyl Ergothionine	23.22 ± 10.40	23.75 ± 15.71	29.49 ± 22.22	0.693	0.672	0.457
Ergothionine	1.43 ± 0.46	1.54 ± 0.48	1.85 ± 1.15	0.328	0.191	0.738
Isovalerylcarnitine	0.02 ± 0.02	0.02 ± 0.01	0.02 ± 0.01	0.060	0.133	0.421
Propionylcarnitine	0.18 ± 0.09	0.24 ± 0.09	0.24 ± 0.08	0.040*	0.014*	0.872
Butyrylcarnitine	0.04 ± 0.04	0.11 ± 0.16	0.11 ± 0.17	0.002**	0.012*	0.511
F2-isoprostane, pg/ml	7.89 ± 4.95	5.62 ± 2.78	6.62 ± 4.20	0.134	0.399	0.524
Phenlyalanine	55.64 ± 23.17	81.83 ± 23.63	79.45 ± 32.64	0.003**	0.011*	0.965
Proline	409.10 ± 178.34	410.48 ± 184.31	471.28 ± 200.46	0.737	0.287	0.249
Tyrosin	49.63 ± 28.58	52.77 ± 14.47	57.53 ± 25.31	0.405	0.343	0.474
Arginine	60.29 ± 33.89	66.79 ± 22.52	74.26 ± 29.20	0.487	0.048*	0.138
Glutamic acid	47.92 ± 17.93	69.49 ± 25.60	66.23 ± 22.84	0.010*	0.013*	0.693
Leucine	226.13 ± 173.03	167.04 ± 77.29	192.26 ± 141.85	0.609	0.609	0.827
Serine	111.70 ± 134.20	150.03 ± 98.80	152.46 ± 88.09	0.013*	0.007*	0.737
Isoleucine	73.76 ± 22.62	67.60 ± 17.59	65.23 ± 21.98	0.194	0.088	0.474
Threonine	88.50 ± 63.76	73.34 ± 41.02	135.11 ± 125.02	0.672	0.300	0.133
Glutamine	39.29 ± 15.55	35.82 ± 12.21	50.69 ± 32.69	0.671	0.136	0.064
Pyridoxine	12.23 ± 0.004	12.23 ± 0.008	12.24 ± 0.02	0.301	0.693	0.215
Pyridoxamine	34.82 ± 16.30	44.92 ± 31.63	38.64 ± 20.92	0.457	0.343	0.895

Note: Data are presented as mean ± SD and metabolite concentrations (μM). Multiple comparison between the timepoint was performed by Kruskal–Wallis tests with Dunn–Bonferroni post hoc.

Abbreviations: APAP, acetaminophen; LC-MS/MS, liquid chromatography tandem mass spectrometry; NAC, *N*-acetylcysteine.

*$p < 0.05$, **$p < 0.005$, ***$p < 0.0001$.

TABLE 3 Concentration of metabolites in blood samples from 26 paracetamol overdose patients collected at predose, 12 h, and 20.25 h following modified NAC regimen

