Untargeted Proteomics Reveals Regeneration-associated Serum Biomarkers that can Predict Death in Acute Liver Failure Patients

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Significance & background

Acute liver failure (ALF) is a syndrome of encephalopathy, coagulopathy, and multi-organ damage caused by loss of liver function secondary to acute liver injury. Estimates of the annual incidence of ALF in the US vary from 6 to 32 cases per million population, or roughly 2,000 to 10,000 cases per year (Hoofnagle et al., 1995; Bower et al., 2007). Although rare, ALF is devastating, with overall mortality of 25-30% (Reuben et al., 2016).

Currently, the only life-extending treatment for ALF is a liver transplant, but current approaches to determine which patients need a new liver to survive have limited clinical utility. On one hand, the liver injury biomarkers ALT, AST, and others are sensitive for injury but do not correlated with outcomes. On the other hand, liver function tests like bilirubin and prothrombin time do correlate with outcomes, but rise too late to be useful. Prognostic scores are helpful but insufficient to influence care alone. New biomarkers to guide transplant decisions are needed.

Surprisingly, untargeted proteomics has never been applied directly to samples from ALF patients. Untargeted analytical methods can measure thousands of compounds simultaneously and without bias, so they are powerful tools for biomarker discovery. Here, we used untargeted proteomics analysis to identify biomarker candidates to predict poor outcomes in patients with acetaminophen (APAP)-induced ALF. We then used a reverse translational approach with Ingenuity Pathway Analysis® of our proteomics data to explore the mechanistic significance of our results, with confirmation in a mouse model of APAP hepatotoxicity.

Results

Discovery patients information

Table 1. Untargeted proteomics cohort demographics and laboratory data.

Parameter	Control volunteers	Survivors (S)	Non-survivors (NS)
N	10	10	10
Sex (% female)	5 (50)	5 (50)	5 (50)
Age (median, range)	45 (23 – 66)	32 (19 – 46)	33 (18 – 67)
Race and ethnicity			
White, non-Hispanic (%)	8 (80)	8 (80)	7 (70)
Black, non-Hispanic (%)	2 (20)	1 (10)	2 (20)
White, Hispanic (%)	0 (0)	1 (10)	1 (10)
Other (%)	0 (0)	0 (0)	0 (0)
Peak ALT (U/L) (mean±SE)	17±2	8275±1494	7493±992

Untargeted proteomics revealed 23 novel biomarker candidates

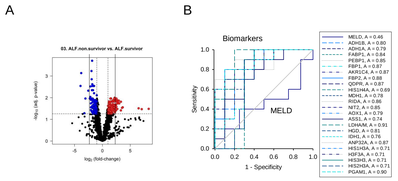

Figure 1. Untargeted proteomics revealed 23 proteins that were ≥4-fold elevated in serum from non-survivors compared to survivors. Day 1 serum samples from survivors (n=10) and non-survivors (n=10) of APAP-induced ALF and healthy controls (n=10) were subjected to untargeted proteomics. (A) Volcano plot displaying results for non-survivors vs. survivors. Each dot represents one serum protein. Numerous proteins were elevated ≥4-fold (right-side solid vertical line) in non-survivors compared to survivors. We focused on 23 that were also ≥4-fold elevated in ALF patients overall compared to control subjects for further workup to ensure robust results. (B) Receiver operating characteristic (ROC) curves showing sensitivity and specificity for the 23 biomarker candidates at different cutoffs. A: area under the curve.

Confirmation patients information

Table 2. Confirmation cohort demographics and laboratory data.

Parameter	Survivors (S)	Non-survivors (NS)
N	28	30
Sex (% female)	19 (68)	20 (67)
Age (median, range)	31 (19 – 70)	36 (18 – 67)
Race and ethnicity		
White, non-Hispanic (%)	27 (93)	26 (90)
Black, non-Hispanic (%)	1 (3.5)	2 (6.7)
White, Hispanic (%)	1 (3.5)	1 (3.3)
Other (%)	0 (0)	0 (0)
Peak ALT (U/L) (mean±SE)	4513±920	3406±531
Peak Tbili (mg/dL) (mean±SE)	5.5±0.6	8.6±1.2
Peak Cre (mg/dL) (mean±SE)	3.4±0.6	3.4±0.3
Peak MELD (mean±SE)	28±2	39±1

Targeted measurement confirmed utility of LDH in confirmation patients

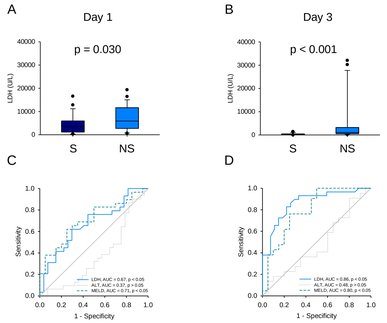

Figure 2. Serum LDH activity was greater in the non-survivors of APAP-induced ALF compared to survivors. Total LDH activity and ALT were measured in serum from all non-survivors (NS) and survivors (S) on study days 1 and 3. MELD scores were calculated when possible (n = 20 for survivors, 29 for non-survivors). (A) LDH activity on day 1. (B) LDH activity on day 3. (C) Receiver operating characteristic (ROC) curves for LDH, ALT, and MELD score on day 1. (D) Receiver operating characteristic (ROC) curves for LDH, ALT, and MELD score on day 3. Boxes show the 25th to 75th percentiles. Whiskers show the 10th and 90th percentile values. Lines show median values. Dots represent outliers.

