Supplementary Material

Blood (Cohorts A and B) and Ascitic Fluid (Cohort A) Sampling and Storage

Routine laboratory tests were performed on the day of blood (cohorts A and B) and ascitic fluid (AF; cohort A) sampling.

For experimental analyses (cohort B), aliquots of AF and plasma were centrifuged ($3{,}000 \times g$ for 20 minutes) and stored at −80°C until measurements were performed in series.

Extracellular Vesicle-Associated Tissue Factor Activity in Cohort A

The extracellular vesicle-associated tissue factor activity was determined with a functional factor Xa generation assay, as previously described.[1]

Routine Laboratory Tests, α2-Antiplasmin, Plasminogen Activity, and Plasminogen Activator Inhibitor-1 in Cohort A

Routine laboratory parameters, α2-antiplasmin, plasminogen activity, and plasminogen activator inhibitor-1 were determined in the central laboratory of the Vienna General Hospital according to protocols that were implemented in the clinical routine (http://www.kimcl.at/).

Determination of Coagulation Factor Activities in Ascitic Fluid and Thrombin–Antithrombin as well as Plasmin–α2-Antiplasmin Assessments in Cohort A

Coagulation factor activities in ascites were assessed using similar coagulometric assays as compared with the determination of coagulation factor activities in plasma.

Thrombin–antithrombin (TAT) complex (Enzygnost TAT Micro, Siemens, Munich, Germany) and plasmin–α2-antiplasmin (PAP) complex (Technoclone, Vienna, Austria) were determined with commercially available enzyme-linked immunosorbent assay kits according to the manufacturers' instructions.

Clotting Assays in Cohort A

The potential of AF, ascitic fluid-derived extracellular vesicles, and ascitic fluid supernatant to induce plasma clot formation was investigated in different experimental set-ups.

First, we incubated ascites (27.5 µL) with citrated platelet-free normal pooled plasma (NPP, centrifuged for 10 minutes at $3{,}000 \times g$ and 2 minutes at $13{,}400 \times g$, 55 µL) and initiated clotting by addition of 27.5 µL HBSA buffer + $CaCl_2$ (137 mM NaCl, 5.38 mM KCl, 5.55 mM glucose, 10 mM HEPES, 0.1% bovine serum albumin, 20 mM $CaCl_2$, pH 7.5).

In another clotting experiment, extracellular vesicles were pelleted from 27.5 µL of ascitic fluid by centrifugation at $20{,}000 \times g$ for 15 minutes at 4°C, washed twice with HBSA, resuspended in 27.5 µL of HBSA, and added to extracellular vesicle-depleted NPP (centrifuged for 1 hour at $150{,}000 \times g$, 55 µL). Clotting was initiated by addition of HBSA + $CaCl_2$ (27.5 µL). Ascites supernatant (derived from ascites that was centrifuged at $20{,}000 \times g$ for 15 minutes) and ascites that was filtered through a membrane with 0.1-µm pore size (Merck Millipore, Tullagreen, Carrigtwohill, County Cork, Ireland) were also investigated. Clotting experiments were performed in the presence and absence of a tissue factor-blocking antibody (hTF1, 4 µg/L, 3 µL; BD Biosciences, San Jose, California, United States) or a control antibody (mouse immunoglobulin G, 4 µg/mL, 3 µL; Sigma-Aldrich, St. Louis, California, United States). The lag phase of the turbidity curve, reflecting the time until the onset of clot formation, was recorded with a Multiskan Spectrum microplate reader (Thermo Scientific, Bremen, Germany) at a wavelength of 405 nm. Measurements were performed for 120 minutes in duplicates.