	Modified regimen			*p* value		
Metabolite	Predose ($n = 26$)	12 h ($n = 26$)	20.25 h ($n = 26$)	Predose vs. 12 h	Predose vs. 20.25 h	12 h vs. 20.25 h
APAP clinical	127.54 ± 69.96	8.68 ± 6.04	8.52 ± 14.88	<0.0001***	<0.0001***	0.381
APAP LC-MS/MS	129.93 ± 93.77	7.35 ± 8.55	2.32 ± 4.15	<0.0001***	<0.0001***	0.153
APAP-sulfate	72.84 ± 51.21	33.82 ± 34.95	9.34 ± 12.29	<0.0001***	<0.0001***	0.001**
APAP-glucuronide	277.02 ± 168.28	52.96 ± 69.78	14.35 ± 19.88	<0.0001***	<0.0001***	0.002**
APAP-cysteine	1.40 ± 0.65	1.06 ± 0.52	0.81 ± 0.14	0.014*	<0.0001***	0.001**
APAP-glutathione	0.38 ± 0.15	0.30 ± 0.20	0.30 ± 0.21	0.487	0.113	0.148
APAP-NAC	1.40 ± 0.86	0.54 ± 0.80	0.18 ± 0.08	<0.0001***	<0.0001***	0.026*
NAC	0.00 ± 0.00	20.99 ± 14.14	6.72 ± 0.71	<0.0001***	<0.0001***	<0.0001***
Cysteine (Cys)	47.27 ± 10.96	332.29 ± 358.57	51.43 ± 19.50	<0.0001***	<0.0001***	0.754
Cystine (CySS)	28.82 ± 4.04	25.68 ± 4.16	26.49 ± 5.82	0.004**	0.008*	0.830
Cys/CySS ratio	1.67 ± 0.45	12.54 ± 11.03	1.97 ± 0.71	<0.0001***	0.188	<0.0001***
Methionine	8.75 ± 4.83	7.91 ± 3.75	9.74 ± 4.90	0.660	0.453	0.257
Betaine	6.44 ± 3.25	9.62 ± 7.45	7.44 ± 2.84	0.122	0.133	0.880
Choline	3.13 ± 1.84	3.21 ± 2.21	3.14 ± 1.81	0.869	0.985	0.869
Oxoproline	29.85 ± 33.41	35.78 ± 53.93	19.94 ± 16.55	0.812	0.096	0.124
Cystathione	6.40 ± 0.05	6.42 ± 0.08	6.40 ± 0.05	0.430	0.639	0.275
Taurine	35.74 ± 10.84	32.82 ± 11.88	33.91 ± 11.18	0.148	0.370	0.583
S-Adenosyl Homocysteine	13.09 ± 4.58	12.44 ± 5.73	13.55 ± 7.08	0.393	0.865	0.411
Hypoxanthine	208.77 ± 293.28	150.06 ± 143.51	178.68 ± 212.71	0.679	0.851	0.880
Xanthine	3.46 ± 7.23	6.18 ± 18.96	3.67 ± 8.15	0.443	0.534	0.910
Uric acid	290.71 ± 88.70	265.07 ± 73.05	273.33 ± 74.79	0.368	0.221	0.678
Allantoin	0.34 ± 0.29	0.45 ± 0.73	0.31 ± 0.20	0.342	0.464	0.572
Histidine	81.00 ± 20.62	91.86 ± 26.89	80.96 ± 21.12	0.050	1.000	0.065
Hercynine	40.49 ± 16.59	40.43 ± 17.57	40.51 ± 22.25	0.733	0.639	0.536
S-Methyl Ergothionine	32.98 ± 17.19	34.08 ± 26.39	34.59 ± 21.17	0.558	0.985	0.570
Ergothionine	1.89 ± 0.84	1.76 ± 0.74	2.00 ± 0.96	0.583	0.728	0.421
Isovalerylcarnitine	0.01 ± 0.01	0.02 ± 0.02	0.02 ± 0.01	0.158	0.178	0.442
Propionylcarnitine	0.22 ± 0.17	0.28 ± 0.20	0.25 ± 0.10	0.023*	0.014*	0.964
Butyrylcarnitine	0.06 ± 0.07	0.10 ± 0.04	0.07 ± 0.03	<0.0001***	0.002**	0.008*
F2-isoprostane, pg/ml	6.10 ± 2.72	5.02 ± 2.21	5.92 ± 3.59	0.271	1.000	0.522
Phenlyalanine	67.44 ± 29.86	70.72 ± 24.64	74.73 ± 27.87	0.421	0.314	0.728
Proline	379.70 ± 158.42	405.59 ± 216.31	345.51 ± 106.50	0.798	0.742	0.546
Tyrosine	48.30 ± 18.43	54.74 ± 57.56	48.17 ± 18.52	0.742	0.855	0.784
Arginine	66.17 ± 22.88	58.92 ± 30.34	69.42 ± 22.65	0.377	0.589	0.168
Glutamic acid	72.64 ± 54.45	75.43 ± 43.61	79.66 ± 62.54	0.400	0.855	0.400
Leucine	231.73 ± 413.17	123.63 ± 59.47	180.12 ± 168.43	0.170	0.884	0.170
Serine	82.98 ± 41.24	120.36 ± 87.17	108.85 ± 66.66	0.095	0.093	0.821
Isoleucine	68.87 ± 24.43	57.79 ± 20.59	64.37 ± 19.99	0.052	0.546	0.120
Threonine	94.56 ± 92.81	111.86 ± 68.13	104.36 ± 108.73	0.116	0.608	0.220
Glutamine	38.79 ± 20.11	35.36 ± 18.32	38.77 ± 14.13	0.170	0.913	0.107
Pyridoxine	12.24 ± 0.01	12.24 ± 0.04	12.23 ± 0.01	0.280	0.101	0.380
Pyridoxamine	35.72 ± 10.90	36.93 ± 22.42	36.48 ± 21.88	0.241	0.241	0.770

Note: Data are presented as mean ± SD and metabolite concentrations (μM). Multiple comparison between timepoint was performed by Kruskal–Wallis tests with Dunn–Bonferroni post hoc.

Abbreviations: APAP, acetaminophen; LC-MS/MS, liquid chromatography tandem mass spectrometry; NAC, *N*-acetylcysteine.

*$p < 0.05$, **$p < 0.005$, ***$p < 0.0001$.

Effect 12 h after starting NAC

Both regimens delivered an NAC dose of 300 mg/kg but the rate of drug delivery was different. In the SNAP trial, NAC was stopped in all patients at the end of the modified NAC regimen, however, in clinical practice, NAC would continue in those with evidence of liver injury or detectable APAP levels. Blood was drawn at the end of the modified NAC regimen (12 h after starting) and compared with the same timepoint in the three-bag regimen. The serum NAC concentration was higher in the patients treated with the modified regimen than the standard regimen (standard regimen 9.88 ± 2.83 μM vs. modified regimen 20.99 ± 14.14, $p < 0.001$). The standard NAC regimen resulted in 3.9-fold (42.20 ± 11.03 vs. 164.91 ± 116.50 μM, $p < 0.0001$) increase in plasma cysteine at the 12 h timepoint. However, there was a higher plasma cysteine concentration in the modified regimen with sevenfold (47.27 ± 10.96 vs. 332.29 ± 358.57 μM, $p < 0.0001$) increase from baseline at the 12 h timepoint (12 h: standard; 164.91 ± 116.50, modified; 332.29 ± 358.57 μM, $p = 0.02$). Because NAC can reduce cystine (the oxidized, dimeric form of cysteine) to cysteine in the plasma, we measured the plasma cystine concentration. At 12 h, the reduction in cystine after NAC was significant and of similar magnitudes with the two regimens.

