Circulating proteomic panels for diagnosis and risk stratification of acute-on-chronic liver failure in patients with viral hepatitis B

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Supplementary Materials and Methods

Inclusion and exclusion criteria

Patients dropped from the 3-month follow-up were also excluded. HBV reactivation was defined by ≥ 2 log increase of HBV-DNA from previously stable baseline level or ≥ 100 IU/mL in patients with previously undetectable level, or ≥ 20000 IU/ml with no baseline HBV-DNA. Diagnostic criteria for ACLF related complications and organ failures (OFs) were summarized in Table S4. Exclusion criteria included concurrent viral infections (e.g. hepatitis virus A/C/D/E, human immunodeficiency virus, cytomegalovirus, Epstein-Barr virus); liver failure by other causes including autoimmune, alcohol- or drug-related liver diseases; malignant tumors; other severe systematic or mental diseases before ACLF onset.

Treatment schedule.

All enrolled CHB or HBV-ACLF patients were hospitalized and received standard medical treatments including bed rest, antiviral therapy (lamivudine alone 100 mg, telbivudine alone 600 mg, entecavir alone 0.5 mg, or lamivudine 100 mg plus adefovir 10 mg daily), and nutrition support (glucose, vitamins, electrolytes, glutathione, adenosylmethionine. etc). HBV-ACLF associated complications were treated accordingly. Briefly, variceal bleeding was treated with somatostatin, proton pump inhibitors and antibiotic prophylaxis; bacterial infection was treated with antibiotics (adjusted based on bacteria culture and antibiotic sensitivity test); ascites was treated with restriction of sodium intake and/or diuretics (aldosterone antagonist and/or furosemide), or paracentesis for severe cases; hepatorenal syndrome was treated with intravenous infusion of albumin or plasma, vasoconstrictors (noradrenaline, dopamine or terlipressine); hepatic encephalopathy was treated with lactulose, L-ornithine aspartate and antibiotics. Fluid replacement was given to patients with MAP <70 mmHg, and vasoactive agents were used as necessary. Oxygen therapy (nasal catheter, or venturi mask oxygen inhalation) was performed in patients with decreased PaO_2 or SpO_2. Several ACLF patients who developed respiratory failure and cannot be improved by oxygen therapy were transferred to ICU in which mechanical ventilation was provided. HBV-ACLF patients with deterioration of complications after medical treatments, yet without contraindications, were given the option of receiving liver transplantation (LT) operated by the Division of Hepatobiliary and Pancreatic Surgery at The First Affiliated Hospital of the College of Medicine, Zhejiang University. All patients waiting for LT were registered in the China Liver Transplant Registry (CLTR) network, and were given priority for available livers from deceased-donors based on utilitarian ethics, i.e. allocation based on LT urgency and necessity.

Discovery comparative proteomic analyses.

To elucidate the incremental changes in circulation proteome related to CHB and HBV-ACLF progression, patients in two sub-clinical phases of CHB, the mild CHB (CHB-M with increase of ALT but normal Tbil level) and severe CHB (CHB-S with significant increase of ALT and mild jaundice, Tbil level ≥ 2 mg/dL, INR≤1.5), and

two sub-clinical phases of HBV-ACLF (ACLF-M with 2>INR≥1.5, and ACLF-S with INR≥2) were included for high-throughput comparative proteomic analyses (Table S1).

Four parallel comparative proteomics experiments that employed triplex-2MEGA labeling and 2D-RPLC prefractionation coupled with high-throughput MS/MS experiment to compare protein abundances within each group. Two parallel shotgun proteomic experiments were performed to compare low abundant proteins (LAP) from all 5 groups. In the first experiment, LAP peptides from pooled CHB-M (n=10) and CHB-S (n=6) samples were tagged by medium and heavy dimethyl label, respectively, using a 3-plex N-terminal dimethylation after lysine guanidination (2MEGA) protocol described later. In the second experiment, LAP peptides from ACLF-M (n=9) and ACLF-S (n=10) samples were tagged by medium and heavy dimethyl label, respectively. The control samples (n=10) were pooled and tagged by light dimethyl label and used as a common reference in both experiments. Analogously, another two parallel shotgun proteomic experiments were performed to compare high abundant proteins (HAP) from 5 groups with the identical labeling scheme. The overall design of all four 3-plex 2MEGA-2DLC-MS/MS experiments was illustrated in Figure S1.

Plasma samples from patients within the same group were pooled before proteomic analysis. The pooled plasma was divided into high and low abundant protein fractions by combinatorial peptide ligands library (CPLL) method with a modified protocol described previously [1]. Briefly, the ProteoMiner column (Catalog 163-3006, Bio-Rad, USA) was conditioned three times by 200 μL phosphate-buffered saline (PBS) supplemented with 0.1% sodium dodecyl sulfate (SDS). Before sample loading, 540 μL of pooled plasma was mixed with 60 μL PBS buffer, and SDS was added to a final concentration of 0.1%. The column was then loaded with 200 μL prepared plasma and incubated in room temperature (RT) with constant vortex for 1 h. After the loading, the column was centrifuged at 1,000 g for 2 min and then washed with 1 mL of PBS with 0.1% SDS and centrifuged for 2 min. Three loading-washing cycles were performed to load all 600 μL prepared plasma. The flow-through eluents from 3 loading cycles were combined and designated as high abundant protein (HAP) fraction. After removal of the residual wash buffer by 0.8 mL ddH_2O, the CPLL bound proteins, i.e. low abundant proteins (LAP), were eluted with 1 mL of elution buffer (8 M urea, 2% SDS).

Both HAP and LAP proteins were subjected to digestion procedure modified from the FASP protocol described previously [2]. Briefly, proteins (~200 μg) were denatured and reduced with 25 mM dithiothreitol at RT for 30 min. The sample was centrifuged at 16,000 x g for 5 min. Sample was then applied to a 30 kDa MWCO spin filter (MRCPRT030, Millipore) and centrifuged. The sample was then wash by 200 μL 50 mM triethylammonium bicarbonate (TEAB). Protein alkylation was performed at dark with 30 mM iodoacetamide for 30 min in RT. After centrifugation, the sample was washed by 200 μL 50 mM TEAB twice. Trypsin (V5111, Promega) in 100 μL 50 mM TEAB was added in 1:50 enzyme to protein ratio. After 14 h digestion at 37°C, the tryptic peptides were collected in a new tube by centrifugation. All

centrifugation steps in FASP protocol were carried out at 14,000 x g for 15 min.