Novel MELD-LDH score outperformed MELD on study day 3

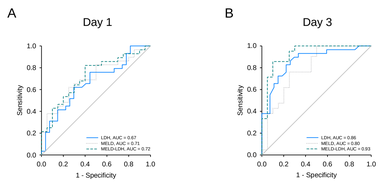

Figure 3. Serum LDH activity improved prediction of death in APAP-induced ALF. Total LDH activity and ALT were measured in serum from all non-survivors and survivors on study days 1 and 3. MELD scores were calculated when possible (n = 20 for survivors, 29 for non-survivors). (A) Receiver operating characteristic (ROC) curves showing sensitivity and specificity for LDH, MELD, and MELD-LDH on day 1. (B) Receiver operating characteristic (ROC) curves showing sensitivity and specificity for LDH, MELD, and MELD-LDH on day 3. MELD-LDH score was generated from multiple logistic regression. The score performed significantly better (p<0.05) on day 3.

Upstream analysis revealed 23 altered signaling pathways

Table 3. Altered signaling pathways in non-survivors compared to survivors.

Regulator	z-score	p-value
LKB1/STK11	3.201	$3.32x10^{-6}$
AHR	3.133	$3.44x10^{-5}$
FOXO3	2.939	$1.52x10^{-3}$
CD38	2.433	$8.41x10^{-3}$
IL-5	2.345	$4.59x10^{-3}$
CD28	2.333	$1.62x10^{-2}$
HIF1α	2.332	$4.06x10^{-3}$
MLXIPL	2.253	$2.20x10^{-4}$
INSR	2.216	$2.78x10^{-4}$
Alpha-catenin	2.215	$1.04x10^{-2}$
SRF	2.190	$1.44x10^{-2}$
MYC	2.182	$5.70x10^{-11}$
CSF1	2.160	$8.27x10^{-3}$
THRB	2.138	$6.69x10^{-5}$
ACOX1	2.111	$5.56x10^{-7}$
CBX5	-2.000	$3.89x10^{-2}$
CLPP	-2.000	$5.47x10^{-3}$
TNFSF11	-2.182	$9.60x10^{-3}$
CEBPB	-2.198	$1.22x10^{-5}$
MAP4K4	-2.236	$9.39x10^{-3}$
KDM5A	-2.236	$1.52x10^{-2}$
OGA	-2.496	$7.43x10^{-5}$
GDF2	-2.588	$7.31x10^{-6}$

Mouse studies revealed a critical role for LKB1-AMPK signaling in repair

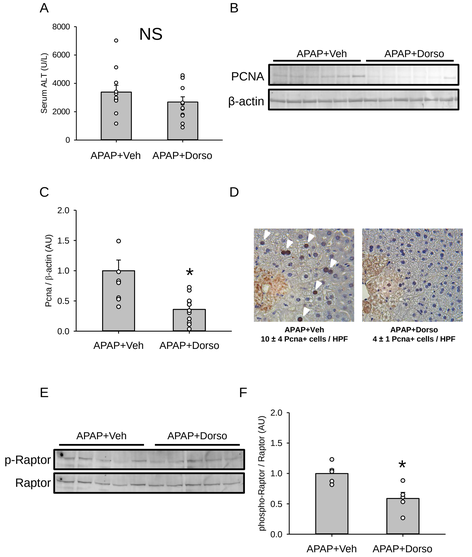

Figure 4. Late post-treatment with dorsomorphin reduced survival and liver regeneration after APAP overdose in mice. Mice were treated with 300 mg/kg APAP at 0 h followed by 20 mg/kg either dorsomorphin (Dorso) or vehicle control (Veh) at 6 h. Blood and liver tissue were collected at 24 h. (A) Serum ALT. (B) Densitometry and representative blot for proliferating cell nuclear antigen (Pcna) in liver tissue. (C) Immunohistochemistry for Pcna in liver tissue with counts (cell per 400x high-power field [HPF]). Arrowheads point to Pcna-positive nuclei (dark brown, punctate staining pattern). Data are expressed as mean±SE for n = 11-12. *p<0.05 vs. APAP+Veh.