Effect 20.25 h after starting NAC

This timepoint was at the end of the standard NAC regimen and 8.25 h after stopping the modified NAC regimen. At this time, NAC was higher with the standard regimen but it remained elevated in the modified regimen treated patients (standard 9.13 ± 1.64 vs. modified 6.72 ± 0.71 μM, $p < 0.001$). Cysteine was elevated compared with baseline in the standard treatment group but had returned to baseline concentration after NAC was stopped in the modified treatment group (standard 131.95 ± 68.19 vs. modified 51.43 ± 19.50 μM, $p = 0.001$). Cystine remained lower than the pre-NAC concentration with both regimens.

Effect of NAC regimen on plasma thiol biomarkers

Because APAP-induced liver injury increases oxidative stress in the mitochondria, elevation of plasma acylcarnitines can indicate early hepatocytes injury in APAP overdose.[9] We observed elevation of plasma butyrylcarnitine and propionylcarnitine concentrations in standard (Table 2) and modified regimens (Table 3). However, plasma butyrylcarnitine and propionylcarnitine were not significantly different between the two regimens (Table S1). In addition to alterations in plasma acylcarnitines, NAC administration also resulted in elevated levels of several amino acids (serine, glutamic acid, and phenylalanine), with the two timepoints being significantly different to pretreatment in the standard regimen, but not in the modified regimen. However, plasma concentration of other thiol biomarkers from purine, allantoin, ergothioneine, and F2-isoprostane pathways were not significantly different in the standard (Table 2) and the modified regimens (Table 3).

Effect of NAC regimen on APAP metabolites

Given the differences in NAC and cysteine concentrations between the two regimens we optimized LC-MS/MS to examine plasma APAP parent drug and metabolite concentrations to explore if there was a treatment regimen effect on drug metabolism. The concentrations of APAP and its five major metabolites at predose, 12 h, and 20.25 h postinitiation of NAC are presented in the standard (Table 2) and the modified regimens (Table 3). There was no difference in the concentrations of the metabolites between the two regimens predose, at 12 h, or 20.25 h.

APAP metabolites and ALI

It has been reported that elevated plasma APAP-cysteine predicts ALI after APAP overdose.[10] In agreement with previous findings, we found that the plasma concentration of APAP-cysteine (2.07 ± 0.80 vs. 1.20 ± 0.46 μM, $p = 0.002$) and the ratio of APAP-cysteine/APAP (%) (4.89 ± 2.56 vs. 1.09 ± 0.77, $p < 0.0001$; Table 4) was higher in patients who developed ALI than those without (no ALI). Plasma ALT (189.75 ± 167.78 vs. 22.43 ± 15.38 U/L, $p < 0.0001$) and APAP-cysteine/APAP (log-transformed) at baseline were significantly higher in patients who developed ALI (4.89 ± 2.56 vs. 1.09 ± 0.77, $p < 0.0001$; Table 4), compared with those who did not developed ALI (Figure 1a,b). The area under curve ($AUC_{0–20.25\ h}$) of APAP-cysteine/APAP fraction was significantly higher in patients with ALI compared with without ALI (Figure 1c). The $AUC_{0–20.25\ h}$ of plasma APAP-cysteine/APAP fraction was positively correlated with plasma ALT at admission (Figure 1d). We used ROC analysis to evaluate the predictive values of the five APAP-metabolites measured at baseline for ALI (Table 5). We demonstrated the APAP metabolites derived from CYP-mediated pathway were predictive for ALI: APAP-cysteine (APAP-cysteine/APAP % with ROC-AUC of 0.85 [95% CI = 0.71–0.99, $p = 0.002$]), APAP-glutathione (APAP-glutathione/APAP % with ROC-AUC of 0.90 [95% CI = 0.74–1.00, $p = 0.009$]), and APAP-NAC (APAP-NAC/APAP % with ROC-AUC of 0.80 [95% CI =0.64–0.95, $p = 0.018$]). Non-CYP-mediated

TABLE 4 Baseline levels of clinical variables, APAP, and purine metabolites categorized by patients with ALI ($n = 4$) or no ALI ($n = 40$)