Tryptic digests from different groups were then multiplexed by 2MEGA labeling protocol described previously [3]. Briefly, 100 µg (~1 µg/µL) peptides from each sample were adjusted to pH 11 by 2M NaOH. The amino group of lysine side chain was blocked via guanidination reaction at 37 °C for 1 h with 20 µL 6 M O-methylisourea. The pH was then adjusted to 5 with trifluoroacetic acid (TFA). Dimethylation was carried out at RT for 30 min, with either 4 µL 4% (v/v) CH_2O (light, L), CD_2O (medium, M), or $^{13}CD_2O$ (heavy, H) as N-terminal tagging reagent, and with 4 µL 0.6 M $NaBH_3CN$ to light, medium labeled samples and $NaBD_3CN$ to heavy labeled sample as reductive reagent. The reaction was stopped by 16 µL 1% (v/v) NH_4OH solution.

2DLC-MS/MS and data analysis.

The labeled peptides were then mixed according to the scheme in Figure S1 and further fractionated with high pH reverse phase chromatography on a 1260 HPLC System (Agilent) with an XBridge RP column (5 µm, 150 Å, 4.6*250 mm, Waters). Mobile phases A and B contain 2% and 98% acetonitrile (ACN), respectively, and were both adjusted to pH 10.0 using NH_4OH. The solvent gradient was set as follows: 2–5% B in 2 min; 5–18% B in 11 min; 18–32% B in 9 min; 32–95% B in 1 min; maintained at 95% B for 5 min. The tryptic peptides were separated at a flow rate of 0.5 mL/min and monitored by UV at 214 nm. Concatenation pooling strategy [4] was used to generate a total of 20 and 4 fractions from LAP and HAP digest, respectively. The resolved peptides were dried by Speed Vac (Labconco) and reconstituted in 20 µl of 0.1% formic acid (FA), 2% ACN for subsequent LC-MS/MS analyses.

The peptides from each fraction were separated on an Acclaim PepMap RSLC nanoViper column (C18, 2 µm, 100 Å, 50 µm i.d. x 15 cm, Thermo Scientific) with a flow rate at 250 nL/min on an Easy nanoLC 1000 system (Thermo Scientific). The peptides were subsequently eluted with a five-step linear gradient (buffer A: 0.1% FA, buffer B: ACN with 0.1% FA): 0-10min, 3-8% B; 10-120 min, 8-20% B; 120-137 min, 20-30% B; 137-143 min, 30-90% B; 143-150 min, 90% B. The nanoLC was coupled to a Linear Trap Quadrupole-Orbitrap Elite hybrid mass spectrometer (Thermo Fisher Scientific, Waltham, MA) under positive mode. The source was operated at 1.8 kV. The data-dependent analysis (DDA) scheme included a full MS survey scan from 400 to 1,200 Th at the resolution of 70,000 FWHM (at m/z 200 Th), followed by 20 MS/MS scan of most intensive precursors selected with 1.0 Th isolation width for fragmentation by collision induced dissociation (CID) with 35% collision energy.

The overall data processing procedures for discovery proteomics study were illustrated in Figure S2. For each 3-plex 2MEGA 2DLC-MS/MS experiment within the discovery comparative proteomic study, a consensus identification strategy with 2MEGA quantitation within the TransProteomic Pipeline environment (TPP, v.4.8) [5] was employed to process the data. Briefly, peak lists in mzXML format were generated from RAW files by ProteoWizard (v.3.0.5655) [6] for peak deisotope and charge state determination. Peak lists were then searched by Comet (2014.02 rev.2) [7]

and MS-GF+ (beta.v10089) [8] in parallel against an Uniprot database contain both human and HBV proteins (downloaded at 2013.6, 88504 sequences) concatenated with reverse sequences as decoys. The search parameters were set to carboxyamidomethylation (+57.02) on cysteine, guanidination (+42.02) on lysine as fixed modifications, and oxidation (+15.99) on methionine as variable modification. To prepare search results for ASAPratio quantitation using isotopic 2MEGA triplet peak clusters, separate searches each using one isotopic dimethylation on peptide N-term (L+28.03 or M+32.06 or H+36.08) as fixed modification was performed. Other search parameters included one missed cleavage during trypsin digestion, a MS mass tolerance of 50 ppm, and MS/MS mass tolerance of 0.8 Da on monoisotopic mode.

For each separate search with different 2MEGA modification settings using either Comet or MS-GF+ algorism, all peptide-spectra matches (PSMs) from all fractions were compiled and filtered (probability>0.8) by PeptideProphet [9] using the non-parametric scoring model. Then results from both engines were combined by iProphet [10] to generate a consensus list of matched peptides. The combined peptide identifications were then subjected to two pair-wise (M vs. L, H vs. L) isotopic peak ratio calculations using the ASAPRatioPeptideParser [11], with the following settings: use fixed scan range for light and heavy isotopic peaks; precursor m/z tolerance of 0.05 Da; only use static modification for quantitation. The resulting peptide lists with pair-wise 2MEGA ratio were assembled to give protein identification and quantitation by ProteinProphet in coupled with ASAPRatioProteinParser. For each protein, a p-value was calculated by ASAPRatioPvalueParser to estimate the probability of not changing as compared to the overall 2MEGA ratio distribution of all proteins according to the *Lowess* theorem [11]. The false discovery rate (FDR) was calculated by dividing the number of false hits by the number of all hits. Individual identification search results were compiled by ProteinProphet for pair-wise 2MEGA comparison.

Western Blot Analysis.

Liver tissue specimens were obtained from HBV-ACLF patients following total hepatectomy for subsequent liver transplantation. Liver biopsy specimens from healthy liver transplantation donor and CHB patients were used for comparison. The liver tissues were washed with ice-cold PBS. Proteins were extracted using RIPA lysis buffer containing protease inhibitor PMSF. Total protein was determined by BCA method (Thermo Scientific, USA). The proteins (20 μg) were separated on a 12% SDS-PAGE gel and then transferred to a polyvinylidene difluoride membrane (Millipore, USA). The membrane was blocked by 1% BSA in TBST and then incubated with primary antibodies at 4°C overnight. The primary antibodies used were rabbit polyclonal anti-HNF1α (#ab96777, 1:500 dilution, Abcam, USA), goat polyclonal anti-HNF4α (#sc-6557, 1:100 dilution, Santa Cruz, CA, USA) and rabbit monoclonal anti-β-actin (#4970, 1:1000 dilution, CST, USA). After washing, the membrane was then incubated with horseradish peroxidase conjugated secondary antibodies (#7074 anti-rabbit IgG, 1:2000 dilution, CST USA; #ab6741 anti-goat IgG, 1:2000 dilution, Abcam, USA) for 1h at RT. The membrane was visualized with

enhanced ECL (Thermo Scientific, USA) following exposure to X-ray films. The software Image J (NIH, USA) was used to quantify the relative band intensity.