Methods

ALFSG samples. Serum samples from 30 random survivors and 30 random non-survivors of APAP-induced ALF were obtained from the Acute Liver Failure Study Group (ALFSG) biorepository. ALF was diagnosed by ALFSG investigators and defined as INR ≥ 1.5, hepatic encephalopathy, duration of illness <26 weeks, and absence of chronic liver disease. APAP toxicity was determined to be the etiology based on a combination of patient-reported history of APAP overdose, a detectable APAP level documenting ingestion, and aminotransferase level of ≥ 1,000 IU/L. Due to hepatic encephalopathy, consent was obtained from next of kin. Samples were centrifuged at each ALFSG study site to obtain serum and stored at −80°C for later distribution and analysis. Demographic and laboratory data provided with the samples included daily values for serum ALT, AST, total bilirubin (Tbili), prothrombin time (PT), and creatinine (Cre) during hospitalization; age; sex; race; and ethnicity. Internal review board (IRB) approval was obtained at each ALFSG study site and the study was conducted in accordance with the 1975 Declaration of Helsinki.

Volunteer subjects. Ten volunteers without liver disease and with recent therapeutic APAP exposure were recruited at the University of Arkansas for Medical Sciences (UAMS) in Little Rock, AR, USA. Each subject was informed of the potential risks and benefits of the study and signed a consent form. After enrollment, a blood sample was collected from each subject and serum was separated by centrifugation. The study protocol was reviewed and approved by the UAMS IRB and the study was conducted in accordance with the 1975 Declaration of Helsinki.

Untargeted Proteomics. Abundant serum proteins were depleted with HighSelect Top14 resin (Thermo) according to the manufacturer's instructions. Proteins were reduced and alkylated prior to digestion with sequencing grade modified porcine trypsin (Promega) using S-Trap columns (Protifi). Tryptic peptides were then separated by reverse phase XSelect CSH C18 2.5 um resin (Waters) on an in-line 150 x 0.075 mm column using an UltiMate 3000 RSLCnano system (Thermo). Peptides were eluted using a 60 min gradient from 98:2 to 65:35 buffer A:B ratio (buffer A = 0.1% formic acid, 0.5% acetonitrile; buffer B = 0.1% formic acid, 99.9% acetonitrile). Eluted peptides were ionized by electrospray (2.2 kV) followed by mass spectrometric analysis on an Orbitrap Exploris 480 mass spectrometer (Thermo). Proteins with an FDR adjusted p-value < 0.05 and a fold change >2 were considered significant. Pathway Analysis and subsequent Upstream Analysis of the untargeted proteomics data were performed using Ingenuity Pathway Analysis® software (Qiagen, Germantown, MD). LogFC cutoffs of -1 to 1 and a p-value cutoff of 0.05 were used in the initial core analysis.

Lactate dehydrogenase measurement. Lactate dehydrogenase (LDH) activity was measured using a standard kinetic assay based on the loss of NADH absorbance in the reaction mixture.

Animal study. Wild-type male C57Bl/6J mice were acquired from the Jackson Laboratory (Bar Harbor, ME) and housed in a temperature-controlled facility with a 12 h light-dark cycle at the University of Arkansas for Medical Sciences (UAMS). All mice were used in experiments at 8-9 weeks of age. The animals were allowed free access to food and water until the night before APAP administration. Briefly, food was removed overnight beginning at -12 to -16 h, followed by i.p. treatment with 300 mg/kg APAP dissolved in warm 1x phosphate-buffered saline (PBS) at 0 h the next morning, and finally either 20 mg/kg dorsomorphin (Dorso) dissolved in DMSO vehicle or an equal volume of DMSO control at 6 h. Food was returned at the time of Dorso treatment. Blood and liver tissue were collected at 24 h. Alanine aminotransferase (ALT) was measured in serum from the mice using a kit from MedTest Dx (Canton, MI), according to the manufacturer's instructions. Hematoxylin and eosin (H&E) staining and both immunohistochemistry and immunoblotting for proliferating cell nuclear antigen (Pcna) were performed as previously described (Clemens et al., 2019).

Statistical analyses. Sensitivity, specificity, likelihood ratios, and post-test probabilities were calculated using standard equations. Data normality was tested using the Shapiro-Wilk test. For normally distributed data, groups were compared using Student's t-test. For non-normally distributed data, groups were compared using a t-test on ranks. Logistic regression was used to screen for associations between biomarkers and outcome and receiver operating characteristic (ROC) curves were used to visualize the associations. Optimal biomarker cutoffs were determined using logistic regression with sensitivity set at 90%. The equation to combine MELD score and LDH values was MELD-LDH = -1.981 + (0.00008*LDH) + (0.0698*MELD), derived using multiple logistic regression. All statistical analyses were performed in SigmaPlot 12.5 (Systat, San Jose, CA).

Conclusions

- Proteomics revealed 23 potentially promising prognostic biomarkers in patients
- LDH, in particular, is a readily-available biomarker in clinical laboratories that may be useful to predict death in patients
- LKB1-AMPK signaling likely supports liver repair in both patients and mice

References

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