Baseline level	ALT rise < 50%	ALT rise > 50%	*p* value
Clinical variables			
ALT (U/L) at admission	22.43 ± 15.38	189.75 ± 167.78	<0.0001***
APAP clinical (at admission)	141.21 ± 66.06	50.00 ± 38.09	0.012*
INR	0.97 ± 0.08	1.13 ± 0.05	0.004*
Bilirubin	9.50 ± 9.62	18.50 ± 5.97	0.075
AlkPhos	80.55 ± 59.88	67.25 ± 14.03	0.663
GGT	60.38 ± 104.49	21.75 ± 16.27	0.469
Urea	4.04 ± 2.29	4.67 ± 1.27	0.591
Creatinine	68.73 ± 12.76	86.00 ± 23.05	0.021*
Hb, g/L	140.87 ± 16.01	135.00 ± 15.13	0.543
MCV	91.79 ± 4.92	93.00 ± 7.00	0.693
WBC	8.27 ± 2.36	9.56 ± 4.64	0.398
Ingestion-time to NAC treatment			0.006*
Number of patients <8 h ingestion to starting NAC	28 (70)	0 (0)	
Number of patients >8 h ingestion to starting NAC	12 (30)	4 (100)	
APAP overdose			0.497
<16 g	23 (57.50)	3 (75.00)	
≥16 g	17 (42.50)	1 (25.00)	
APAP metabolite			
APAP LC-MS/MS	143.71 + 95.19	61.50 + 62.41	0.079
APAP-cysteine	1.20 + 0.46	2.07 + 0.80	0.002**
APAP-glucuronide	265.90 + 154.46	243.98 + 187.40	0.791
APAP-glutathione	0.38 + 0.15	0.45 + 0.01	0.358
APAP-sulfate	69.00 + 45.26	46.24 + 21.96	0.330
APAP-mercapturate	1.27 + 0.78	1.04 + 0.56	0.567
APAP-sulfate/APAP	106.65 + 224.23	125.73 + 106.41	0.868
APAP-glucuronide/APAP	320.68 + 484.97	472.13 + 163.00	0.018*
APAP-cysteine/APAP	1.09 + 0.77	4.89 + 2.56	<0.0001***
APAP-glutathione/APAP	0.34 + 0.34	1.22 + 0.66	0.009*
Purine metabolite			
Hypoxanthine	161.94 + 196.53	230.74 + 122.03	0.027*
Xanthine	1.94 + 3.57	25.50 + 29.42	<0.0001***
Uric acid	264.11 + 84.25	315.36 + 56.35	0.023*
Allantoin	0.32 + 0.38	0.39 + 0.24	0.151
Hypoxanthine/total	31.00 + 19.00	38.00 + 11.00	0.160
Xanthine/total	0.40 + 1.00	4.40 + 5.00	<0.0001***
Uric acid/total	68.80 + 19.50	13.00 + 8.00	0.041*
Allantoin/total	0.10 + 0.10	0.10 + 0.10	0.822

Note: ALI is defined as ALT rise >50% at 20.25 h compared with the hospital admission value. Data are presented as mean ± SD and frequency *n* (%). All measurements were assessed at baseline. Plasma purine metabolite is expressed as absolute concentration (μM) and as ratio of each metabolite formed relative to total purine metabolites (ratio (%). Plasma APAP metabolites is expressed as concentrations (μM) and as ratio of each APAP metabolite formed relative to APAP drug (ratio (%). Pairwise comparison was performed by Mann–Whitney *U* test.

Abbreviations: ALI, acute liver injury; ALT, alanine aminotransferase; APAP, acetaminophen; GGT, gamma glutamyltransferase; INR, International Normalized Ratio; LC-MS/MS, liquid chromatography tandem mass spectrometry; NAC, *N*-acetylcysteine, WBC, white blood cell.

*$p < 0.05$, **$p < 0.005$, ***$p < 0.0001$.