Selection of potential CHB and ACLF related biomarkers.

We used the following steps to select biomarker candidates for downstream validation (also summarized in Figure 2B):

1, Proteins with convincing evidence of presence, using the identification criteria detailed in previous section, in any one of the four 2MEGA-2DLC-MS/MS experiments were considered as detected, thus resulting a list of 255 HAP and 1087 LAP (Table S6) which were subjected to 2MEGA quantitative analyses.

2, Proteins shown expression differences (p-value < 0.05) at least in one comparison as determined by ASAPRatioPvalueParser were kept as DEP. Proteins with p-value of "N/A" or > 0.05 in all comparisons were discarded. For each protein deemed as differentially expressed, its original LC-MS1 isotopic peak pairs and chromatograms were manually inspected to filter out any mis-matches, interferences. After this step, 43 and 154 differentially expressed HAP and LAP (Table S6) were retained.

3, Potential markers found in HAP and LAP fractions were then combined. Proteins were kept as long as no contradictory quantitation were shown, e.g. proteins upregulated in HAP fraction but downregulated in LAP, or verse vice, in one pair-wise comparison were discarded. In addition, proteins that are highly expressed in liver according to the annotation on transcriptomic or proteomic level provided by www.genecards.org and http://www.proteinatlas.org were kept. After this step, 52 liver specific DEPs (Table S6) were retained.

4, Plasma samples were treated as described later in the "Targeted protein assay on an nLC-Orbitrap Q-Exactive MS" section to build peptide-spectra match library. After this step, 42 proteins with peptides successfully detected were kept as the final candidates for targeted proteomic validation (Table S7).

5, The quantitative data from the targeted proteomic study were then filtered to remove proteins with missing value in more than 60% samples (~146 samples), thus resulted in a list of 28 quantified proteins (Table S8).

Targeted proteomics.

To build the MS/MS spectra library for reference, a small set of samples (n=40) were first analyzed using LC-MSMS under the DDA scheme. Peptides (~500 ng) were reconstituted in phase A (2% ACN, 0.1% FA) and then enriched on a Symmetry C18 nanoACQUITY Trap Column (100 Å, 5µm, 180 µm x 20 mm). Peptide separation was carried out by a BEH C18 nanoACQUITY Column (130Å, 1.7 µm, 75 µm x 250 mm) on an nanoACQUITY UPLC system (Waters, Milford, MA) at a flow rate of 200 nL/min. The gradient started with 3% of mobile phase B (98% ACN, 0.1% FA) and increased to 8% in 4 min, then reached 18% B in 36 min and 32% B in 20 min. The gradient finally reached 90% B in 1 min and was then held for 14 min before it return to 3% B in 1 min and kept for 19 min re-equilibration until next injection. The total analysis time per injection was 95 min. The nanoLC was coupled

to an Orbitrap Q-Exactive mass spectrometer (Thermo). The source was operated at 2.0 kV. The DDA analysis included a full MS survey scan from 400 to 1,200 Th at the resolution of 70,000 FWHM (at m/z 200) with automatic gain control (AGC) Th target set to 3e6, followed by 20 most intense peaks were selected for fragmentation by higher-energy collision dissociation (HCD). The MS/MS spectra were acquired with 17,500 FWHM resolution with AGC set to 2e5. Search results from MaxQuant against the human Uniprot database were loaded to Skyline (v3.5.0.9319) for selection of peptide ions according to the targeted proteomic workflow protocol described previously [12].

A list of 222 peptides derived from 42 protein markers (Table S7) was generated for the targeted proteomic survey. Thereafter, all samples were analyzed using the same chromatographic conditions while the MS operated under the parallel-reaction monitoring (PRM) acquisition scheme, which included a full MS survey scan from 300 to 1,800 at the resolution of 17,500 FWHM (at m/z 200) with AGC set to 3e6, followed by HCD events to collect MS/MS spectra scheduled for a list of pre-defined 222 peptide targets. The isolation window was set to 2 Th. All MS/MS spectra were acquired at the resolution of 17,500 FWHM with AGC set to 1e6. The HCD normalized collision energy was set to 27%.

Data and materials availability

All mass spectrometric raw files are uploaded to iProX reservoir (http://www.iprox.cn/index, project ID: IPX0000769000) by this link: http://www.iprox.org/page/PSV023.html;?url=1533606841953rIHy, password: SoIJ.

Supplementary Figures

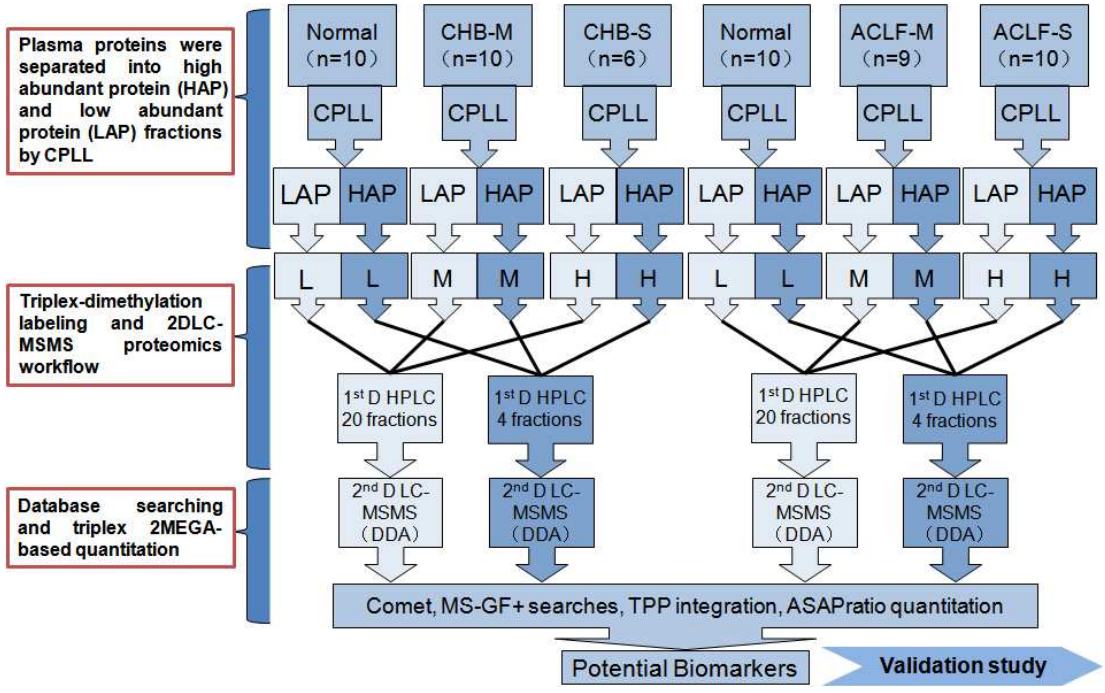


Figure S1. Schematic diagram summarizing the workflow for comparative proteomics study at discovery stage.