Fibrinolysis Assays in Cohort A

Fibrinolytic capacity of AF was assessed by immersing fibrinogen clots into AF using a methodology previously used to assess the lytic activity of pancreatic fluid.[2] In short, spherical clots were generated by mixing 100 µL of fibrinogen concentrate (Haemocomplettan P, CSL Behring, Marburg, Germany, 20 mg/mL) with 10 µL of thrombin (Enzyme Research Laboratories, Swansea, United Kingdom; 20 IU/mL, final concentration) on a sheet of plastic paraffin film. After 15 minutes, the clots were suspended in 100 µL of AF, washed extracellular vesicles from AF, the supernatant of AF spun down to obtain washed extracellular vesicles, AF in the presence of aprotinin (26,000 kallikrein inhibitor units/mL), or normal saline, and incubated for 24 hours at 37°C. The clots were removed and the remaining fluid was spun down ($20{,}000 \times g$ for 10 minutes), after which an aliquot of the supernatant was taken. In samples taken before addition of the clot and after 24 hours, D-dimer levels were determined on an ACL300 coagulation analyzer using reagents from the manufacturer (Werfen, Breda, the Netherlands)

Thrombin Generation Potential in Cohort A

The thrombin generation (TG) potential was measured with a commercially available assay kit (Technothrombin TGA kit, Technoclone, Vienna, Austria) on a microplate reader (FL800, BioTek, Winooski, Vermont, United States) using the fluorogenic substrate Z-Gly-Gly-Arg-AMC (Bachem, Bubendorf, Switzerland) according to the manufacturer's instructions.

Routine Laboratory Tests in Cohort B

D-dimer levels and additional routine laboratory parameters were assessed in the central laboratory of the Vienna General Hospital according to protocols that were implemented in the clinical routine (http://www.kimcl.at/).

HVPG Measurement in Cohorts A and B

The hepatic venous pressure gradient (HVPG) was measured in clinical routine for diagnostic or prognostic purposes, or HVPG-guided nonselective β-blocker therapy,[3] as supported by the Austrian consensus recommendations for the

treatment of portal hypertension.[4–6] HVPG measurements were performed in the absence of portal pressure-lowering medications (i.e., nonselective beta-blockers and nitrates) and according to a standardized protocol, as previously described.[7,8] HVPG $\geq$10 mmHg denoted clinically significant portal hypertension.

Statistical Analyses

IBM SPSS Statistics 25 (IBM, Armonk, New York, United States) and GraphPad Prism 8 (GraphPad Software, La Jolla, California, United States) were used for statistical analyses. Categorical variables are reported as numbers and proportions of patients, while continuous variables are shown as mean $\pm$ standard deviation or median (interquartile range [IQR]), as appropriate. Differences in categorical variables were analyzed using the chi-squared or Fisher's exact test. Student's *t*-test, Mann–Whitney U test, or Kruskal–Wallis one-way analysis of variance (applying Dunn's multiple comparisons test) was used to compare continuous variables, as appropriate. Simple and multiple linear regression analyses were performed to evaluate factors independently associated with TG parameters as well as plasma TAT complex, prothrombin fragment $1+2$, D-dimer, and PAP complex levels. Variables showing a trend in univariate analysis ($p < 0.1$) as well as the factor of interest (ascites severity) were included into the multivariate models. We abstained from including composite measures of hepatic function, as they include the coagulation parameter international normalized ratio (model for end-stage liver disease [MELD] and Child–Turcotte–Pugh [CTP] score) and ascites (CTP score). Moreover, if similar variables showed associations in univariate analyses (i.e., different markers of systemic inflammation), only one was included. Multicollinearity was detected by variable inflation factor. *p*-Values $\leq$0.05 were considered as statistically significant.

Ethics

Both substudies were conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committee of the Medical University of Vienna (No. 1881/2014 and 1446/2018). Written informed consent was obtained from all patients included in the prospective study, while the requirement of a written informed consent for the retrospective analysis was waived by the Ethics Committee.

Patient Characteristics According to the Presence or Absence of Ascites (Cohort A)

Alcoholic liver disease (ALD) was the most common etiology (44%) and mean HVPG and median MELD were 20.6 $\pm$4.4 mmHg and 13 points (IQR: 4), respectively (►**Table 1**). CTP stages A, B, and C were observed in 26, 46, and 28%, respectively.

