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Neutrophil Extracellular Traps Are Not Linked to Decompensation, ACLF, or Death in Clinically Stable Patients With ACLD

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ABSTRACT

Background and Aims: Neutrophil extracellular traps (NETs) are part of the body's innate immune response. In animal models, NETs aggravated liver injury and promoted disease progression/portal hypertension by the formation of (micro)thrombi leading to parenchymal extinction.

This study aimed to investigate NETosis in patients with clinically stable advanced chronic liver disease (ACLD).

Methods: We evaluated stable ACLD patients undergoing hepatic venous pressure gradient (HVPG) measurement in whom an extensive panel of laboratory tests related to coagulation and NET biomarkers was assessed in plasma. Hepatic decompensation/liver-related death (LRD) as well as the development of ACLF/LRD were the outcomes of interest.

Results: 194 patients (70 compensated/124 decompensated; mean Child-Turcotte-Pugh score (CTP): 7 ± 2 points; mean HVPG: 17 ± 6 mmHg) were included.

Abbreviations: ACLD, advanced chronic liver disease; ACLF, acute-on-chronic liver disease; AD, acute decompensation; ArLD, alcohol-related liver disease; cf-DNA, cell-free deoxyribonucleic acid; CTP, Child-Turcotte-Pugh score; CI, confidence interval; CLT, clot lysis time; DIC, disseminated intravascular coagulation; HVPG, hepatic venous pressure gradient; IL-6, interleukin-6; IQR, interquartile range; LRD, liver-related death; MPO-DNA, myeloperoxidase deoxyribonucleic acid; NAP-2, neutrophil-activating peptide-2; NET, neutrophil extracellular traps; PAP, plasmin antiplasmin complex; PF4, platelet-factor 4; PVT, portal vein thrombosis; sP-selectin, soluble platelet-selectin; TAT, thrombin antithrombin complex; TM-TGA, thrombomodulin-modified thrombin generation assay; UNOS MELD, United Network for Organ Sharing Model for End-stage Liver Disease; VWF-Ag, von Willebrand-factor antigen.

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Compared to healthy controls ($n = 29$), levels of cell-free DNA (cf-DNA) were higher (0.88 [IQR 0.84–0.93] vs. 0.94 [IQR 0.88–1.03] $\mu\text{g/mL}$; $p = 0.001$) in ACLD, whereas myeloperoxidase-DNA (MPO-DNA) values were similar (0.32 [IQR 0.17–0.54] vs. 0.39 [IQR 0.18–0.76] AU; $p = 0.400$).

Factor XIII activity levels, soluble P-selectin, and cf-DNA but not MPO-DNA levels were linked to HD/LRD and/or ACLF/LRD in univariable analysis. However, none of these tests were associated with the aforementioned outcomes after adjusting for established prognostic indicators.

Conclusion: Patients with stable ACLD showed increased cf-DNA levels (i.e., NETosis, but also apoptosis/necrosis). However, MPO-DNA as a NETosis-specific marker was comparable to healthy controls. NETosis does not appear to drive disease progression in clinically stable ACLD as it was not linked to endpoints, thereby questioning whether findings obtained in animal models are translatable to humans.

1 | Introduction

Advanced chronic liver disease (ACLD) represents a major and growing cause of morbidity world-wide and is responsible for the second-highest number of years of working life lost in Europe [1]. The ‘congestive escalator’ hypothesis summarising the evolution of vascular obstruction to sinusoidal destruction and finally extinction of functional parenchyma provides a potential aetiology-independent explanation for liver disease progression [2].

Neutrophil extracellular traps (NETs) are generated from activated neutrophils and link inflammation, a key disease-driving mechanism in patients with ACLD [3], and thrombosis development [4, 5]. Indeed, preclinical studies found that NETs promote thrombus development via endothelial cell activation [6], platelet activation [7], activation of coagulation [8], and contribute to micro- and macrovascular thrombus formation [8]. NETs have been hypothesised to be crucial for the development of pathological thrombosis (i.e., ‘immunothrombosis’) in the intrahepatic microvasculature [9], which may, in the context of ACLD, lead to parenchymal extinction and further progression of liver disease [2]. Moreover, patients with ACLD may be particularly susceptible to intrahepatic NET formation due to von Willebrand factor (VWF) mediated NETs anchoring to the liver vasculature [10]. Indeed, a recent study compared levels of NET markers (cell-free [cf-]DNA and myeloperoxidase [MPO-]DNA), activation of coagulation and inflammation between patients with acute decompensation (AD), acute-on-chronic liver failure (ACLF), and healthy controls. All evaluated markers were higher in patients compared to controls and highest in patients with ACLF (i.e., those with extrahepatic organ dysfunction and/or failure). Furthermore, circulating cf-DNA and interleukin-6 (IL-6) levels were associated with death in this study [11]. Finally, the level of cf-DNA was also associated with disseminated intravascular coagulation (DIC) [12] and mortality in liver-healthy patients with sepsis [13, 14], who share several similarities in regard to pathophysiological mechanisms with ACLF patients.

Interestingly, a recent small study showed that markers for NETs were significantly elevated in plasma derived from patients undergoing liver transplantation. In addition, nucleosome levels were correlated with markers of coagulation activation [15].

Previous clinical evidence on NETs has been obtained in liver-healthy patients with sepsis [13, 14], patients with AD/ACLF [11], or patients with very advanced ACLD (i.e., patients undergoing liver transplantation) [15], but not in patients with clinically stable ACLD complicated by portal hypertension, i.e., by far the largest subgroup of ACLD patients. In these patients, bacterial translocation from the gut and systemic inflammation have been postulated to aggravate extracellular matrix remodelling and portal hypertension [16]. Notably, systemic inflammation in patients with stable ACLD is substantially less pronounced than in AD/ACLF, questioning whether findings on immunothrombosis and NET formation in the latter population are generalisable.

The aim of this study was to evaluate the levels and significance (i.e., association with laboratory tests related to coagulation) of NET markers in patients with stable ACLD and their impact on the development of hepatic decompensation/ACLF and macrovascular thrombosis in the splanchnic venous system. As a single test reliably assessing haemostatic capacity does not exist, we performed an extensive profiling of the haemostatic system by a combination of functional tests, markers of *in vivo* activation of haemostasis, and plasma levels of key haemostatic proteins as we have described previously in various populations of patients with liver disease [17–19].

2 | Methods

2.1 | Study Design and Patients

We performed a retrospective, single-centre cohort study in patients with ACLD undergoing hepatic venous pressure gradient (HVPG) measurement at the Vienna Hepatic Hemodynamic Lab between April 2017 and July 2020. Inclusion criteria were (i) HVPG ≥ 6 mmHg and (ii) availability of citrate plasma for NET marker quantification and laboratory tests of coagulation. Patients were excluded if any of the following criteria were present: history of orthotopic liver transplantation, non-ACLD aetiology (e.g., porto-sinusoidal vascular disorder), active malignancy, presence of portal vein thrombosis (PVT), evidence of bacterial infection, ACLF at study inclusion, unreliable HVPG, missing information on clinical follow-up, or unstable course of liver disease (acute decompensation/non-elective liver-related hospitalisation within 90 days of HVPG measurement) at the time of characterisation (Figure S1).

Summary

- Neutrophil extracellular traps (NETs) are part of the body's innate immune response that were linked to liver disease progression in animal models by aggravating liver injury through the formation of (micro) thrombi leading to parenchymal extinction.
- In patients with stable advanced chronic liver disease (ACLD), cell-free DNA levels were increased (i.e., NETosis/apoptosis/necrosis).
- However, myeloperoxidase-DNA (a more specific NETosis-marker) was comparable to healthy controls, and therefore, NETosis does not appear to drive disease progression in clinically stable ACLD as it was not linked to clinical endpoints.

2.2 | Healthy Controls

Healthy controls aged > 18 years without a history of thrombotic or liver-related diseases, medical conditions known to be associated with haemostatic alterations, or current use of anticoagulants, platelet function inhibitors, or oral contraceptives were enrolled.

2.3 | HVPG Measurement

HVPG measurement was performed in the absence of NSBB therapy and in adherence to a standard operating procedure [20]. Briefly, a catheter introducer sheath was inserted into the right internal jugular vein under local anaesthesia and ultrasound guidance. Subsequently, a hepatic vein was cannulated using a dedicated balloon catheter [21] and the free and wedged hepatic venous pressures were obtained at least as triplicate measurements, according to our standard operating procedure [20] which is in line with the more recent Baveno VII [22] and Billroth IV [20] consensus statements.

2.4 | Laboratory Assessments

Analyses were performed using blood samples obtained via a central venous line (i.e., the side-port of the catheter introducer sheath) at the time of HVPG measurement.

Routine laboratory tests (i.e., all parameters except for those explicitly mentioned below) were conducted by the ISO-certified Department of Laboratory Medicine of our institution using commercially available methods that are applied in clinical routine.

2.4.1 | Endothelium/Platelet Markers

Soluble-P-selectin (sP-selectin; DY137, R&D Systems, Minneapolis, USA), platelet-factor 4 (PF4; DY795, R&D Systems), neutrophil-activating peptide-2 (NAP-2; DY393, R&D Systems), and syndecan-1 (DY2780, R&D Systems) were measured using commercially available ELISAs.