Samples were pooled within each groups and separated into low abundant (LAP) and high abundant (HAP) protein fractions. Totally 4 parallel 2MEGA-2DLC-MS/MS experiments were performed to compare: LAP from CHB-S, CHB-M and control; LAP from ACLF-S, ACLF-M and control; HAP from CHB-S, CHB-M and control; HAP from ACLF-S, ACLF-M and control.

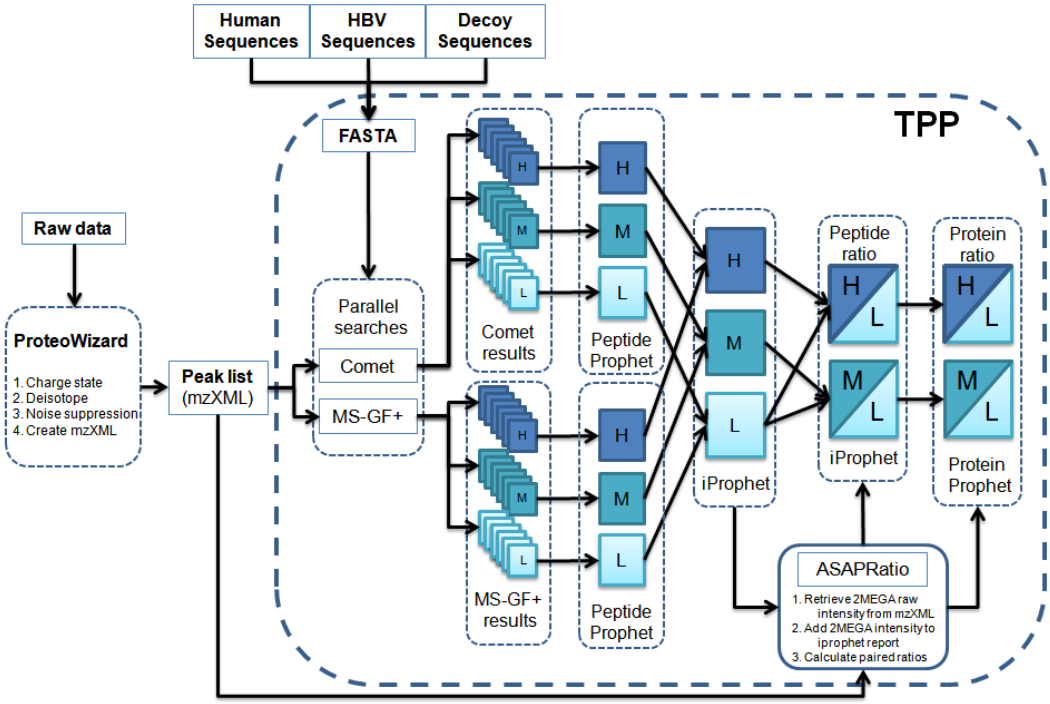


Figure S2. Overall bioinformatics workflow to process proteomics data collected at discovery stage.

A consensus identification strategy using two parallel search algorithms was used to process each 3-plex 2DLC-MS/MS dataset in 3 runs, each setting the H, M and L dimethylation, respectively, as fixed modification. The parallel search results were combined by iProphet while using ASAPRatio to calculate 2MEGA M/L or H/L ratio. ProteinProphet was used to generate the final protein level identification and quantitation results.

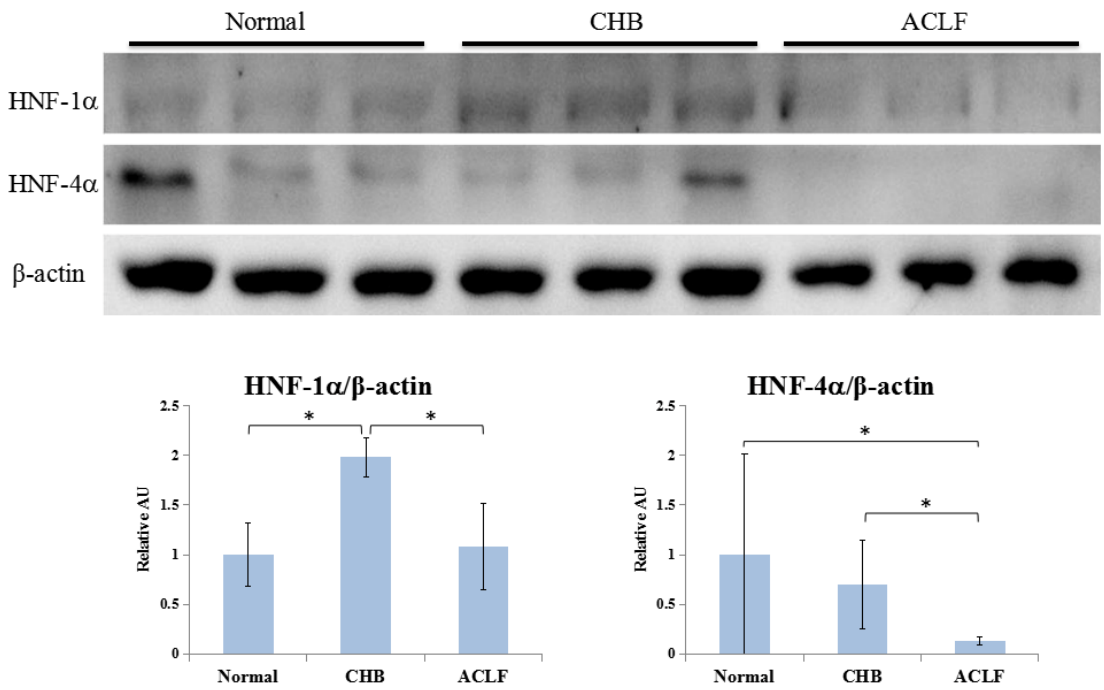


Figure S3. Western blotting analysis of liver HNF-1α and HNF-4α expression. Downregulations of both HNF-1α and HNF-4α in ACLF liver as compared to CHB and healthy donors were confirmed. Immunoblots showing expression differences of HNF-1α and HNF-4α in 3 normal, 3 CHB and 3 HBV-ACLF liver specimens were shown in the upper panels while using β-actin as the loading control. Semi-quantitative densitometric measurement of blot bands were summarized in the lower panels. * p-value (*t*-test) of <0.05.