ALD was more common among patients with grade 3 ascites, who also more commonly had hepatic encephalopathy. As expected, patients with grade 3 ascites had higher CTP scores. While there was no difference in MELD score, serum albumin levels were lower in patients with grade 3 ascites. Moreover, serum creatinine and C-reactive protein, interleukin 6, and lipopolysaccharide-binding protein levels were higher in patients with ascites, as compared with HVPG-matched patients without ascites.

References (Supplementary Information Only)

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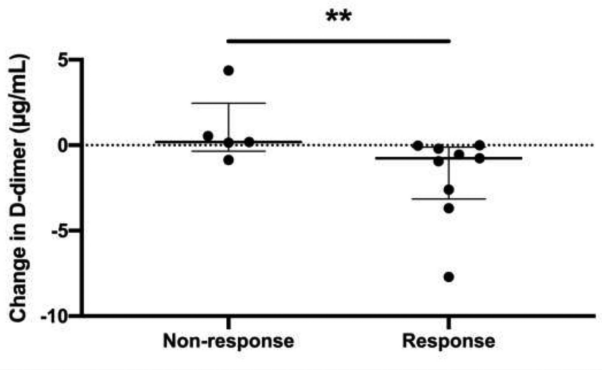

Supplementary Fig. S1 Comparison of changes in D-dimer levels between patients in whom ascites improved ("Response") following transjugular intrahepatic portosystemic shunt (TIPS) placement for recurrent/refractory ascites versus patients in whom TIPS did not affect ascites control ("Non-response"). $^{**}p < 0.05$. Data are presented as median with interquartile range and were analyzed by the Mann–Whitney U test.

Supplementary Table S1 Parameters associated with plasma peak thrombin generation in cohort A

	A		B		C	
Patient characteristics	**B**	***p*-Value**	**B**	***p*-Value**	**B**	***p*-Value**
Age, y	1.95	0.107	–	–	–	–
Male sex	16.2	0.631	–	–	–	–
ALD, vs. other etiologies	−7.89	0.789	–	–	–	–
Varices	52.7	0.138	–	–	–	–
History of variceal bleeding	40.1	0.231	–	–	–	–
Hepatic encephalopathy	47.7	0.105	–	–	–	–
HVPG, mmHg	4.28	0.231	–	–	–	–
MELD, points	−7.95	0.004	–	–	–	–
CTP score, points	−0.981	0.885	–	–	–	–
Albumin, g/L	0.666	0.813	–	–	–	–
Bilirubin, mg/dL	−8	<0.001	−9.18	<0.001	−9.18	<0.001
INR	−138	0.001	–	–	–	–
Creatinine, mg/dL	71.9	0.067	86	0.012	86	0.012
CRP, mg/L	21	0.155	–	–	–	–
IL-6, µg/mL	0.866	0.13	–	–	–	–
LBP, µg/mL	6.15	0.114	–	–	–	–
Grade 3 ascites	71.3	0.012	50.2	0.036	50.2	0.036

Abbreviations: ALD, alcoholic liver disease; CRP, C-reactive protein; CTP, Child–Turcotte–Pugh score; HVPG, hepatic venous pressure gradient; IL-6, interleukin 6; INR, international normalized ratio; LBP, lipopolysaccharide binding protein; MELD, model for end-stage liver disease.
Note: (A) Univariate analysis, (B) multivariate analysis, and (C) final step of backward selection of linear regression analysis.

Supplementary Table S2 Parameters associated with plasma thrombin generation lag phase in cohort A

	A		B		C	
Patient characteristics	**B**	***p*-Value**	**B**	***p*-Value**	**B**	***p*-Value**
Age, y	−0.112	0.146	–	–	–	–
Male sex	−1.98	0.339	–	–	–	–
ALD, vs. other etiologies	−1.27	0.492	–	–	–	–
Varices	−2.36	0.299	–	–	–	–
History of variceal bleeding	−2.02	0.342	–	–	–	–
Hepatic encephalopathy	−1.61	0.392	–	–	–	–
HVPG, mmHg	−0.468	0.028	−0.418	0.045	−0.418	0.045
MELD, points	0.028	0.88	–	–	–	–
CTP score, points	−0.538	0.198	–	–	–	–
Albumin, g/L	0.157	0.378	–	–	–	–
Bilirubin, mg/dL	0.142	0.353	–	–	–	–
INR	1.18	0.681	–	–	–	–
Creatinine, mg/dL	−1.65	0.505	–	–	–	–
CRP, mg/L	−0.796	0.396	–	–	–	–
IL-6, µg/mL	−0.046	0.206	–	–	–	–
LBP, µg/mL	−0.068	0.785	–	–	–	–
Grade 3 ascites	−3.52	0.051	−3.04	0.083	−3.04	0.083