2.4.2 | Coagulation Activation

Thrombin antithrombin complex levels (TAT; NC0480020; Siemens, Den Haag, The Netherlands) were measured using commercially available ELISAs. Factor XIII activity was measured as previously described [23].

2.4.3 | Thrombin Generation Potential

Thrombomodulin-modified thrombin-generation assays (TM-TGAs) were performed using a fluorometric method as previously described [24–26].

2.4.4 | Fibrinolysis Activation

Plasmin antiplasmin complex levels (PAP; TC12060; Bio-Techne, Minneapolis, USA) were measured using commercially available ELISAs.

2.4.5 | Fibrinolytic Potential

Fibrinolytic capacity in plasma was assessed by monitoring changes in turbidity during clot formation and lysis of a tissue factor-induced clot by exogenous tissue plasminogen activator, as described previously [27]. Clot lysis time (CLT) was defined as the time from the midpoint of the clear to maximum turbid transition, representing clot formation, to the midpoint of the maximum turbid to the clear transition, representing clot lysis.

2.4.6 | NET Markers

The concentration of cf-DNA was measured using the Quant-iT PicoGreen double strand DNA assay kit (Fisher Scientific, Landsmeer, the Netherlands). In short, 10 µL of plasma was added to 90 µL of Tris-EDTA (TE) buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5) in a 96-well microtiter plate. Next, 100 µL of PicoGreen solution (diluted 1:200 in TE buffer) was added to the wells. After 10 min of incubation in the dark at room temperature, fluorescence was measured at 480 nm excitation and 520 nm emission using a Victor3 (PerkinElmer, Groningen, the Netherlands) spectrophotometer. Complexes of MPO-DNA were quantified by ELISA as previously described [28] using a commercially available monoclonal antibody (clone 4A4) to MPO (Sanbio, Uden, the Netherlands) and the detection antibody derived from the cell death detection ELISA (Sigma-Aldrich, Zwijndrecht, The Netherlands).

2.4.7 | Bacterial Translocation/Systemic Inflammation

Bacterial DNA was determined by broad-range PCR of the 16S rRNA gene and categorised according to a concentration threshold of ≥ 5 pg/mL [29]. C-reactive protein (CRP) and interleukin-6 (IL-6) levels were measured using commercially available methods that are applied in clinical routine (see above).

2.5 | Statistical Analyses

All statistical analyses were performed using R 4.3.1 (R Core Team, R Foundation for Statistical Computing, Vienna, Austria) or GraphPad Prism 8 (GraphPad Software, CA, USA). Categorical variables were reported as absolute (n) and relative frequencies (%), whereas continuous variables were reported as mean $\pm$ SD or median (IQR), as appropriate. Student's t -test was used for group comparisons of normally distributed variables and Mann-Whitney U -test for non-normally distributed variables.

Univariable linear regression analyses were performed to decipher factors associated with NET markers. Correlation analyses were performed between key factors related to coagulation and/or inflammation parameters and plotted using the 'corrplot' package in R. Variables were non-normally distributed and hence, Spearman's ρ was used.

Time-dependent event rates were obtained by the Kaplan-Meier method. Events of interest were either (i) hepatic decompensation (according to clinical stage at baseline; i.e., for cACLD patients first decompensation, for dACLD patients further decompensation) or liver-related death and (ii) development of acute-on-chronic liver failure (ACLF) or liver-related death, or (iii) development of non-tumorous portal vein thrombosis (PVT). Patients were censored at the time of liver transplantation, non-liver-related death, or last follow-up. Data was transformed into long formatting to account for the removal/suppression of the primary aetiological factor as a time-dependent covariable (initiation of antiviral therapy for HCV or HBV without HDV coinfection/long-term alcohol abstinence, as defined by Baveno VII).

Variable selection for the multivariable model was based on parameters having prognostic implications as well as parameters which we considered of particular importance for the endpoint of interest (i.e., age, indicators of hepatic dysfunction, and HVPg). Variables were included as continuous variables and log-transformed if variables were skewed. Due to the low number of PVT events, analyses on PVT incidence were only adjusted for CTP score. After performing a first model including all factors considering expert opinion and collinearity aspects, we used backward elimination with a p -value threshold of $p > 0.157$ for eliminating co-factors for our final/last model [30].

The level of significance was set at a 2-sided p -value < 0.05 .

2.6 | Ethics

The study was conducted in accordance with the principles of the Declaration of Helsinki and was approved by the local ethics committee of the Medical University of Vienna (EK1262/2017). Patients were consented for inclusion in the Vienna Cirrhosis Study (VICIS), i.e., a prospective registry with a biobank which provided the samples for all laboratory analyses except for routine laboratory tests.

3 | Results

3.1 | Patient Characteristics

After applying in- and exclusion criteria, 194 patients were included in this study (Figure 1 and Figure S1). Mean age was 56.6 ± 11.7 years, with the majority being male ($n = 125$, 64.4% vs.

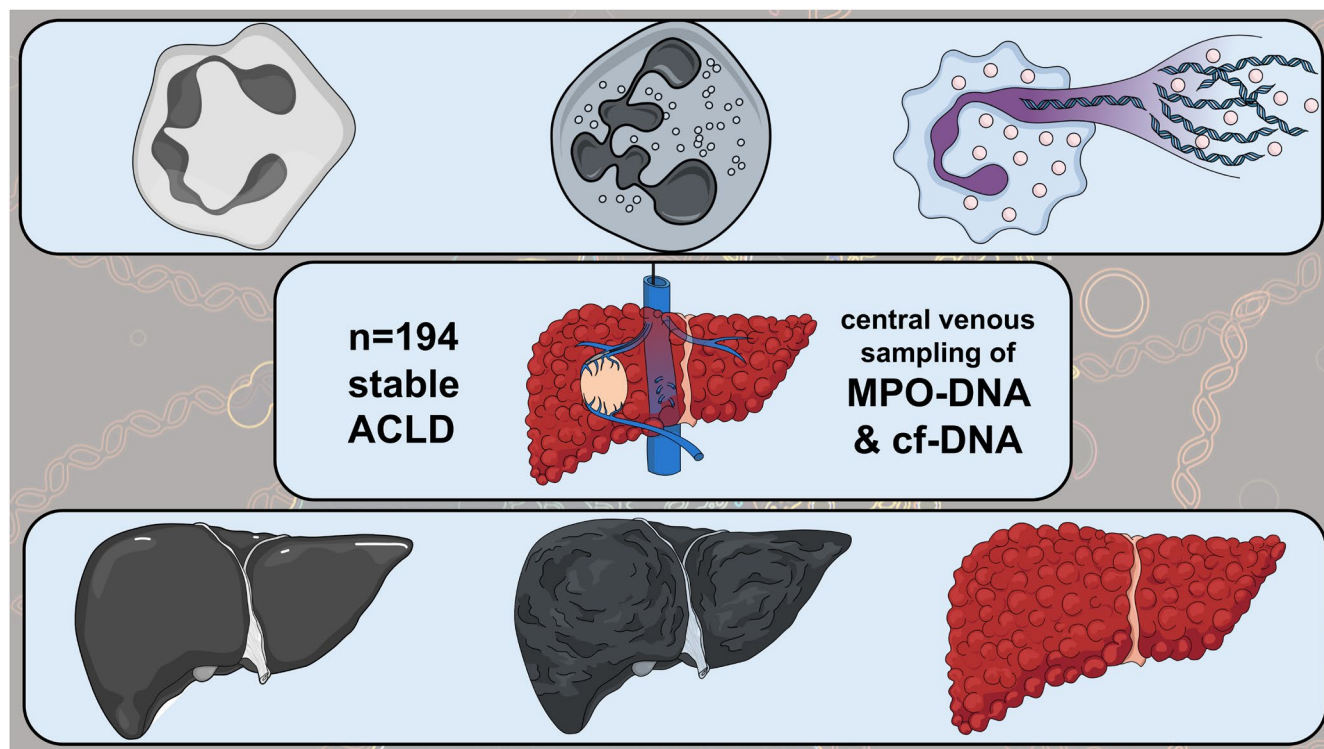


FIGURE 1 | Study description.

female: $n = 69$, 35.6%), and having alcohol-related liver disease (ArLD) ($n = 115$, 59.3%, followed by viral hepatitis: $n = 37$, 19.1%). Regarding portal hypertension severity, almost two thirds of patients ($n = 123$, 63.4%) presented with clinically significant portal hypertension (i.e., HVPG ≥ 10 mmHg), whereas subclinical portal hypertension (i.e., HVPG 6–9 mmHg) was comparatively uncommon ($n = 20$, 10.3%). In line, 63.9% had varices at endoscopy ($n = 124$). Mean HVPG was 17.2 ± 5.9 mmHg, mean CTP 6.7 ± 1.7 points and mean United Network for Organ Sharing Model for End-stage Liver Disease (UNOS MELD) (2016) was 12.4 ± 4.3 points. Overall, 107 (55.2%) patients achieved aetiological cure/removal or suppression of the primary aetiological factor before study inclusion ($n = 87$ abstinent ALD, $n = 20$ removal/suppression of HCV/HBV). Median time from aetiological cure/removal or suppression of the primary aetiological factor before study inclusion was 11.2 (IQR: 3.8–33.2, range: 0.8–395.4) months.