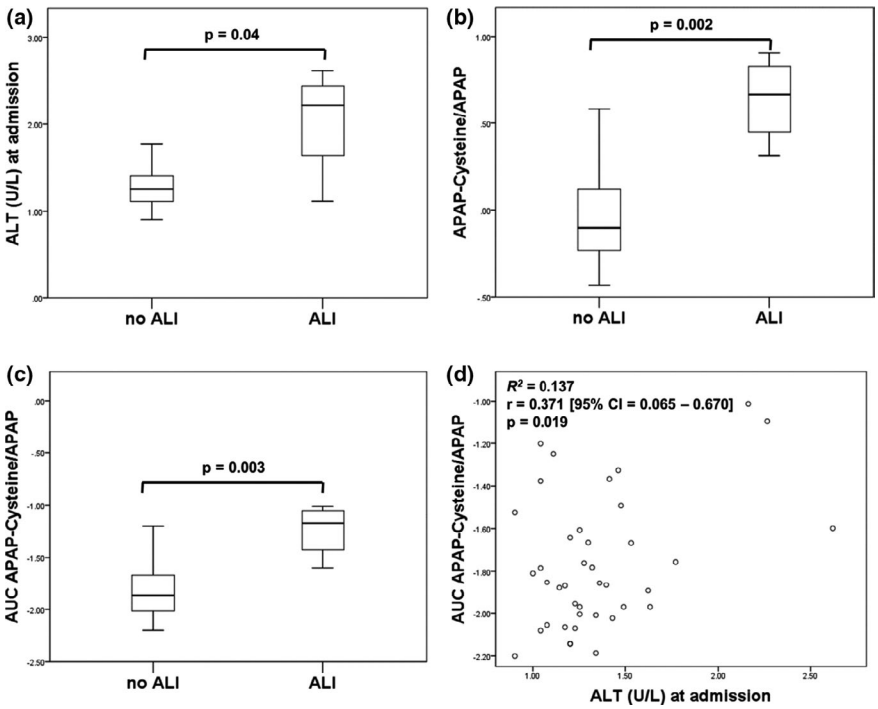

FIGURE 1 Alanine aminotransferase (ALT) and acetaminophen (APAP)-cysteine levels in patients with acute liver injury (ALI; $n = 4$), as defined by >50% ALT rise at 20.25 h, and patients without ALI (no ALI; $n = 40$). (a) Serum ALT level at admission in patients stratified by ALI ($n = 4$) and no ALI ($n = 40$). (b) Plasma APAP-cysteine level is expressed as a ratio of metabolite formed by APAP-cysteine relative to APAP drug (APAP-CYS/APAP) (%) at admission in ALI ($n = 4$) and no ALI ($n = 40$) patients. (c) Area under the curve (AUC) for APAP-CYS is expressed as a fraction of APAP-CYS (APAP-CYS/APAP) (%) from time 0 to 20.25 h ($AUC_{0–20.25\ h}$) after initiation of *N*-acetylcysteine (NAC) in ALI ($n = 4$) and no ALI ($n = 40$) patients. (d) Correlation between the AUC of APAP-cysteine expresses as a fraction of APAP-CYS (APAP-CYS/APAP) (%) from time 0 to 20.25 h ($AUC_{0–20.25\ h}$) after initiation of NAC and serum ALT at admission in ALI ($n = 4$) and no ALI ($n = 40$) patients. All measurements were log-transformed and reported by geometric means and ratios. (a–c) Box plots represent median (interquartile range) and whisker represents range. Pairwise comparison was performed by Mann–Whitney *U* test. (d) Spearman correlation was performed. *$p < 0.05$, **$p < 0.005$. CI, confidence interval

pathway: APAP-glucuronide (APAP-Glucuronide/APAP % with ROC-AUC of 0.86 [95% CI = 0.75–0.96, $p = 0.018$]). A combination of both the APAP non-CYP/CYP metabolites and xanthine in the ROC model had superior predictive power for ALI with ROC-AUC of 0.98 (95% CI = 0.94–1.00, $p < 0.0001$; Table 5), compared with individual metabolites in predicting ALI. Collectively, our data suggest assessment of non-CYP/CYP-APAP and xanthine metabolites to predict risk of subsequent development of ALI at presentation to the hospital.

Purine metabolites and ALI

In humans, xanthine oxidase (XO) enzyme produces uric acid by breakdown of hypoxanthine to xanthine in the purine metabolism pathway. These endogenous metabolites and reactive oxygen species can be cytotoxic. APAP can trigger XO to produce exogenous cytotoxic metabolites.[11] We examined purine metabolites in the SNAP cohort, and found that patients with ALI had a markedly higher plasma xanthine concentration (no-ALI: 1.94 ± 3.57, ALI: 25.50 ± 29.42 μM, $p < 0.0001$), and a higher ratio of xanthine/total purine metabolites (0.40 ± 1.00 vs. 4.40 ± 5.00, $p < 0.0001$; Table 4). The $AUC_{0–20.25\ h}$ of total purine metabolites (8322.49 ± 3209.30 vs. 11644.76 ± 2440.88, $p = 0.031$; Figure 2a) and of xanthine, expressed as a fraction relative to total purine metabolites, xanthine/total (%) from time 0–20.25 h after initiation of NAC, (0.39 ± 0.24 vs. 3.22 ± 2.79, $p = 0.0001$; Figure 2b) were also significantly higher in patients with ALI compared with those with no ALI. The $AUC_{0–20.25\ h}$ of xanthine significantly correlated with ALT at admission (r = 0.497, 95% CI = 0.386–0.705, $p < 0.0001$; Figure 2c). The performance of each purine metabolite for ALI at baseline was evaluated using ROC analysis (Table 5). Plasma xanthine and its ratio (xanthine/total purine metabolites [%]) predicted ALI with ROC-AUC of 0.90 (95% CI = 0.79–1.00, $p < 0.0001$; Table 5). Taken together, our data suggest that plasma xanthine is elevated in overdose patients with ALI. There was no difference in xanthine concentration between the modified and standard NAC regimens.