Abbreviations: ALD, alcoholic liver disease; CRP, C-reactive protein; CTP, Child–Turcotte–Pugh score; HVPG, hepatic venous pressure gradient; IL-6, interleukin 6; INR, international normalized ratio; LBP, lipopolysaccharide binding protein; MELD, model for end-stage liver disease.
Note: (A) Univariate analysis, (B) multivariate analysis, and (C) final step of backward selection of linear regression analysis.

Supplementary Table S3 Parameters associated with plasma thrombin generation velocity index in cohort A

	A		B		C	
Patient characteristics	**B**	***p*-Value**	**B**	***p*-Value**	**B**	***p*-Value**
Age, y	0.434	0.246	–	–	–	–
Male sex	1.1	0.915	–	–	–	–
ALD, vs. other etiologies	8.64	0.337	–	–	–	–
Varices	14.9	0.173	–	–	–	–
History of variceal bleeding	7.61	0.461	–	–	–	–
Hepatic encephalopathy	11.4	0.21	–	–	–	–
HVPG, mmHg	1.33	0.209	–	–	–	–
MELD, points	−1.35	0.124	–	–	–	–
CTP score, points	1.01	0.628	–	–	–	–
Albumin, g/L	−0.382	0.658	–	–	–	–
Bilirubin, mg/dL	−1.56	0.032	−1.97	0.004	−2.07	0.003
INR	−31.2	0.021	–	–	–	–
Creatinine, mg/dL	21.1	0.081	19.7	0.087	23	0.041
CRP, mg/L	7.76	0.085	–	–	–	–
IL-6, µg/mL	0.414	0.016	0.313	0.074	0.394	0.014
LBP, µg/mL	2.5	0.034	–	–	–	–
Grade 3 ascites	21.8	0.012	9.67	0.281	–	–

Abbreviations: ALD, alcoholic liver disease; CRP, C-reactive protein; CTP, Child–Turcotte–Pugh score; HVPG, hepatic venous pressure gradient; IL-6, interleukin 6; INR, international normalized ratio; LBP, lipopolysaccharide binding protein; MELD, model for end-stage liver disease.
Note: (A) Univariate analysis, (B) multivariate analysis, and (C) final step of backward selection of linear regression analysis.

Supplementary Table S4 Parameters associated with plasma thrombin–antithrombin levels in cohort A

	A		B		C	
Patient characteristics	**B**	***p*-Value**	**B**	***p*-Value**	**B**	***p*-Value**
Age, y	0.155	0.576	–	–	–	–
Male sex	−20.1	0.005	−14.9	0.061	−18.1	0.08
ALD, vs. other etiologies	−12.1	0.062	−1.26	0.854	–	–
Varices	−3.05	0.699	–	–	–	–
History of variceal bleeding	1.84	0.81	–	–	–	–
Hepatic encephalopathy	−15.6	0.016	−3.12	0.697	–	–
HVPG, mmHg	0.05	0.947	–	–	–	–
MELD, points	−0.634	0.329	–	–	–	–
CTP score, points	−3.02	0.04	–	–	–	–
Albumin, g/L	1.13	0.07	0.463	0.473	–	–
Bilirubin, mg/dL	−0.391	0.477	–	–	–	–
INR	2.76	0.789	–	–	–	–
Creatinine, mg/dL	−17.9	0.044	−2.78	0.771	–	–
CRP, mg/L	−8.41	0.01	−4.94	0.17	−7.4	0.016
IL-6, µg/mL	−0.52	0.692	–	–	–	–
LBP, µg/mL	−1.6	0.07	–	–	–	–
Grade 3 ascites	−15.6	0.014	−3.73	0.645	–	–

Abbreviations: ALD, alcoholic liver disease; CRP, C-reactive protein; CTP, Child–Turcotte–Pugh score; HVPG, hepatic venous pressure gradient; IL-6, interleukin 6; INR, international normalized ratio; LBP, lipopolysaccharide binding protein; MELD, model for end-stage liver disease.
Note: (A) Univariate analysis, (B) multivariate analysis, and (C) final step of backward selection of linear regression analysis.