While 70 patients (36.1%) had cACLD, 124 patients (63.9%) had dACLD. Of these, the most common previous/current decompensating events were ascites (mild/moderate: $n = 86$, 44.3% vs. severe: $n = 14$, 7.2%), followed by a previous episode of overt hepatic encephalopathy ($n = 37$, 19.1%) or a history of variceal bleeding ($n = 23$, 11.9%). Detailed information of laboratory parameters of interest can be found in Table 1.

Additionally, we included 29 healthy controls meeting the inclusion criteria for this study. While healthy controls were significantly younger (32.9 ± 9.1 years; $p < 0.001$) and presented with a slightly lower body mass index (24.0 ± 2.9 vs. 26.5 ± 5.1 kg/m² in the study cohort; $p < 0.001$), the distribution of sex was similar ($p = 0.544$; Table S1).

3.2 | NET Parameters Across Disease Severity Strata and Compared to Healthy Controls

When stratifying patients according to disease stages, there were no differences in MPO-DNA (cACLD: 0.38 [IQR 0.19–0.64] vs. dACLD: 0.4 [IQR 0.18–0.82] AU; $p = 0.688$) and cf-DNA levels (cACLD: 0.94 [IQR 0.89–1.02] vs. dACLD: 0.94 [IQR 0.89–1.03] μ g/mL; $p = 0.673$; Table 1).

Compared to healthy controls, cf-DNA was increased, whereas MPO-DNA was comparable to the study cohort (cf-DNA: 0.88 [IQR 0.84–0.93] vs. 0.94 [IQR 0.88–1.03] μ g/mL; $p = 0.001$; MPO-DNA: 0.32 [IQR 0.17–0.54] vs. 0.39 [IQR 0.18–0.76] AU; $p = 0.400$; Figure 2).

Notably, in patients with/without detection of bacterial DNA, median levels of cf-DNA were comparable, indicating that cf-DNA is not of bacterial origin (Figure 3).

Syndecan-1, PF4, and PAP levels were significantly higher, while factor XIII activity was lower in dACLD compared to cACLD patients (Table 2). However, when correcting for platelet count, a determinant of PF4, the differences in PF4 disappeared. While a similar pattern was observed when stratifying according to disease severity indices (i.e., CTP

score), there were no differences when stratifying according to HVPG (Table 2).

3.3 | Associations of Haemostasis Parameters With Markers of NET Formation

Given the role of NETs in modulating endothelial cells, platelet, coagulation, and fibrinolysis, we assessed various indicators of these haemostatic processes in relation to markers of NET formation. Univariable linear regression analyses of MPO-DNA/cf-DNA according to disease stage are depicted in the Data S1 (Table S2; Table S3; Figure S2). Importantly, none of the investigated parameters yielded a statistically significant association with both myeloperoxidase-DNA and cell-free DNA in the stratified analysis. Figure 4 plots the correlation matrices of parameters of interest. While MPO-DNA showed a weak but significant positive correlation with Syndecan-1 (Spearman's $\rho = 0.208$; $p = 0.004$) and VWF-antigen (0.142; $p = 0.048$), cf-DNA levels positively correlated with D-Dimer (0.309; $p < 0.001$), PAP (0.176; $p = 0.014$), sP-selectin (0.146; $p = 0.043$), NAP-2 (0.142; $p = 0.048$), and showed a negative correlation with ADAMTS13 (-0.201 ; $p = 0.005$; Table S4).

3.4 | Outcome Analyses

Median estimated follow-up was 43.4 (95% CI 37.8–49.0) months. While 16 patients (8.2%) received a liver transplantation, whereas 54 patients (27.8%) died (liver-related $n = 41$, 21.1%; Table S5). During follow-up, 21 patients achieved aetiological cure ($n = 14$ abstinent ALD, $n = 7$ removal/suppression of HCV/HBV). The median time from study inclusion to aetiological cure was 4.3 (IQR: 2.2–16.6, range: 0.8–42.4) months.

In the Cox regression model including hepatic decompensation and/or liver-related death as outcomes of interest, 94 events of interest were recorded during follow-up. While MPO-DNA was not associated, cf-DNA levels were associated with the outcome of interest in univariable analyses (hazard ratio [HR] per AU: 4.19 [95% CI: 1.13–15.56]; $p = 0.032$). Moreover, factor XIII activity, sP-selectin, and NAP-2 levels were associated with the outcome of interest. However, after adjusting for important prognostic indicators (age, HVPG, CTP), the association of cf-DNA as well as factor XIII activity, sP-selectin, and NAP-2 levels with the outcome of interest did not attain statistical significance (Table 3).

Moreover, we analysed the second outcome of interest, i.e., the development of ACLF/liver-related death ($n = 49$ events, median time to events: 27.7 [95% CI 9.6–45.8] months). In line with results on hepatic decompensation, cf-DNA (HR: 7.52 [95% CI 1.23–46.03]; $p = 0.029$) and sP-selectin were associated with the outcome of interest in univariate analyses. However, associations were lost when adjusting for disease severity indicators in multivariable analysis (Table 4).

These findings were confirmed in a sensitivity analysis only including patients with CSPH (Table S6 and Table S7).

TABLE 1 | Baseline characteristics.

	Overall (<i>n</i> = 194)	Compensated ACLD (<i>n</i> = 70)	Decompensated ACLD (<i>n</i> = 124)	<i>p</i>
<i>Patient characteristics</i>				
Age, years, mean (SD)	56.6 (11.7)	55.6 (12.5)	57.1 (11.3)	0.408
Body mass index, mean (SD)	26.8 (5.1)	27.2 (5.2)	26.6 (5.1)	0.416
Sex, <i>n</i> (%)				
Male	125 (64.4%)	43 (61.4%)	82 (66.1%)	0.511
Female	69 (35.6%)	27 (38.6%)	42 (33.9%)	
Aetiology, <i>n</i> (%)				
ArLD	115 (59.3%)	24 (34.3%)	91 (73.4%)	< 0.001
Viral	37 (19.1%)	26 (37.1%)	11 (8.9%)	
MASLD	24 (12.4%)	12 (17.1%)	12 (9.7%)	
Other/unknown	18 (9.3%)	8 (11.4%)	10 (8.1%)	
Varices, <i>n</i> (%)				
None	70 (36.1%)	31 (44.3%)	39 (31.5%)	0.026
Small	72 (37.1%)	28 (40.0%)	44 (35.5%)	
Large	52 (26.8%)	11 (15.7%)	41 (33.1%)	
History of variceal bleeding, <i>n</i> (%)	23 (11.9%)	—	23 (18.5%)	—
Ascites, <i>n</i> (%)				
None	94 (48.5%)	70 (100%)	24 (19.4%)	—
Mild/moderate	86 (44.3%)	—	86 (69.4%)	
Severe	14 (7.2%)	—	14 (11.3%)	
History of overt hepatic encephalopathy, <i>n</i> (%)	37 (19.1%)	—	37 (29.8%)	—
HVPG, mmHg	17.2 (5.9)	14.3 (5.7)	18.9 (5.3)	< 0.001
6-9 mmHg	20 (10.3%)	15 (21.4%)	5 (4.0%)	< 0.001
10-15 mmHg	51 (26.3%)	24 (34.3%)	27 (21.8%)	
≥ 16 mmHg	123 (63.4%)	31 (44.3%)	92 (74.2%)	
UNOS MELD (2016), points	12.4 (4.3)	10.2 (2.9)	13.6 (4.5)	< 0.001
CTP score, points	6.7 (1.7)	5.4 (0.8)	7.4 (1.6)	< 0.001
A	105 (54.1%)	64 (91.4%)	41 (33.1%)	—
B	74 (38.1%)	6 (8.6%)	68 (54.8%)	
C	15 (7.7%)	—	15 (12.1%)	
Aetiological treatment prior to baseline, <i>n</i> (%)	107 (55.2%)	35 (50%)	82 (66%)	—
<i>Laboratory parameters, mean (SD) or median (IQR)</i>				
Mean arterial pressure, mmHg	101 (15)	107 (15)	98 (14)	< 0.001
PLT, G/L	96 (70–131)	98 (78–126)	94 (61–133)	0.351
Sodium, mmol/L	138 (3.8)	140 (2.2)	137 (4)	< 0.001
Albumin, g/L	36.4 (5.2)	39.1 (4)	34.9 (5.2)	< 0.001

(Continues)

TABLE 1 | (Continued)