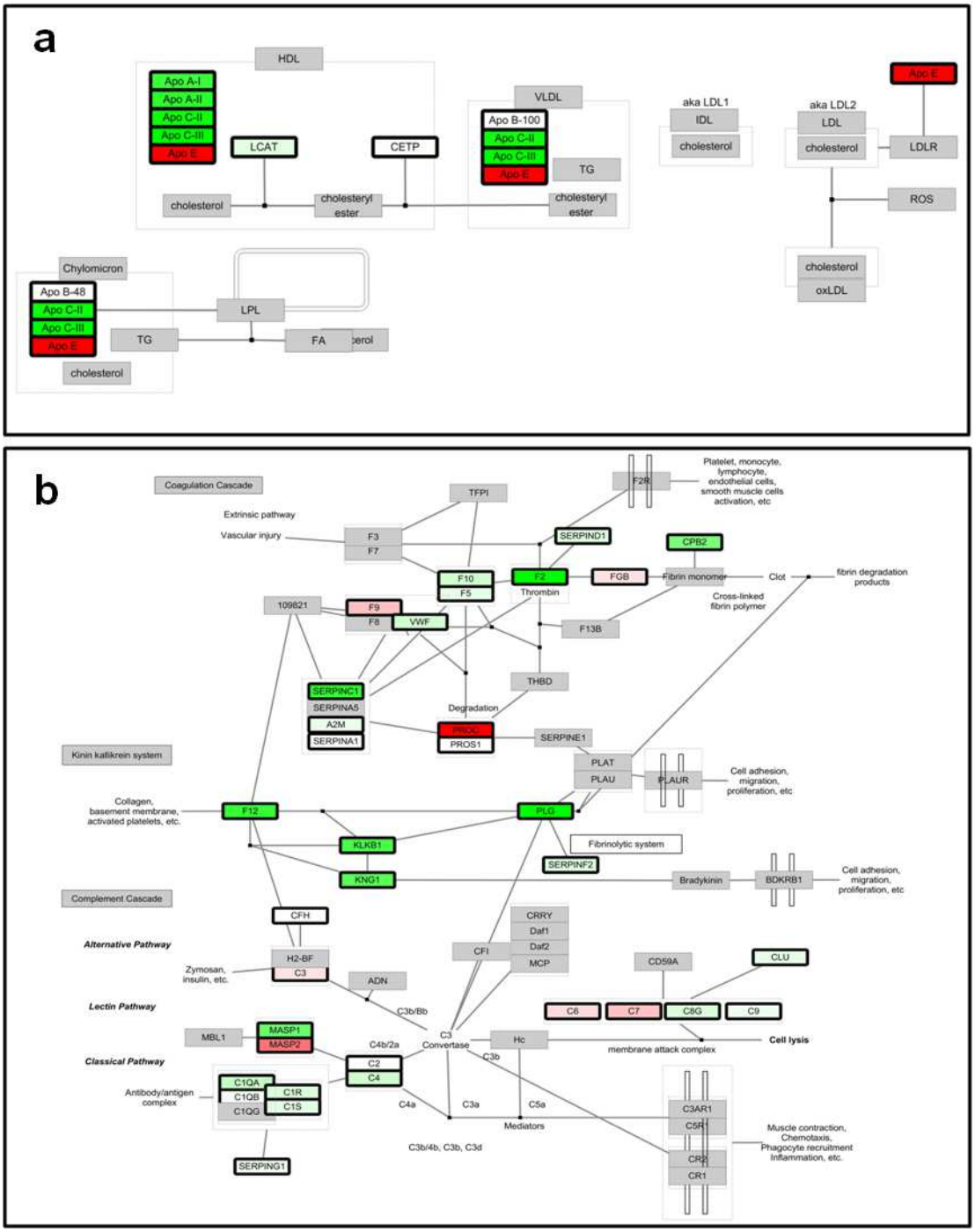
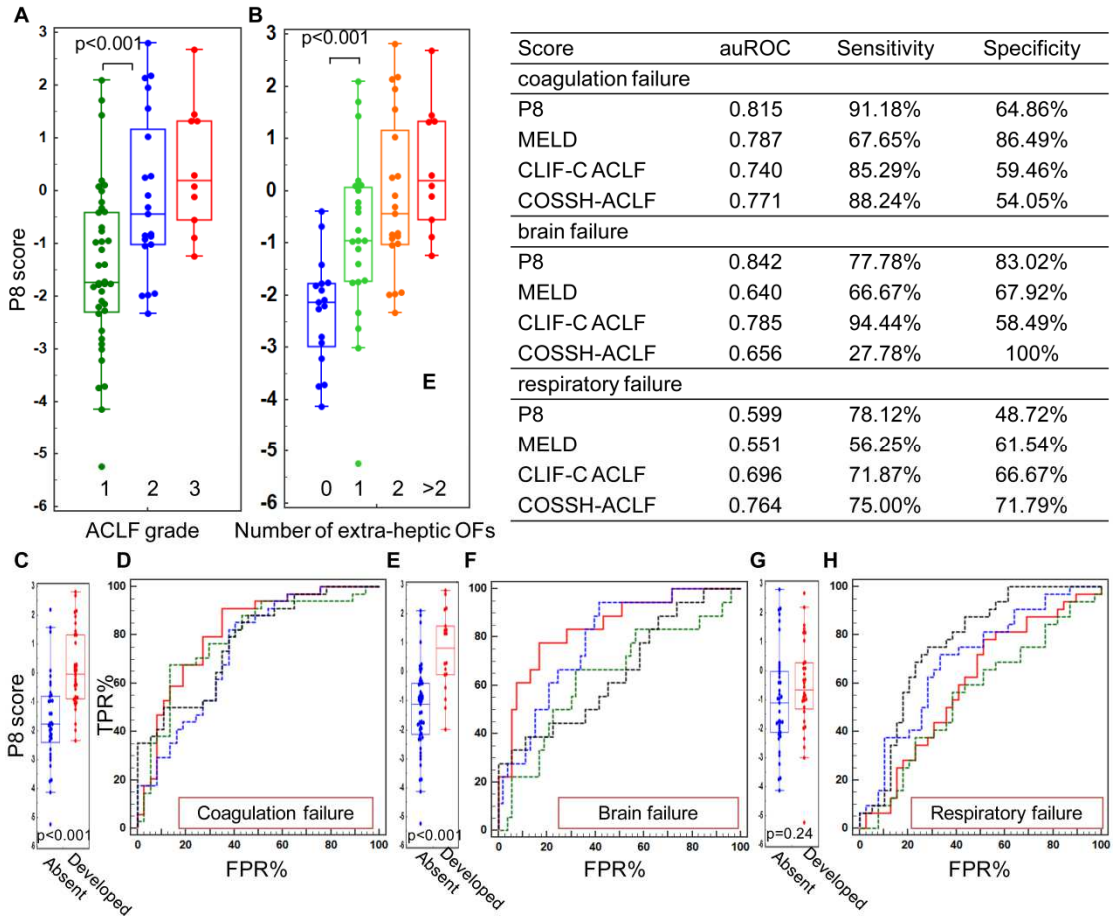