Supplementary Table S5 Parameters associated with plasma prothrombin fragment 1 + 2 levels in cohort A

	A		B		C	
Patient characteristics	**B**	***p*-Value**	**B**	***p*-Value**	**B**	***p*-Value**
Age, y	5.82	0.195	–	–	–	–
Male sex	−103	0.42	–	–	–	–
ALD, vs. other etiologies	−193	0.069	−251	0.012	−309	0.001
Varices	−34.8	0.786	–	–	–	–
History of variceal bleeding	271	0.025	113	0.287	–	–
Hepatic encephalopathy	7.39	0.946	–	–	–	–
HVPG, mmHg	−15.1	0.22	–	–	–	–
MELD, points	−3.51	0.745	–	–	–	–
CTP score, points	−3.44	0.891	–	–	–	–
Albumin, g/L	−8.24	0.421	–	–	–	–
Bilirubin, mg/dL	−10.9	0.219	–	–	–	–
INR	−208	0.215	–	–	–	–
Creatinine, mg/dL	431	0.002	475	0.001	473	<0.001
CRP, mg/L	−18.9	0.731	–	–	–	–
IL-6, µg/mL	4.23	0.044	4.34	0.032	3.89	0.033
LBP, µg/mL	5	0.736	–	–	–	–
Grade 3 ascites	−3.98	0.971	−100	0.346	–	–

Abbreviations: ALD, alcoholic liver disease; CRP, C-reactive protein; CTP, Child–Turcotte–Pugh score; HVPG, hepatic venous pressure gradient; IL-6, interleukin 6; INR, international normalized ratio; LBP, lipopolysaccharide binding protein; MELD, model for end-stage liver disease.
Note: (A) Univariate analysis, (B) multivariate analysis, and (C) final step of backward selection of linear regression analysis.

Supplementary Table S6 Parameters associated with plasma D-dimer levels in cohort A

	A		B		C	
Patient characteristics	**B**	***p*-Value**	**B**	***p*-Value**	**B**	***p*-Value**
Age, y	0.032	0.483	–	–	–	–
Male sex	−0.397	0.761	–	–	–	–
ALD, vs. other etiologies	2.72	0.011	0.801	0.359	–	–
Varices	0.051	0.969	–	–	–	–
History of variceal bleeding	−0.71	0.576	–	–	–	–
Hepatic encephalopathy	1.8	0.101	–	–	–	–
HVPG, mmHg	0.061	0.631	–	–	–	–
MELD, points	0.135	0.216	–	–	–	–
CTP score, points	1.01	<0.001	–	–	–	–
Albumin, g/L	−0.297	0.003	−0.128	0.128	−0.154	0.06
Bilirubin, mg/dL	0.019	0.831	–	–	–	–
INR	−0.416	0.809	–	–	–	–
Creatinine, mg/dL	1.74	0.26	–	–	–	–
CRP, mg/L	1.27	0.02	–	–	–	–
IL-6, µg/mL	0.066	0.002	0.02	0.277	–	–
LBP, µg/mL	0.238	0.111	–	–	–	–
Grade 3 ascites	4.96	<0.001	3.74	<0.001	4.41	<0.001

Abbreviations: ALD, alcoholic liver disease; CRP, C-reactive protein; CTP, Child–Turcotte–Pugh score; HVPG, hepatic venous pressure gradient; IL-6, interleukin 6; INR, international normalized ratio; LBP, lipopolysaccharide binding protein; MELD, model for end-stage liver disease.
Note: (A) Univariate analysis, (B) multivariate analysis, and (C) final step of backward selection of linear regression analysis.