	Overall (n = 194)	Compensated ACLD (n = 70)	Decompensated ACLD (n = 124)	p
Bilirubin, mg/dL	1.1 (0.8–1.8)	1.0 (0.7–1.5)	1.3 (0.8–1.9)	0.019
INR, %	1.4 (0.3)	1.3 (0.2)	1.5 (0.3)	<0.001
Creatinine, mg/dL	0.7 (0.6–0.9)	0.7 (0.6–0.8)	0.8 (0.6–1.0)	0.013
Aspartate aminotransferase, IU/L	40 (30–54)	40 (28–56)	40 (30–53)	0.936
Alanine aminotransferase, IU/L	27 (19–40)	31 (21–50)	25 (18–36)	0.008
<i>Endothelium/platelet markers</i>				
von Willebrand factor antigen, %	276 (93)	234 (82)	300 (90)	<0.001
Soluble P-selectin, ng/mL	21.8 (8.16)	20.7 (7.09)	22.4 (8.67)	0.157
Syndecan-1, ng/mL	1.36 (0.82–2.92)	1.06 (0.72–2.05)	1.64 (0.89–4.05)	0.017
Platelet factor 4, ng/mL	238 (150–421)	322 (188–454)	221 (143–410)	0.028
Platelet factor 4/PLT ratio	2.74 (1.75–4.02)	2.91 (1.83–4.19)	2.66 (1.71–3.84)	0.358
Neutrophil-activating peptide-2, ng/mL	150 (91–222)	172 (104–237)	137 (83–211)	0.074
<i>NET markers</i>				
Myeloperoxidase-DNA, AU	0.39 (0.19–0.76)	0.38 (0.19–0.64)	0.4 (0.18–0.82)	0.688
Cell-free DNA, µg/mL	0.94 (0.89–1.03)	0.94 (0.89–1.02)	0.94 (0.89–1.03)	0.673
Median values of cf-DNA in patients with detection of bacterial DNA (≥ 5 pg/mL) ^a	0.95 (0.90–1.05)	0.96 (0.90–1.04)	0.95 (0.89–1.06)	0.994
Median values of cf-DNA in patients without detection of bacterial DNA (< 5 pg/mL) ^a	0.93 (0.88–1.02)	0.94 (0.89–1.02)	0.93 (0.87–0.99)	0.892
Detection of bacterial DNA ≥ 5 pg/mL, n (%) ^a	62 (41.3%)	24 (44.4%)	38 (39.5%)	0.562
<i>Inflammation markers</i>				
C-reactive protein, mg/dL	0.3 (0.1–0.6)	0.2 (0.1–0.4)	0.3 (0.1–0.8)	<0.001
Interleukin-6, pg/mL	8 (5–13)	6 (3–10)	10 (6–15)	<0.001
<i>Coagulation activation</i>				
Thrombin antithrombin complex, µg/mL	16.6 (9.1–37.5)	17.4 (10.2–40.9)	14.7 (8.5–35.9)	0.262
F XIII activity, %	69.6 (36.0)	79.0 (38.4)	64.3 (33.6)	0.006
<i>Thrombin generation potential</i>				
TM-TGA endogenous thrombin potential, nM IIa * min	551 (410–675)	505 (336–651)	560 (439–692)	0.045
TM-TGA peak thrombin generation, nM IIa	145 (70.7)	139 (74.1)	149 (68.8)	0.372
TM-TGA lagtime, min	2.45 (0.76)	2.41 (0.74)	2.48 (0.77)	0.587
TM-TGA velocity index, nM IIa/min	70.3 (42.6–91.5)	63.4 (36.6–88.8)	72.5 (47.8–93.4)	0.187
<i>Fibrinolysis activation</i>				
Plasmin antiplasmin complex, ng/mL	912 (492–1850)	613 (446–1470)	1060 (525–2110)	0.010
<i>Fibrinolytic potential</i>				
TM-TGA CLT, min	48.8 (38.9–59.6)	50.8 (40.5–60.2)	48.4 (38.1–58.7)	0.555

Abbreviations: ArLD, alcohol-related liver disease; CLT, clot lysis time; CTP, Child-Turcotte-Pugh score; HVPg, hepatic venous pressure gradient; INR, international normalised ratio; IQR, interquartile range; MASLD, metabolic-dysfunction associated steatotic liver disease; n, number; PLT, platelet count; SD, standard deviation; TM-TGA, thrombomodulin-modified thrombin generation assay; UNOS MELD (2016), United Network for Organ Sharing model for end-stage liver disease (2016).

^aData available in 150 patients (77%). P-values < 0.05 denotes statistical significance.

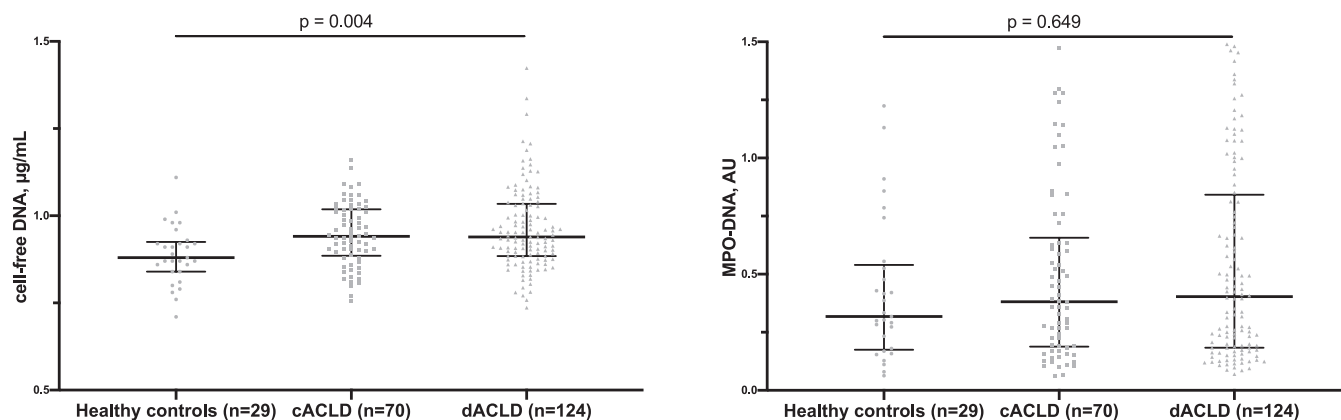


FIGURE 2 | Comparison of cell-free (left) and myeloperoxidase-DNA (MPO-DNA; right) in the compensated and decompensated stable advanced chronic liver disease (ACLD) patients versus healthy controls.

3.5 | Association With Non-Tumorous Portal Vein Thrombosis During Follow-Up

During follow-up, 15 patients (7.7%) developed a non-tumorous portal vein thrombosis (median time to events: 10.2 [95% CI: 1.0–20.2] months). While TAT was associated with the development of non-tumorous portal vein thromboses in univariable analysis (log-transformed, HR: 1.72 [95% CI 1.03–2.86]; $p=0.039$), this association disappeared when adjusting for hepatic dysfunction (i.e., CTP) in multivariable analysis (Table S8 and Table S9).

4 | Discussion

This study is the first study to evaluate the association of NET biomarkers with laboratory tests related to coagulation as well as long-term clinical outcomes in clinically stable patients with ACLD. While (non-NET-specific) cf-DNA levels were increased compared to healthy controls, MPO-DNA (a more specific marker of NETosis) was not statistically significantly different. Moreover, NETosis was not associated with clinical outcomes, and hence, it is questionable whether NETs play a disease-modifying role in stable ACLD.

The functionality of neutrophils (i.e., the first wall of in vivo defence) is impaired in patients with cirrhosis as a consequence of dysfunctions in phagocytosis, chemotaxis, reactive oxygen species production and degranulation, killing capacities, as well as increased apoptosis [31]. Formation of NETs, i.e., filamentous/web-like structures comprising extracellular DNA, histones, and granular proteins that are released by neutrophils [32], is triggered by various pathways (e.g., bacterial products [33], immune complexes, and platelets [34]). Nevertheless, due to the complex interplay of portal hypertension and bacterial translocation/systemic inflammation, patients with ACLD may be particularly susceptible to intrahepatic NET formation [35].

NET formation in response to phorbol-12-myristate-13-acetate in samples of patients with decompensated ACLD has been found to be decreased [36, 37], whereas levels of H3Cit-DNA [38] and MPO-DNA [11, 38] were increased as compared to healthy controls. In line with Tritto et al. [39], levels of NET markers were not different in patients with vs. without bacterial DNA in our study, indicating that cf-DNA itself is not of bacterial origin. MPO-DNA levels in our study cohort were comparable to healthy controls, whereas cf-DNA levels were increased. When stratifying according to disease stage, there were no differences in median levels of both markers. Blasi et al. observed that there were no correlations of circulating DNA as well as markers of systemic inflammation and oxidative stress with markers of activation of coagulation in AD/ACLF [11]. Notably, cf-DNA but not MPO-DNA levels were associated with organ failures and prognosis. They concluded that systemic inflammation and—more downstream—cf-DNA release rather than neutrophil activation with NETosis per se or NETosis-associated activation of coagulation/immunothrombosis, may act as a disease-driving mechanism in AD/ACLF. In line, in unadjusted Cox regression analyses, cf-DNA was also associated with liver-related outcomes in our study whereas MPO-DNA was not. However, this association was lost after adjusting for important prognostic co-factors.

Importantly, data indicating a role of NETosis linked to activation of coagulation in different stages of ACLD mainly originate from experimental studies in animal models [40, 41]. It has been proposed that an overactive coagulation system, perhaps in part fuelled by NET formation, might drive disease progression in patients with cirrhosis by inducing a so-called congestive escalator (microthrombi promoting the activation of hepatic stellate cells; also known as the ‘vascular hypothesis’) [2]. This overactivation of the coagulation system leads to an intrahepatic compartment syndrome causing additional vascular lesions and parenchymal extinction. Following this hypothesis, loss of hepatocytes is the most obvious feature,

FIGURE 3 | Comparison of cell-free DNA levels in the overall cohort (A), compensated (B) and decompensated ACLD patients (C) according to the presence/detection of bacterial DNA versus without.