TABLE 5 Predictive accuracy of APAP and purine metabolites for ALI

Baseline level	AUC [95% CI]	*p* value
APAP metabolite		
APAP LC-MS/MS	0.17 [0.00–0.39]	0.031*
APAP-sulfate	0.32 [0.08–0.55]	0.236
APAP-glucuronide	0.41 [0.10–0.72]	0.568
APAP-cysteine	0.91 [0.81–1.00]	0.007*
APAP-NAC	0.35 [0.08–0.62]	0.380
APAP-glutathione	0.73 [0.48–0.97]	0.142
APAP-sulfate/APAP	0.69 [0.46–0.92]	0.206
APAP-glucuronide/APAP	0.86 [0.75–0.96]	0.018*
APAP-cysteine/APAP	0.85 [0.71–0.99]	0.002**
APAP-NAC/APAP	0.80 [0.64–0.95]	0.018*
APAP-glutathione/APAP	0.90 [0.74–1.00]	0.009*
Purine metabolite		
Hypoxanthine	0.72 [0.59–0.85]	0.027*
Xanthine	0.91 [0.82–1.00]	<0.0001***
Uric acid	0.73 [0.60 −0.85]	0.025*
Allantoin	0.64 [0.45–0.83]	0.157
Hypoxanthine/total	0.64 [0.51–0.78]	0.151
Xanthine/total	0.90 [0.79–1.00]	<0.0001***
Uric acid/total	0.35 [0.11–0.58]	0.395
Allantoin/total	0.48 [0.26–0.69]	0.822
Sum of APAP metabolites panel and xanthine/total	0.98 [0.94–1.00]	<0.0001***

Note: Receiver operating characteristic- area under the curve with 95% CI for APAP and purine metabolites measured at baseline (predose) shows predictive value of each metabolite for ALI (alanine aminotransferase rise > 50% at 20.25 h compared with the hospital admission value). Sum of APAP Non-CYP/CYP metabolites panel (APAP-glucuronide/APAP + APAP-cysteine/APAP + APAP-NAC/APAP + APAP-glutathione/APAP).

Abbreviations: ALI, acute liver injury; APAP, acetaminophen; CI, confidence interval; LC-MS/MS, liquid chromatography tandem mass spectrometry; NAC, *N*-acetylcysteine.

*$p < 0.05$, **$p < 0.005$, ***$p < 0.0001$.

DISCUSSION

This study investigated the effects of the standard and modified NAC regimens on a wide range of thiol-based antioxidants and APAP metabolites. We compared the efficacy of identical total doses of NAC (300 mg/kg) administered by i.v. infusion of either 20.25 h duration (standard regimen) or 12 h duration (modified regimen) on total antioxidant-capacity, in a subgroup of patients with single APAP overdose (SNAP cohort).[8] We evaluated phenotyping profiles of plasma APAP metabolism and thiol concentrations at baseline and two timepoints after initiation of NAC. All patients were admitted to the hospital within 36 h of a single APAP overdose and received either standard or modified NAC and had three blood samples collected for biochemical and LC-MS/MS analyses. The standard regimen begins with a large loading dose then gradually decreasing concentrations up to 20.25 h (150 mg/kg in 200 ml, over 15 min, 50 mg/kg in 0.5 L, over 4 h, and 100 mg/kg in 1 L, over 16 h), whereas the modified regimen begins with smaller loading dose (100 mg/kg in 200 ml, over 2 h, 200 mg/kg in 1 L, over 10 h).[4] The modified regimen has reduced adverse reactions associated with NAC treatment,[4] with comparable treatment effectiveness to the standard regimen.[5] In the liver, NAC is metabolized to cysteine, a precursor to minimize GSH depletion due to APAP overdose. At present, the antioxidant potential generated by the modified and standard regimens has not been reported.

Standard and modified NAC regimens

Our main finding was the modified NAC regimen substantially enhanced cysteine/cystine redox thiol response, which would be expected to lead to a greater antioxidant effect within the first 12 h of treatment. Because NAC is a precursor of GSH, NAC infusion enhances the plasma concentration of cysteine for GSH biosynthesis. GSH is a main protein thiol molecule for the conjugation of NAPQI.[12] Timepoint analysis showed the modified NAC regimen had a higher plasma cysteine concentration (7-fold increase at the 12 h timepoint relative to baseline; Table 3), compared with the standard NAC regimen (3.9-fold increase at the 12 h timepoint relative to baseline; Table 2) and markedly increased the cysteine/cystine redox potential (17 mV; Table S2). In the standard regimen, the steady-state redox potential for cysteine ($E_h = -110$ mV) noted an increase ($E_h = -150$ mV) at 12 h, whereas the modified regimen ($E_h = -112$ mV) noted an increase to ($E_h = -169$ mV) at 12 h. A greater increase in plasma cysteine ($E_h = 17$ mV) was observed in the modified as compared with the standard regimen (Table S2). In addition, we demonstrated the expected reduction in plasma cysteine at 20.25 h timepoint in the modified regimen (standard: 332.29 ± 358.57 μM; modified 51.43 ± 19.50 μM, $p < 0.0001$; Table 3) when the infusion of NAC discontinued after 12 h in the modified protocol. However, in clinical practice, in those patients with raised paracetamol concentrations or liver injury after 12 h of the modified protocol, NAC would be continued and this decrease in plasma cysteine would, not only not occur, but based on these data cysteine concentrations would be expect to be higher as a result of further NAC. The normal human plasma concentration of total cysteine/cystine (reduced and oxidized) has been reported to be 89.13–107.29 μM (cited reference range)[13] and the cysteine/cystine redox potential has been reported to be −84.6 mV.[14] The plasma cysteine/cystine concentration and cysteine/cystine redox potential were not significantly affected by APAP administration in healthy human subjects.[15,16]