Figure S4. Schematic illustration of two enriched pathways.

Key participants of the lipid transport pathway (**a**), coagulation & complement systems (**b**), as annotated by Wikipathways, were colored to reflect the molecular changes during the onset of ACLF. Protein with quantitation data were highlighted in bold box. Color shades (red for upregulation, green for downregulation, colorless for insignificant changes, grey for data not available) represent their relative abundance of ACLF-M vs. CHB-S as quantified by 2MEGA log2 ratios



Score	auROC	Sensitivity	Specificity
coagulation failure			
P8	0.815	91.18%	64.86%
MELD	0.787	67.65%	86.49%
CLIF-C ACLF	0.740	85.29%	59.46%
COSSH-ACLF	0.771	88.24%	54.05%
brain failure			
P8	0.842	77.78%	83.02%
MELD	0.640	66.67%	67.92%
CLIF-C ACLF	0.785	94.44%	58.49%
COSSH-ACLF	0.656	27.78%	100%
respiratory failure			
P8	0.599	78.12%	48.72%
MELD	0.551	56.25%	61.54%
CLIF-C ACLF	0.696	71.87%	66.67%
COSSH-ACLF	0.764	75.00%	71.79%

Figure S5. Prediction performance of the P8 score for organ failures

The Prognostic P8 score was associated with the ACLF grade per COSSH prognostic scores (A) and the numbers of OFs in ACLF patients (B), and also with 3 major types of OFs, i.e. coagulation failure (C), brain failure (E) but not respiratory failure (G). ROC analyses of prediction of developing each type of OFs within 90 days by Prognostic P8, MELD,CLIF-C ACLF and COSSH-ACLF score at admission (D,F,H).

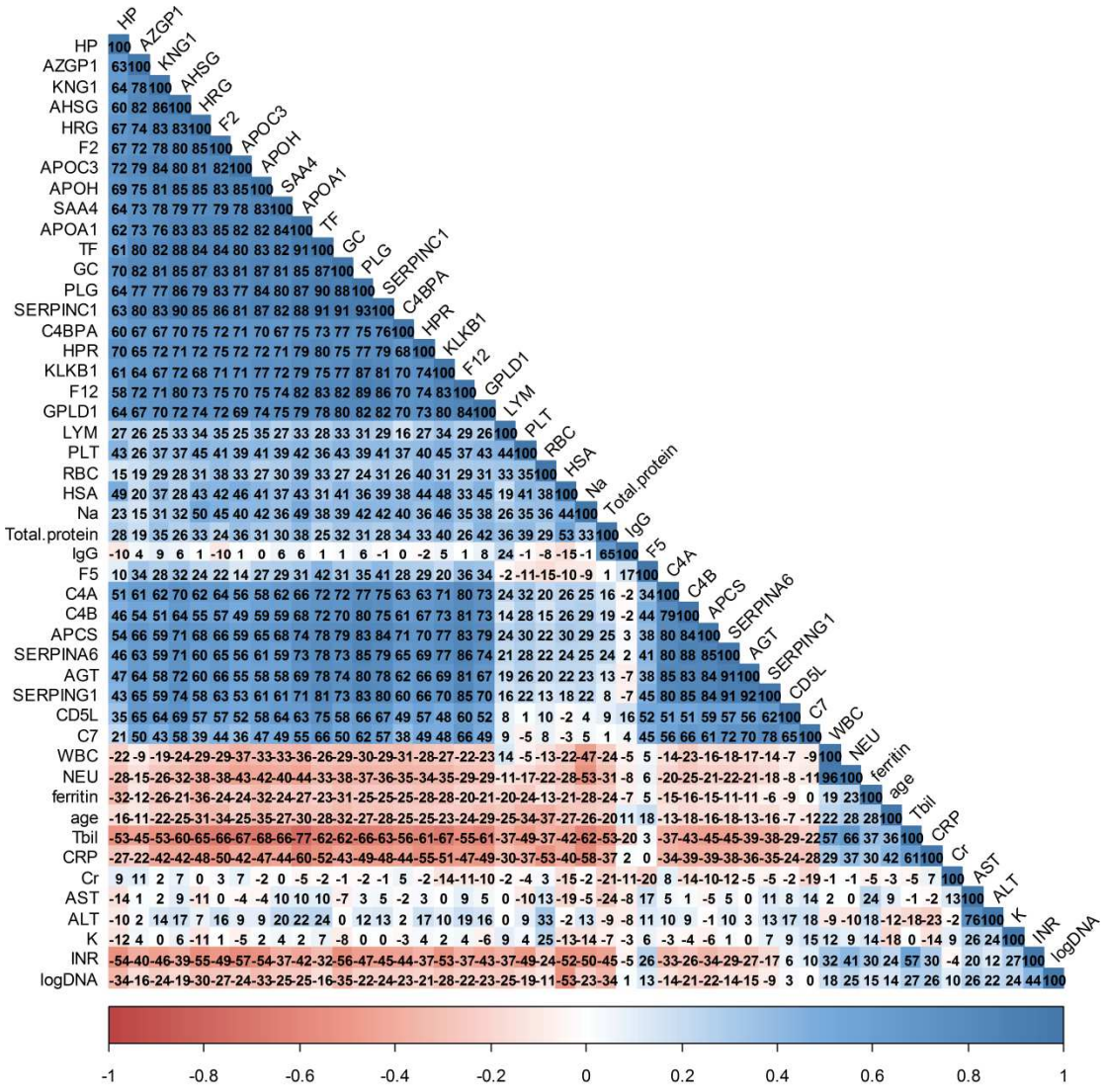


Figure S6. Biomarker correlation matrix.