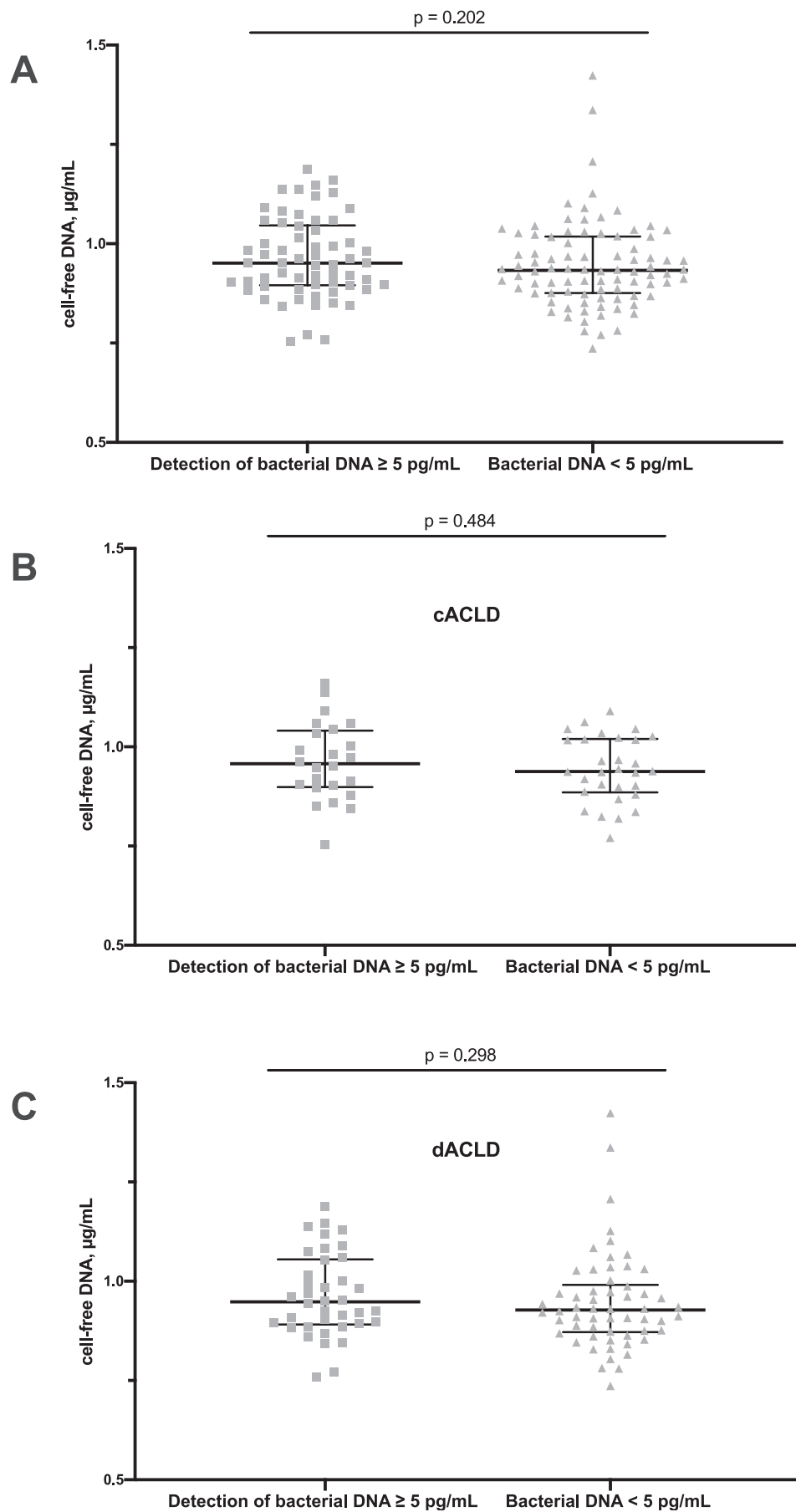


FIGURE 3 | Legend on previous page.

TABLE 2 | Factors according to liver disease severity.

Child-Turcotte-Pugh Score	A (n = 105)	B (n = 74)	C (n = 15)	p
<i>Endothelium/platelet markers</i>				
Von Willebrand factor antigen, %	233 (171–294)	320 (251–400)	372 (326–420)	<0.001
Soluble P-selectin, ng/mL	19.6 (6.7)	24.4 (9.2)	24.3 (8)	<0.001
Platelet factor 4, ng/mL	271 (160–444)	229 (147–426)	181 (117–278)	0.421
Neutrophil-activating peptide-2, ng/mL	160 (98–223)	151 (90–238)	85.6 (59–134)	0.642
Syndecan-1, ng/mL	1.03 (0.69–2.39)	1.64 (0.92–2.79)	2.86 (1.48–15.8)	0.047
<i>NET markers</i>				
Myeloperoxidase-DNA, AU	0.38 (0.17–0.67)	0.37 (0.20–0.78)	0.77 (0.34–1.05)	0.500
Cell-free DNA, µg/mL	0.93 (0.87–1)	0.96 (0.90–1.06)	0.96 (0.9–1.07)	0.013
<i>Coagulation activation</i>				
Thrombin antithrombin complex, µg/mL	17.1 (10.6–38.2)	15.6 (7.3–39.8)	10 (7.7–18.7)	0.282
F XIII activity, %	77.5 (35.6)	61.5 (35.8)	54.3 (27)	0.004
<i>Fibrinolysis activation</i>				
Plasmin antiplasmin complex, ng/mL	690 (454–1630)	1140 (588–2140)	929 (646–1620)	0.020
<i>Fibrinolytic potential</i>				
TM-TGA CLT, min	51.9 (39.9–61.6)	48.2 (37.9–57.3)	44.7 (40.3–52.3)	0.235
<i>Thrombin generation potential</i>				
TM-TGA endogenous thrombin potential, nM IIa * min	493 (351–633)	590 (464–695)	669 (558–726)	0.004
TM-TGA peak thrombin generation, nM IIa	139 (71.7)	156 (73.7)	137 (36.3)	0.104
HVPG strata	6–9 mmHg (n = 20)	10–15 mmHg (n = 51)	≥16 mmHg (n = 123)	p
<i>Endothelium/platelet markers</i>				
Von Willebrand factor antigen, %	175 (108–256)	225 (178–302)	299 (239–377)	0.022
Soluble platelet selectin, ng/mL	19.6 (4.7)	19.2 (6.7)	23.2 (8.8)	0.825
Platelet factor 4, ng/mL	267 (158–436)	276 (171–411)	226 (144–426)	0.919
Neutrophil-activating peptide-2, ng/mL	166 (113–249)	153 (100–219)	135 (85–216)	0.502
Syndecan-1, ng/mL	0.96 (0.41–1.46)	1.09 (0.63–2.65)	1.69 (0.91–3.49)	0.182
<i>NET markers</i>				
Myeloperoxidase-DNA, AU	0.33 (0.14–0.76)	0.4 (0.18–0.7)	0.41 (0.19–0.83)	0.474
Cell-free DNA, µg/mL	0.95 (0.9–1.01)	0.94 (0.88–1.01)	0.94 (0.89–1.04)	0.600

(Continues)

TABLE 2 | (Continued)

HVPG strata	6–9 mmHg (n = 20)	10–15 mmHg (n = 51)	≥ 16 mmHg (n = 123)	p
<i>Coagulation activation</i>				
Thrombin antithrombin complex, µg/mL	26.0 (16.2–55.2)	17.2 (10.3–39.1)	14.2 (8–33.8)	0.081
F XIII activity, %	70.0 (35.0)	81.3 (35.5)	64.7 (35.5)	0.230
<i>Fibrinolysis activation</i>				
Plasmin antiplasmin complex	561 (435–1910)	788 (470–1600)	939 (519–2130)	0.980
<i>Fibrinolytic potential</i>				
TM-TGA CLT, min	45.2 (29.6–56.0)	52.2 (42.7–65.6)	47.5 (39.0–57.8)	0.059
<i>Thrombin generation potential</i>				
TM-TGA endogenous thrombin potential, nM IIa * min	461 (275–621)	494 (346–615)	581 (462–695)	0.788
TM-TGA peak thrombin generation, nM IIa	135 (84.2)	132 (68.2)	153 (68.9)	0.863
UNOS MELD (2016) strata	< 10 points (n = 59)	10–15 points (n = 83)	≥ 16 points (n = 52)	p
<i>Endothelium/platelet markers</i>				
Von Willebrand factor antigen, %	233 (173–304)	251 (213–310)	344 (281–420)	0.187
Soluble platelet selectin, ng/mL	21.4 (8)	20.4 (7.5)	24.5 (8.9)	0.443
Platelet factor 4, ng/mL	332 (198–507)	230 (153–413)	197 (134–333)	0.075
Neutrophil-activating peptide-2, ng/mL	181 (112–291)	142 (96–212)	130 (63–187)	0.044
Syndecan-1, ng/mL	0.97 (0.61–2.43)	1.17 (0.75–2.53)	1.96 (1.32–5.44)	0.334
<i>NET markers</i>				
Myeloperoxidase-DNA, AU	0.38 (0.17–0.72)	0.35 (0.20–0.73)	0.49 (0.19–0.87)	0.730
Cell-free DNA, µg/mL	0.95 (0.88–1.02)	0.93 (0.89–0.99)	0.96 (0.88–1.08)	0.522
<i>Coagulation activation</i>				
Thrombin antithrombin complex, µg/mL	19.7 (12.8–42.4)	16.7 (9.7–33.8)	10.4 (7–25.6)	0.119
F XIII activity, %	84.1 (38)	67.4 (35.7)	56.7 (28.1)	0.008
<i>Fibrinolysis activation</i>				
Plasmin antiplasmin complex	592 (474–1570)	921 (511–1730)	1040 (559–2170)	0.260
<i>Fibrinolytic potential</i>				
TM-TGA CLT, min	51.9 (41.6–60.1)	49.1 (38.1–63.5)	47.5 (38.7–54.5)	0.649
<i>Thrombin generation potential</i>				
TM-TGA endogenous thrombin potential, nM IIa * min	461 (296–643)	551 (431–660)	620 (496–696)	0.034
TM-TGA peak thrombin generation, nM IIa	136 (75.8)	152 (78.6)	146 (47.6)	0.218

Abbreviations: HVPG, hepatic venous pressure gradient; TM-TGA, thrombomodulin-modified thrombin generation assay; UNOS MELD (2016), United Network for Organ Sharing model for end-stage liver disease (2016). P-value < 0.05 denotes statistical significance.