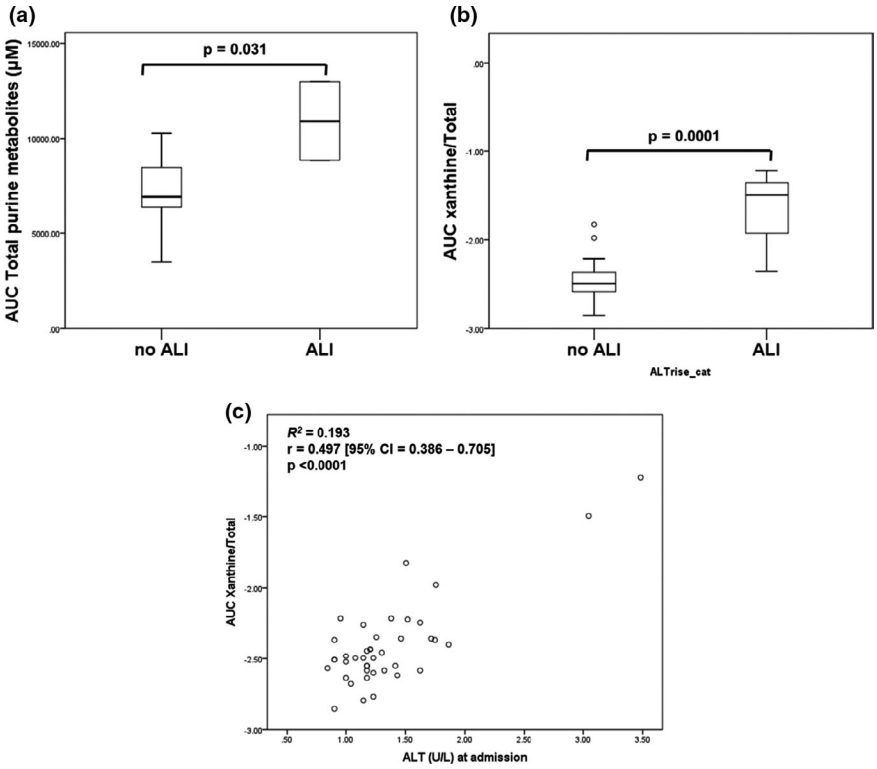

FIGURE 2 Purine metabolites level in patients with acute liver injury (ALI) ($n = 4$), as defined by >50% alanine aminotransferase (ALT) rise at 20.25 h, and patients without ALI (no ALI; $n = 40$). (a) Plasma purine metabolites concentration (μM) are expressed as the total metabolites formed (hypoxanthine, xanthine, uric acid, and allantoin) from time 0 to 20.25 h after initiation of NAC in ALI ($n = 4$) and no ALI ($n = 40$) patients. (b) Area under the curve (AUC) for xanthine is expressed as a fraction of xanthine metabolite relative to total purine metabolites (xanthine/total) (%) from time 0 to 20.25 h after initiation of NAC ($AUC_{0–20.25\ h}$) in ALI ($n = 4$) and no ALI ($n = 40$) patients. (c) Correlation between the AUC for xanthine expressed as a fraction of xanthine metabolite relative to total purine metabolites (xanthine/total) (%) from time 0 to 20.25 h after initiation of NAC ($AUC_{0–20.25\ h}$) and serum ALT) at admission in all patients ($n = 44$). All measurements were log-transformed and reported by geometric means and ratios. (a–b) Box plots represent median (interquartile range) and whisker represents range. Pairwise comparison was performed by Mann–Whitney *U* test. (c) Spearman correlation was performed. $^*p < 0.05$, $^{**}p < 0.005$, $^{***}p < 0.0001$. CI, confidence interval

Compared with the normal range, we observed a slight reduction in plasma concentration of total cysteine/cystine at predose of 69.75 ± 16.91 μM (standard NAC regimen) (Table 2) and 76.09 + 15 μM (modified NAC regimen; Table 3), possibly a consequence of APAP overdose that oxidized plasma cysteine/cystine.[15] The significant increase in plasma cysteine/cystine at 12 h and 20.25 h timepoints is likely to be a consequence of NAC treatment.

Effect of NAC on plasma amino acids

APAP-induced oxidative stress and GSH depletion can have significant impact on amino acid metabolism.[16–20] APAP exposure depletes amino acid and decreases plasma amino acid concentrations.[16,21] Amino acids, such as leucine, isoleucine, valine, lysine, methionine, tyrosine, and phenylalanine, have important anti-oxidant activity.[22] Several amino acids, serine, methionine, glutamic acid, and cysteine are precursors for GSH synthesis, and any deviation from normal plasma range of these amino acids can be an indicator of oxidative stress.[16,23–25] These amino acids play different roles regarding their function as scavengers of reactive oxygen species. In fact, cysteine is a main precursor for GSH, controlling rate-limiting synthesis via the γ-glutamyl cycle. Serine (a donor of carbon atom) and methionine (donor of –SH) are important molecules for endogenous synthesis of cysteine.[26,27] Phenylalanine regulates APAP sulfation in which its deficiency reduces APAP metabolism via CYPP450-mediated and non-CYPP450-mediated pathways.[28] At present study, we showed significant differences in plasma concentrations of amino acids at 12 h and 20.25 h timepoints. Because baseline plasma concentrations of amino acids were not significantly affected by APAP,[16,22] our result suggests the increase in plasma concentrations of amino acids may be a consequence of NAC treatment.