Abundance profiles of 28 proteins quantified in validation study and available clinical parameters across all samples were ordered via hierarchical clustering. Shades of blue (positively correlated) or red (negatively correlated) represent low-to-high correlation coefficient between markers.

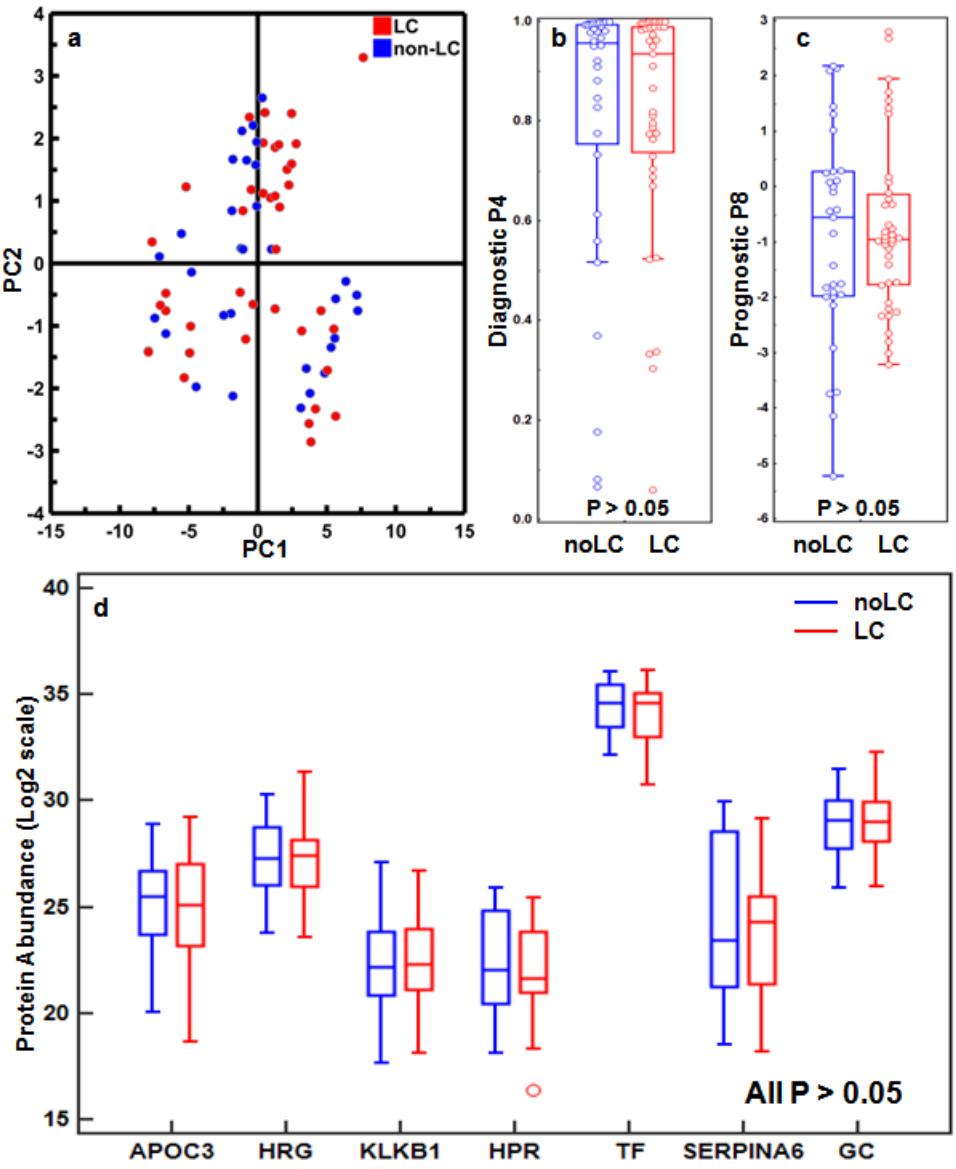


Figure S7. Summary of the impact of liver cirrhosis on the 28 candidate markers.

(**a**) PCA score plot based on 28 protein levels across all 79 ACLF samples analyzed in targeted proteomics validation study showing no separation of ACLF patients with (LC) and without cirrhosis (noLC). Model statistics: R^2X= 0.72, Q^2= 0.65. No significant differences of P4 (**b**), P8 (**c**) or biomarkers associated with the scores (**d**) were found between LC and none-LC patients (all P > 0.05).

Supplementary Tables

Table S1: Clinical characteristics of patients in the discovery proteomics study.

	Controls (n=10)	CHB-M (n=10)	CHB-S (n=6)	ACLF-M (n=9)	ACLF-S (n=10)
Age (yrs)	39.0±8.1	33.6±11.8	34.7±6.5	50.8±10.6	40.0±7.5
3 month survival/death	10/0	10/0	6/0	5/4	4/6

ALT (U/L)	22.7±7.4	104.9±52.4	469.3±401.8	246.1±130.1	603.9±462.3
AST (U/L)	20.0±3.8	62.0±36.0	288.7±260.4	195.7±64.6	433.9±300.1
Albumin (g/L)	45.3±1.7	43.5±5.4	34.0±5.3	29.8±2.9	31.5±3.9
Tbil (mg/dL)	0.64±0.1	1.0±0.4	7.8±4.8	20.1±9.4	24.3±6.0
PT (sec)	N/A	11.5±0.5	14.6±3.0	19.5±2.7	39.3±8.8
INR	N/A	1.00±0.04	1.26±0.25	1.66±0.22	3.34±0.65
Cr (mg/dL)	0.8±0.1	0.8±0.1	0.9±0.2	0.7±0.1	0.9±0.4
LogHBV-DNA	N/A	7.3±0.9	6.1±2.3	5.4±1.5	6.8±2.1
Ferritin (μg/L)	N/A	308.8±18.9	1247.3±1112.9	2151.9±1609.3	3522.4±2926.6
IgG (g/L)	26.2±2.9	25.9±3.1	31.3±5.2	31.3±8.3	23.6±5.6
CRP (mg/L)	N/A	5.2±5.7	8.0±2.9	15.9±9.8	10.7±9.9
WBC (10^9/L)	N/A	5.8±1.9	5.0±2.8	7.0±4.1	6.9±1.7
NEU (10^9/L)	N/A	3.3±1.5	2.8±1.9	4.6±3.9	4.3±1.4
LYM (10^9/L)	N/A	1.9±0.5	1.7±0.6	1.3±0.5	1.7±0.9
RBC (10^{12}/L)	N/A	5.2±0.9	4.6±0.5	3.8±0.6	4.3±0.8
PLT (10^9/L)	N/A	197±54.2	159±81.3	113±31.6	129±61.4
Total Protein (mg/dL)	N/A	68.2±6.2	64.9±3.2	61.3±7.5	57.0±5.0
K (mmol/L)	N/A	4.1±0.2	4.3±0.3	4.0±0.6	4.2±0.6
Na (mmol/L)	N/A	141±1.1	138±1.7	136±4.4	138±3.5
MELD score	N/A	4.5±1.8	13.8±2.7	20.9±5.1	28.4±3.2