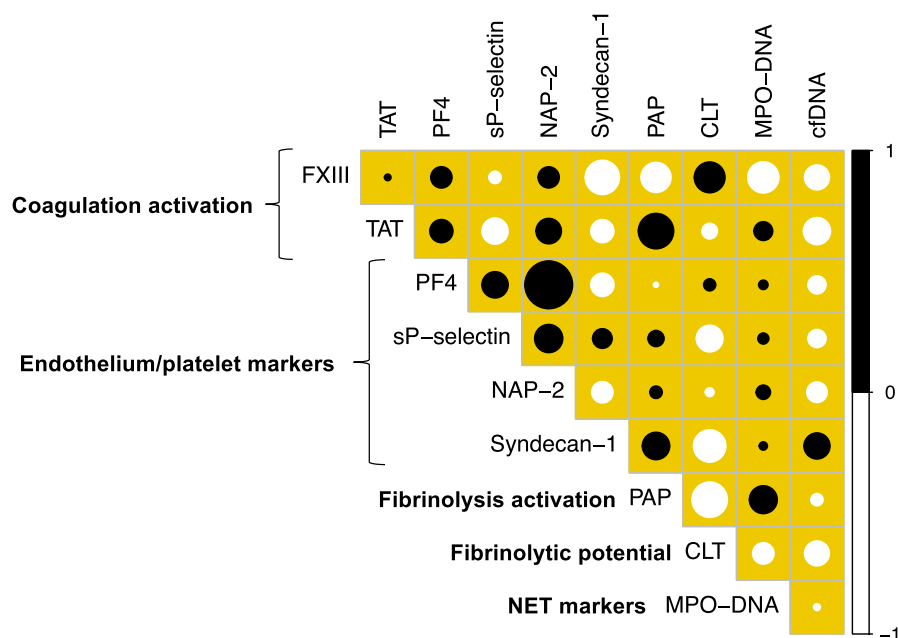


FIGURE 4 | Correlation matrix depicting factors of interest using the ‘corrplot’ package. cfDNA, cell-free DNA; CLT, clot lysis time; MPO-DNA, myeloperoxidase-DNA; NAP-2, neutrophil-activating peptide-2; PAP, plasmin antiplasmin complex; PF4, platelet factor 4; sP-selectin, soluble P-selectin; TAT, thrombin antithrombin complex; VWF, Von Willebrand factor antigen.

but loss of the local microvasculature would probably be more significant for the progression of disease, particularly in the context of portal hypertension. A recent study examining liver tissue from patients with inflammatory liver disease concluded that the clear link between intrahepatic activation of coagulation and intrahepatic formation of NETs derived from animal studies is not evident in humans [42]. Whereas low numbers of intrahepatic NETs were detected in subgroups of patients, intrahepatic fibrin was only occasionally observed. These data are in line with our results and emphasise the difficulty in extrapolating data from animal models to humans.

In different clinical scenarios, NET formation has been associated with (cancer-associated) thromboses [43]. Especially in patients with hepatocellular carcinoma, increased markers of NET formation were linked to activation of contact systems (i.e., a prothrombotic mechanism), compared to healthy subjects [44]. In our study, NET markers were not associated with non-tumorous portal vein thromboses. In line, the association with TAT—as a biomarker for thrombin formation—disappeared when adjusting for hepatic dysfunction as a key risk factor for PVT in multivariable analysis.

While experimental evidence suggests that NETs may contribute to congestive hepatopathy and cholestatic liver disease-induced portal hypertension [41, 45], we did not observe a difference when cross-sectionally stratifying NET parameters across portal hypertension severity strata. However, most of the included patients had ArLD and only 10 patients (5.2%) had cholestatic liver disease, while patients with congestive hepatopathy were excluded. Due to the various potential disease-promoting/

modifying roles of NETs in the spectrum of ACLD, future studies should evaluate an aetiology-specific role of NETs in the pathophysiology of ACLD.

Importantly, patients included in our analysis were derived from the prospective VICIS study and thoroughly characterised in terms of clinical stability as well as portal hypertension, systemic inflammation severity, and importantly, all of these aspects have been considered in our analyses, thereby limiting the possibility of unaccounted confounding. We strongly believe that studies investigating haemostasis/clotting in ACLD should include a comprehensive set of biomarkers—in the context of the present study, we included indicators of endothelial/platelet activation, coagulation activation and thrombin generation potential, fibrinolysis activation and fibrinolytic potential, as well as bacterial translocation/systemic inflammation due to the presumed direct link of the latter with NETosis. While this approach could have led to false-positive findings, the absence of strong/biologically meaningful associations strengthens our conclusions. To account for aetiological cure/removal or suppression of the primary aetiological factor as a major disease-modifying factor, we have adjusted the outcome analyses for aetiological cure. The high rate of aetiological cure reflects the contemporariness of our cohort of stable ACLD patients. Due to the opportunity to consult the nation-wide death registry, we included liver-related death in all composite endpoints to ensure the ascertainment of the most severe disease courses. We cannot rule out selection bias since we only included patients undergoing HVP measurement. However, haemodynamic evaluations are routinely performed for risk stratification and treatment monitoring purposes at our centre, and thus, we are confident that our

TABLE 3 | Uni- and multivariable Cox regression analyses of factors associated with first or further hepatic decompensation (according to clinical stage at baseline) and/or liver-related death ($n = 94$ events).

Patient characteristics	Univariable		Multivariable (first model)		Multivariable (last model)	
	HR (95% CI)	<i>p</i>	aHR (95% CI)	<i>p</i>	aHR (95% CI)	<i>p</i>
Age, year	1.00 (0.98–1.02)	0.863	1.00 (0.98–1.02)	0.819	—	—
HVPG, mmHg	1.11 (1.06–1.15)	<0.001	1.07 (1.02–1.12)	0.006	1.07 (1.03–1.12)	<0.001
CTP score, points	1.35 (1.23–1.49)	<0.001	1.18 (1.03–1.35)	0.017	1.22 (1.10–1.36)	<0.001
Aetiological therapy	0.72 (0.36–1.46)	0.366	0.79 (0.39–1.63)	0.527	—	—
Creatinine, mg/dL	1.00 (0.76–1.33)	0.979	—	—	—	—
Sodium, mmol/L	0.91 (0.87–0.96)	0.001	1.02 (0.94–1.10)	0.633	—	—
C-reactive protein, mg/dL	1.32 (0.99–1.76)	0.061	1.06 (0.78–1.44)	0.705	—	—
PLT, G/L, log	0.73 (0.50–1.08)	0.115	0.75 (0.42–1.34)	0.330	—	—
Aspartate aminotransferase, IU/L, log	2.33 (1.53–3.52)	<0.001	1.60 (0.92–2.78)	0.097	1.67 (1.01–2.75)	0.044
Alanine aminotransferase, IU/L, log	0.90 (0.60–1.36)	0.613	—	—	—	—
<i>Coagulation activation</i>						
FXIII activity, %, log	0.50 (0.30–0.82)	0.007	0.68 (0.41–1.12)	0.128	0.68 (0.43–1.07)	0.094
Thrombin antithrombin complex, log	0.78 (0.60–1.00)	0.052	0.92 (0.71–1.20)	0.550	—	—
<i>Endothelium/platelet markers</i>						
Von Willebrand factor antigen, log	4.49 (2.37–8.50)	<0.001	—	—	—	—
Platelet factor 4, ng/mL, log	0.79 (0.60–1.04)	0.091	1.10 (0.65–1.86)	0.729	—	—
Soluble platelet selectin, ng/mL, log	2.16 (1.20–3.88)	0.010	1.61 (0.68–3.80)	0.275	—	—
Neutrophil-activating peptide-2, ng/mL, log	0.73 (0.55–0.97)	0.031	0.90 (0.51–1.61)	0.731	—	—
Syndecan-1, ng/mL, log	0.97 (0.83–1.13)	0.688	—	—	—	—
<i>Fibrinolysis activation</i>						
Plasmin antiplasmin complex, log	1.11 (0.90–1.38)	0.340	—	—	—	—
<i>Fibrinolytic potential</i>						
TM-TGA CLT, min, log	1.20 (0.68–2.11)	0.522	—	—	—	—
<i>NET markers</i>						
Myeloperoxidase-DNA, AU	1.14 (0.70–1.88)	0.596	1.05 (0.62–1.76)	0.862	—	—
Cell-free DNA, µg/mL	4.19 (1.13–15.56)	0.032	2.49 (0.33–18.94)	0.378	—	—

Abbreviations: aHR, adjusted hazard ratio; CTP, Child-Turcotte-Pugh score; HR, hazard ratio; HVPG, hepatic venous pressure gradient. *P*-value < 0.05 denotes statistical significance.

study population is quite representative. While healthy controls were not comparable in regard to age and body mass index, they were matched regarding sex.