APAP metabolites and ALI

In the liver, APAP parent drug is predominantly metabolized to noncytotoxic conjugates: APAP-glucuronide and APAP-sulfate through glucuronidation and sulfation. A

minor fraction of APAP is oxidized by cytochrome P450 (CYP) enzymes to the toxic metabolite NAPQI. Excess NAPQI can cause liver injury, but it is eliminated by binding to GSH to form APAP-GSH, which is further converted to APAP-cysteine (APAP-CYS) and APAP-NAC (APAP-mercapturate). NAPQI also binds cellular cysteine to form an APAP-cysteine protein adduct, which can be detected before hepatic GSH depletion or liver injury develops.[29] These APAP metabolites are stable in the circulation for hours-to-days after a single APAP overdose.[30–32] Therefore, assessing circulating APAP metabolites may predict risk of subsequent liver injury.[10] In line with previous studies, APAP-CYS was able to predict the subsequent progression to ALI with an AUC-ROC of 0.91 (95% CI = 0.81–1.00, $p = 0.007$; Table 5). In addition, the ROC analysis also revealed that APAP-GSH, APAP-NAC, and APAP-glucuronide could potentially predict ALI risk (Table 5). Therefore, our data suggest that these APAP metabolites could improve ALI risk prediction. Despite the differences in the regimens regarding the protocol for delivery of NAC, and NAC and cysteine concentrations, there was no significant difference in the APAP metabolites. This may be due to only four patients having mild liver injury. A study of increased numbers of patients with toxicity would be expected to enrich these data for excess toxic metabolite production. Thus, although we cannot exclude a signal of a difference between the treatment groups, the lack of difference in the effect on APAP metabolism is consistent with there being no difference in the incidence of liver injury when the modified and standard regimens were compared.[5] Finally, the circulating concentration of APAP metabolites may not faithfully reflect the environment inside the hepatocyte such that any difference in the redox state inside the cell is not reflected in the circulation, at least in the absence of hepatocyte necrosis resulting in membrane rupture and release of the intracellular environment.

Xanthine metabolism and APAP-induced ALI

Increased serum xanthine oxidase has been associated with APAP-induced liver injury.[33] Animal studies report that excess APAP and NAPQI metabolite activate purine oxidation, which leads to an increase in production of purine oxidants and reactive oxygen species.[34,35] This change in purine metabolites, also demonstrated in animals with APAP toxicity, could potentially be a novel indicator of liver injury in man.[36] Thus, inhibiting xanthine oxidation, or the enzyme XO, is potentially a novel therapeutic strategy to prevent APAP-induced liver injury.[37] For example, a preclinical study showed that a high dose of XO inhibitor allopurinol significantly reduced oxidant stress and APAP-induced hepatotoxicity.[37] In addition, elevation of serum XO has been recently reported in patients with liver injury.[4,38] We found that xanthine was significantly elevated in APAP overdose patients who subsequently developed ALI (Table 4). These data suggest that APAP overdose activates XO and production of xanthine can potentially induce APAP-induced hepatotoxicity, independent of CYP-mediated APAP metabolism. Collectively, both purine and APAP metabolites may increase levels of oxidized protein thiols following overdose.

Our study has several limitations. First, we only have samples from two timepoints after the initiation of NAC. Second, the sample size is relatively small to obtain reliable estimates of hard clinical outcomes. Third, all patients in this study were those with single APAP overdose and required NAC therapy. The mean rise in ALT activity (predefined as at least a 50% rise in serum ALT activity at 20.25 h in the SNAP cohort was modest),[8] and there was no significant difference in liver injury between the modified and standard NAC regimens. Therefore, this study was not large enough to accurately describe the metabolite response to severe liver injury.

To conclude, we assessed oxidative stress biomarkers and APAP metabolites in APAP overdose patients treated with either standard or modified NAC regimen. We demonstrated that the 12 h modified NAC regimen used in the SNAP trial may provide greater antioxidant effects, as shown by increased plasma cysteine concentration (and cysteine/cystine redox potential), compared to the 20.25 h standard NAC regimen. Our study also revealed a novel role for purine metabolism in human APAP-induced hepatotoxicity. Further clinical studies are required to translate these findings into clinical practice.

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CONFLICT OF INTEREST

The authors declared no competing interests for this work.

AUTHOR CONTRIBUTIONS

J.W.D., D.N.B., and M.L.N. wrote the manuscript. J.W.D. and D.N.B. designed the research. C.L.D. and P.L.S performed the research. H.C., M.L.N., I.B., and B.J.K. analyzed the data. C.L.D. contributed new reagents/analytical tools.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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