Note: all patients were male. N/A: not available; ALT, Alanine aminotransferase; AST, Aspartate aminotransferase; PT, prothrombin time; Tbil, Total bilirubin; Cr, creatinine; IgG, Immunoglobulin G; CRP, C reactive protein; NEU, Neutrophils; LYM, Lymphocytes; RBC, Red blood cell; PLT, Platelet;

Table S2: Prevalence and number of organ failures (OF) associated with ACLF patients in the validation set

	No. of Patients	**Prevalence**
No. of OFs		
0	0	0%
1	17	23.94%
2	23	32.39%
3	21	29.58%
4	10	14.08%
Type of OFs		
Liver	71	100%
Kidney	6	8.45%
Coagulation	34	47.89%
Cerebral	18	25.35%
Circulatory	5	7.04%
Respiratory	32	45.07%

Table S3: Main causes of endpoint events at 90 days after study enrollment of

ACLF patients in the validation set

Endpoint (within 90days)	Number of cases (n=46)
Multiple organ failure without septic shock and encephalopathy/cerebral edema	14 (30.4%)
Encephalopathy/cerebral edema	13 (28.3%)
Liver transplantation	18 (39.1%)
Unknown	1 (2.2%)

Table S4: Clinical definitions used in this study.

Terms	Definition	reference
Complications		
Liver cirrhosis (LC)	The diagnosis of LC was based on 1), physical examination (hepatic stigmata, spider angioma or splenomegaly) with sonographic evidence of liver nodularity; 2) endoscopic finding of portal hypertension; 3) enlarged spleen diameter and presence of esophageal varices or ascites; 4), medical history.	[13, 14]
Jaundice	Mild and severe jaundice are defined by a total serum bilirubin level ≥ 34 μmol/L (or 2 mg/dL) and ≥ 85 μmol/L (or 5 mg/dL), respectively.	[15]
Ascites	Detection of ascitic fluid by aspiration or radiological examination (ultrasonography, CT or MRI). Grade1 ascites was defined by only detectable via radiological examination. Grade 2 ascites was defined by moderate symmetrical distension of abdomen. Grade 3 ascites was defined by marked abdominal distension.	[13, 16]
Hepatic encephalopathy (HE)	Defined by the acute deterioration of consciousness with previous normal mental status without any other known neurological conditions. Only grade II-IV overt encephalopathy is recorded in this study. Grade II HE was defined by presence of lethargy/apathy, drowsiness, anxiety, restlessness, personality change, mood swings, inappropriate behavior, mild disorientation, dyspraxia /asterixis. Grade III HE was defined by development of severe but rousable drowsiness, somnolence/semistupor, unresponsive to verbal commands, confusion, severe disorientation, bizarre or combative behavior. Grade IV HE was defined by development of decerebrate or decorticate posture, coma, unresponsive to painful stimulus.	[14]
Spontaneous	Ascitic fluid neutrophil/polymorph count >250/μl	[17]

bacterial peritonitis (SBP)	without alternative causes.	
Hepatorenal syndrome	Hepatorenal syndrome was defined by an increased serum creatinine level of ≥2.0 mg/dL (176.8 μmol/L).	[17, 18]
Gastrointestinal hemorrhage	GI hemorrhage including presentation of hematemesis, tarry stool and/or endoscopic signs of active bleeding or oozing from upper and/or lower GI varices; or the presence of variceal fibrin clots or red wale markings.	[19, 20]
Organ Failures (CLIF-OF system)		
Liver	Total serum bilirubin level ≥12 mg/dL	[21]
Kidney	Serum creatinine level of ≥ 2.0 mg/dL	
Coagulation	INR ≥ 2.5	
Cerebral	Grade III-IV HE	
Circulatory	MAP< 70 mmHg or use of vasopressors	
Respiratory	$PaO_2/FiO_2 \leq 200$	

Table S5: Summary of discovery proteomics results and data analysis

Search	Experiment*	CPLL Fraction	Comparison	2MEGA Label ratio	Unique Peptide hits	Unique Protein hits	FDR#
1	1	LAP	CHB-M vs Normal	M vs L	10139	1300	0.46%
2	1	LAP	CHB-S vs Normal	H vs L	11181	2463	1.58%
3	2	LAP	ACLF-M vs Normal	M vs L	6464	1012	0.69%
4	2	LAP	ACLF-S vs Normal	H vs L	7104	1087	0.55%
5	3	HAP	CHB-M vs Normal	M vs L	1269	280	0.71%
6	3	HAP	CHB-S vs Normal	H vs L	2181	341	0.88%
7	4	HAP	ACLF-M vs Normal	M vs L	1387	231	0.43%
8	4	HAP	ACLF-S vs Normal	H vs L	2028	304	0.33%

*Experiment1, 2, 3, 4 refer to triplex comparison of LAP of CHB-SvsCHB-MvsNormal, ACLF-SvsACLF-MvsNormal, HAP of CHB-SvsCHB-MvsNormal, ACLF-SvsACLF-MvsNormal, respectively. Each triplex comparison is comprised of two parallel pair-wise (MvsL, HvsL) database searches in TPP.

#FDR were calculated at protein identification level.

The following tables are in separated Excel files:

Table S6: All plasma proteins quantified in the discovery comparative proteomic survey, including separate sheet of liver-specific differentially expressed proteins that shown high quantitation quality in 2MEGA data.

Table S7: List of proteins and their tryptic peptides surveyed by PRM-MS in the validation study.

Table S8: Abundance level of all 28 proteins quantified by PRM-MS and biochemical measurements of all validation samples.

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