Finally, we did not include histological data on intrahepatic NET formation as liver tissue was not available. Consequently, we do not know if peripherally measured NET parameters are reflecting NET formation in the liver/portal venous system. In fact, there is no generally accepted gold standard for NET quantification [46]. Therefore, we opted for an approach utilised in numerous other

studies assessing NET formation in liver disease to increase the comparability between studies [11, 38, 47].

To conclude, patients with clinically stable ACLD showed increased cf-DNA levels (i.e., NETosis, but also apoptosis/necrosis). However, levels of the more specific marker of NETosis, MPO-DNA, were comparable to healthy controls. NETosis does not appear to drive disease progression in humans with clinically stable ACLD, as it was not linked to clinical endpoints. Thus, findings obtained in animal models may not be translatable to the human setting.

TABLE 4 | Uni- and multivariable Cox regression analyses of factors associated with ACLF or liver-related death ($n = 49$ events).

	Univariable		Multivariable (first model)		Multivariable (last model)	
	HR (95% CI)	<i>p</i>	aHR (95% CI)	<i>p</i>	aHR (95% CI)	<i>p</i>
<i>Patient characteristics</i>						
Age, year	1.00 (0.98–1.03)	0.806	—	—	—	—
HVPG, mmHg	1.08 (1.03–1.13)	0.003	1.04 (0.98–1.10)	0.239	1.05 (0.99–1.11)	0.115
CTP score, points	1.39 (1.19–1.61)	<0.001	1.28 (1.05–1.56)	0.015	1.33 (1.13–1.57)	<0.001
Aetiological therapy	0.73 (0.28–1.86)	0.507	—	—	—	—
Creatinine, mg/dL	1.08 (0.80–1.48)	0.607	—	—	—	—
Sodium, mmol/L	0.92 (0.87–0.96)	<0.001	1.00 (0.93–1.08)	0.975	—	—
C-reactive protein, mg/dL	1.33 (0.97–1.82)	0.078	—	—	—	—
Platelets, G/L, log	0.91 (0.52–1.58)	0.728	—	—	—	—
Aspartate aminotransferase, IU/L, log	2.50 (1.23–5.12)	0.012	1.75 (0.71–4.32)	0.228	—	—
Alanine aminotransferase, IU/L, log	0.77 (0.43–1.36)	0.361	—	—	—	—
<i>Coagulation activation</i>						
FXIII activity, %, log	0.77 (0.40–1.47)	0.424	—	—	—	—
Thrombin antithrombin complex, log	0.76 (0.52–1.10)	0.150	—	—	—	—
<i>Endothelium/platelet markers</i>						
Von Willebrand factor antigen, log	3.31 (1.32–8.32)	0.011	—	—	—	—
Platelet factor 4, ng/mL, log	0.72 (0.49–1.06)	0.093	—	—	—	—
Soluble P-selectin, ng/mL, log	2.09 (1.10–3.97)	0.025	1.07 (0.54–2.12)	0.837	—	—
Neutrophil-activating peptide-2, ng/mL, log	0.73 (0.46–1.15)	0.175	—	—	—	—
Syndecan-1, ng/mL, log	0.99 (0.81–1.21)	0.948	—	—	—	—
<i>Fibrinolysis activation</i>						
Plasmin antiplasmin complex, log	0.91 (0.67–1.25)	0.573	—	—	—	—
<i>Fibrinolytic potential</i>						
TM-TGA CLT, min, log	1.79 (0.77–4.19)	0.178	—	—	—	—
<i>NET markers</i>						
Myeloperoxidase-DNA, AU	0.86 (0.40–1.88)	0.710	—	—	—	—
Cell-free DNA, µg/mL	7.52 (1.23–46.03)	0.029	3.01 (0.39–23.13)	0.289	—	—

Abbreviations: ACLF, acute-on-chronic liver failure; aHR, adjusted hazard ratio; CTP, Child-Turcotte-Pugh score; HR, hazard ratio; HVPG, hepatic venous pressure gradient. *P*-value < 0.05 denotes statistical significance.

Author Contributions

Concept of the study L.B., B.Sc., and M.M., sample and data collection L.B., B.Sc., B.Si., L.H., M.J., G.S., B.S.H., A.F.S., M.P., T.R., and M.M., laboratory analyses T.L., statistical analyses L.B., B.Sc. and M.M., drafting of the manuscript L.B., B.Sc., T.L., and M.M., and revision for important intellectual content and approval of the final manuscript (all authors).

Conflicts of Interest

The authors declare no conflicts of interest. L.B., G.S., M.J., L.H., N.D., A.F.S., and T.L. have nothing to disclose outside of the submitted work.

B.Sc. served as a speaker for Eisai, received travel support from AbbVie, AstraZeneca, Ipsen, Gilead, and Roche as well as grants/research support from AstraZeneca and Eisai. B.Si. received travel support from AbbVie and Gilead. M.S. served as a speaker for Bristol-Myers Squibb and received travel support from AbbVie, Bristol-Myers Squibb, Gilead, MSD, and Sandoz. M.P. served as a speaker and/or consultant and/or advisory board member for Bayer, Bristol-Myers Squibb, Eisai, Ipsen, Lilly, MSD, and Roche and received travel support from Bayer and Bristol-Myers Squibb, Ipsen, and Roche. M.T. received research grants, travel grants, speaker fees and advised for Gilead Sciences; received consultancy fees from AbbVie, Albireo, Agomab, BiomX, Boehringer Ingelheim, Chemomab, Falk, Glaxo Smith Kline, Genfit, Hightide, Intercept, Ipsen, Janssen, Mirum, MSD, Novartis, Pliant, Regulix, Siemens and Shire; research funding from Albireo, Alnylam, Cymabay, Falk, Intercept, MSD,

Takeda and UltraGenyx; travel grants from AbbVie, Falk, Intercept and Janssen; speaker fees from Albireo, BMS, Falk, Intercept, Ipsen, MSD and Madrigal; the Medical Universities of Graz and Vienna have filed patents on the medical use of norUDCA and M.T. is listed as co-inventor. T.R. served as a speaker and/or consultant and/or advisory board member, receiving honoraria from AbbVie, Bayer, Boehringer-Ingelheim, Gilead, Intercept, MSD, Roche, Siemens, and W.L. Gore & Associates, and received travel support from AbbVie, Boehringer-Ingelheim, Gilead, and Roche, as well as grants/research support from AbbVie, Boehringer-Ingelheim, Gilead, Intercept, MSD, Myr Pharmaceuticals, Philips Healthcare, Pliant, Siemens, and W.L. Gore & Associates. M.M. served as a speaker and/or consultant and/or advisory board member for AbbVie, Collective Acumen, Echosens, Falk, Gilead, Ipsen, Takeda, and W.L. Gore & Associates and received travel support from AbbVie and Gilead as well as grants/research support from Echosens.