Supplementary Table S7 Parameters associated with plasma plasmin–α2-antiplasmin levels in cohort A

	A		B		C	
Patient characteristics	**B**	***p*-Value**	**B**	***p*-Value**	**B**	***p*-Value**
Age, y	5.52	0.336	–	–	–	–
Male sex	−196	0.203	–	–	–	–
ALD, vs. other etiologies	216	0.113	–	–	–	–
Varices	27.1	0.869	–	–	–	–
History of variceal bleeding	26.4	0.868	–	–	–	–
Hepatic encephalopathy	286	0.036	−131	0.316	–	–
HVPG, mmHg	3.75	0.816	–	–	–	–
MELD, points	19.8	0.143	–	–	–	–
CTP score, points	111	<0.001	–	–	–	–
Albumin, g/L	−20.4	0.118	–	–	–	–
Bilirubin, mg/dL	0.746	0.948	–	–	–	–
INR	96.8	0.652	–	–	–	–
Creatinine, mg/dL	292	0.117	–	–	–	–
CRP, mg/L	122	0.077	–	–	–	–
IL-6, µg/mL	10.8	<0.001	7.57	0.002	7.26	0.003
LBP, µg/mL	42.4	0.02	–	–	–	–
Grade 3 ascites	550	<0.001	443	0.002	373	0.002

Abbreviations: ALD, alcoholic liver disease; CRP, C-reactive protein; CTP, Child–Turcotte–Pugh score; HVPG, hepatic venous pressure gradient; IL-6, interleukin 6; INR, international normalized ratio; LBP, lipopolysaccharide binding protein; MELD, model for end-stage liver disease.
Note: (A) Univariate analysis, (B) multivariate analysis, and (C) final step of backward selection of linear regression analysis.

Supplementary Table S8 Comparison of patient characteristics between cohort A and cohort B

Patient characteristics	Cohort A, $n = 50$	Cohort B, $n = 317$	*p*-Value
Age, y	57.8 ± 11.9	54.3 ± 11.2	0.047
Sex			
Male	37 (74%)	220 (69%)	0.509
Female	13 (26%)	97 (31%)	
Etiology			
Viral	15 (30%)	116 (37%)	0.595
ALD	22 (44%)	132 (42%)	
NAFLD or cryptogenic	10 (20%)	44 (14%)	
Other	3 (6%)	25 (8%)	
Ascites	25 (50%)	155 (49%)	0.008
Hepatic encephalopathy	20 (40%)	96 (30%)	0.17
HVPG, mmHg	20.5 (17–24)	19 (15–23)	0.026
MELD, points	13 (11–15)	11 (9–14.5)	<0.007
CTP score, points	8 (6–10)	9 (7–11)	<0.001
A	13 (26%)	57 (18%)	0.386
B	23 (46%)	154 (49%)	
C	14 (28%)	106 (33%)	
Albumin, g/L	32.3 (28.7–36.6)	34.8 (30.2–38.8)	0.049
Bilirubin, mg/dL	1.47 (0.99–2.5)	1.37 (0.91–2.26)	0.244
INR	1.4 (1.3–1.53)	1.3 (1.16–1.4)	<0.001
Creatinine, mg/dL	0.76 (0.668–0.915)	0.78 (0.67–0.945)	0.661
CRP, mg/L	0.68 (0.275–1.29)	0.35 (0.13–0.8)	<0.001
D-dimer, µg/mL	2.95 (0.77–5.2)	1.17 (0.55–3.16)	0.005

Abbreviations: ALD, alcoholic liver disease; CRP, C-reactive protein; CTP, Child–Turcotte–Pugh score; HVPG, hepatic venous pressure gradient; INR, international normalized ratio; MELD, model for end-stage liver disease; NAFLD, non-alcoholic fatty liver disease.
Note: Categorical variables are reported as numbers and proportions of patients, while continuous variables are shown as mean ± standard deviation or median (interquartile range [IQR]), as appropriate.