Data Availability Statement

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

References

1. T. H. Karlsen, N. Sheron, S. Zelber-Sagi, et al., "The EASL-Lancet Liver Commission: Protecting the Next Generation of Europeans Against Liver Disease Complications and Premature Mortality," *Lancet* 399, no. 10319 (2022): 61–116.
2. I. R. Wanless, "The Role of Vascular Injury and Congestion in the Pathogenesis of Cirrhosis: The Congestive Escalator and the Parenchymal Extinction Sequence," *Current Hepatology Reports* 19, no. 1 (2020): 40–53.
3. D. Costa, B. Simbrunner, M. Jachs, et al., "Systemic Inflammation Increases Across Distinct Stages of Advanced Chronic Liver Disease and Correlates With Decompensation and Mortality," *Journal of Hepatology* 74, no. 4 (2021): 819–828.
4. K. Martinod and D. D. Wagner, "Thrombosis: Tangled Up in NETs," *Blood* 123, no. 18 (2014): 2768–2776.
5. S. Yang, H. Qi, K. Kan, et al., "Neutrophil Extracellular Traps Promote Hypercoagulability in Patients With Sepsis," *Shock* 47, no. 2 (2017): 132–139.
6. E. J. Folco, T. L. Mawson, A. Vromman, et al., "Neutrophil Extracellular Traps Induce Endothelial Cell Activation and Tissue Factor Production Through Interleukin-1 α and Cathepsin G," *Arteriosclerosis, Thrombosis, and Vascular Biology* 38, no. 8 (2018): 1901–1912.
7. T. A. Fuchs, A. A. Bhandari, and D. D. Wagner, "Histones Induce Rapid and Profound Thrombocytopenia in Mice," *Blood* 118, no. 13 (2011): 3708–3714.
8. T. A. Fuchs, A. Brill, D. Duerschmied, et al., "Extracellular DNA Traps Promote Thrombosis," *Proceedings of the National Academy of Sciences of the United States of America* 107, no. 36 (2010): 15880–15885.
9. S. M. Morris and A. Chauhan, "The Role of Platelet Mediated Thromboinflammation in Acute Liver Injury," *Frontiers in Immunology* 13 (2022): 1037645.
10. C. M. Ward, T. J. Tetaz, R. K. Andrews, and M. C. Berndt, "Binding of the Von Willebrand Factor A1 Domain to Histone," *Thrombosis Research* 86, no. 6 (1997): 469–477.
11. A. Blasi, V. C. Patel, J. Adelmeijer, et al., "Plasma Levels of Circulating DNA Are Associated With Outcome, but Not With Activation of Coagulation in Decompensated Cirrhosis and ACLF," *JHEP Reports* 1, no. 3 (2019): 179–187.
12. M. Levi, T. van der Poll, and M. Schultz, "Systemic Versus Localized Coagulation Activation Contributing to Organ Failure in Critically Ill Patients," *Seminars in Immunopathology* 34, no. 1 (2012): 167–179.
13. A. Rhodes, S. J. Wort, H. Thomas, P. Collinson, and E. D. Bennett, "Plasma DNA Concentration as a Predictor of Mortality and Sepsis in Critically Ill Patients," *Critical Care* 10, no. 2 (2006): R60.
14. M. Levi and T. van der Poll, "Coagulation and Sepsis," *Thrombosis Research* 149 (2017): 38–44.
15. F. A. von Meijenfeldt, L. C. Burlage, S. Bos, J. Adelmeijer, R. J. Porte, and T. Lisman, "Elevated Plasma Levels of Cell-Free DNA During Liver Transplantation Are Associated With Activation of Coagulation," *Liver Transplantation* 24, no. 12 (2018): 1716–1725.
16. B. Simbrunner, I. F. Villesen, P. Königshofer, et al., "Systemic Inflammation Is Linked to Liver Fibrogenesis in Patients With Advanced Chronic Liver Disease," *Liver International* 42, no. 11 (2022): 2501–2512.
17. F. Turon, E. G. Driever, A. Baiges, et al., "Predicting Portal Thrombosis in Cirrhosis: A Prospective Study of Clinical, Ultrasonographic and Hemostatic Factors," *Journal of Hepatology* 75, no. 6 (2021): 1367–1376.
18. T. Lisman, B. Arefaine, J. Adelmeijer, et al., "Global Hemostatic Status in Patients With Acute-On-Chronic Liver Failure and Septics Without Underlying Liver Disease," *Journal of Thrombosis and Haemostasis* 19, no. 1 (2021): 85–95.
19. W. Potze, M. S. Siddiqui, S. L. Boyett, et al., "Preserved Hemostatic Status in Patients With Non-Alcoholic Fatty Liver Disease," *Journal of Hepatology* 65, no. 5 (2016): 980–987.
20. T. Reiberger, P. Schwabl, M. Trauner, M. Peck-Radosavljevic, and M. Mandorfer, "Measurement of the Hepatic Venous Pressure Gradient and Transjugular Liver Biopsy," *Journal of Visualized Experiments* 160 (2020).
21. A. Ferlitsch, S. Bota, R. Paternostro, et al., "Evaluation of a New Balloon Occlusion Catheter Specifically Designed for Measurement of Hepatic Venous Pressure Gradient," *Liver International: Official Journal of the International Association for the Study of the Liver* 35, no. 9 (2015): 2115–2120.
22. R. de Franchis, J. Bosch, G. Garcia-Tsao, et al., "Baveno VII - Renewing Consensus in Portal Hypertension," *Journal of Hepatology* 76, no. 4 (2022): 959–974.
23. R. A. Ariens, H. P. Kohler, M. W. Mansfield, et al., "Subunit Antigen and Activity Levels of Blood Coagulation Factor XIII in Healthy Individuals. Relation to Sex, Age, Smoking, and Hypertension," *Arteriosclerosis, Thrombosis, and Vascular Biology* 19, no. 8 (1999): 2012–2016.
24. S. Bos, B. van den Boom, P. W. Kamphuisen, et al., "Haemostatic Profiles Are Similar Across All Aetiologies of Cirrhosis," *Thrombosis and Haemostasis* 119, no. 2 (2019): 246–253.
25. H. C. Hemker, P. Giesen, R. Al Dieri, et al., "Calibrated Automated Thrombin Generation Measurement in Clotting Plasma," *Pathophysiology of Haemostasis and Thrombosis* 33, no. 1 (2003): 4–15.
26. T. Lisman and J. Adelmeijer, "Preanalytical Variables Affect Thrombomodulin-Modified Thrombin Generation in Healthy Volunteers," *Thrombosis Research* 194 (2020): 237–239.
27. M. E. Meltzer, T. Lisman, C. J. Doggen, P. G. de Groot, and F. R. Rosendaal, "Synergistic Effects of Hypofibrinolysis and Genetic and Acquired Risk Factors on the Risk of a First Venous Thrombosis," *PLoS Medicine* 5, no. 5 (2008): e97.
28. K. Kessenbrock, M. Krumbholz, U. Schönermarck, et al., "Netting Neutrophils in Autoimmune Small-Vessel Vasculitis," *Nature Medicine* 15, no. 6 (2009): 623–625.
29. R. Francés, S. Benlloch, P. Zapater, et al., "A Sequential Study of Serum Bacterial DNA in Patients With Advanced Cirrhosis and Ascites," *Hepatology* 39, no. 2 (2004): 484–491.
30. G. Heinze and D. Dunkler, "Five Myths About Variable Selection," *Transplant International* 30, no. 1 (2017): 6–10.
31. I. Balazs and V. Stadlbauer, "Circulating Neutrophil Anti-Pathogen Dysfunction in Cirrhosis," *JHEP Reports* 5, no. 11 (2023): 100871.

32. V. Brinkmann, U. Reichard, C. Goosmann, et al., "Neutrophil Extracellular Traps Kill Bacteria," *Science* 303, no. 5663 (2004): 1532–1535.
33. H. Huang, S. Tohme, A. B. Al-Khafaji, et al., "Damage-Associated Molecular Pattern-Activated Neutrophil Extracellular Trap Exacerbates Sterile Inflammatory Liver Injury," *Hepatology* 62, no. 2 (2015): 600–614.
34. I. Slaba, J. Wang, E. Kolaczowska, B. McDonald, W. Y. Lee, and P. Kubes, "Imaging the Dynamic Platelet-Neutrophil Response in Sterile Liver Injury and Repair in Mice," *Hepatology* 62, no. 5 (2015): 1593–1605.
35. T. Reiberger, A. Ferlitsch, B. A. Payer, et al., "Non-Selective Beta-blocker Therapy Decreases Intestinal Permeability and Serum Levels of LBP and IL-6 in Patients With Cirrhosis," *Journal of Hepatology* 58, no. 5 (2013): 911–921.
36. J. M. Agraz-Cibrián, V. Delgado-Rizo, J. E. Segura-Ortega, et al., "Impaired Neutrophil Extracellular Traps and Inflammatory Responses in the Peritoneal Fluid of Patients With Liver Cirrhosis," *Scandinavian Journal of Immunology* 88, no. 5 (2018): e12714.
37. J. M. Agraz-Cibrian, J. E. Segura-Ortega, V. Delgado-Rizo, et al., "Alterations in Neutrophil Extracellular Traps Is Associated With the Degree of Decompensation of Liver Cirrhosis," *Journal of Infection in Developing Countries* 10, no. 5 (2016): 512–517.
38. R. Zenlander, S. Havervall, M. Magnusson, et al., "Neutrophil Extracellular Traps in Patients With Liver Cirrhosis and Hepatocellular Carcinoma," *Scientific Reports* 11, no. 1 (2021): 18025.
39. G. Tritto, Z. Bechlis, V. Stadlbauer, et al., "Evidence of Neutrophil Functional Defect Despite Inflammation in Stable Cirrhosis," *Journal of Hepatology* 55, no. 3 (2011): 574–581.
40. Y. Cho, T. N. Bukong, D. Tornai, et al., "Neutrophil Extracellular Traps Contribute to Liver Damage and Increase Defective Low-Density Neutrophils in Alcohol-Associated Hepatitis," *Journal of Hepatology* 78, no. 1 (2023): 28–44.
41. M. B. Hilscher and V. H. Shah, "Neutrophil Extracellular Traps and Liver Disease," *Seminars in Liver Disease* 40, no. 2 (2020): 171–179.
42. F. A. von Meijenfeldt, T. Lisman, A. Pacheco, Y. Zen, and W. Bernal, "Histologic Evidence of Neutrophil Extracellular Traps and Fibrin(O-gen) Deposition in Liver Biopsies From Patients With Inflammatory Liver Disease," *Research and Practice in Thrombosis and Haemostasis* 9, no. 1 (2025): 102666.
43. A. Rosell, K. Martinod, N. Mackman, and C. Thålin, "Neutrophil Extracellular Traps and Cancer-Associated Thrombosis," *Thrombosis Research* 213, no. Suppl 1 (2022): S35–s41.
44. J. D. Seo, J. Y. Gu, H. S. Jung, Y. J. Kim, and H. K. Kim, "Contact System Activation and Neutrophil Extracellular Trap Markers: Risk Factors for Portal Vein Thrombosis in Patients With Hepatocellular Carcinoma," *Clinical and Applied Thrombosis/Hemostasis* 25 (2019): 1076029618825310.
45. M. B. Hilscher, T. Sehrawat, J. P. Arab, et al., "Mechanical Stretch Increases Expression of CXCL1 in Liver Sinusoidal Endothelial Cells to Recruit Neutrophils, Generate Sinusoidal Microthrombi, and Promote Portal Hypertension," *Gastroenterology* 157, no. 1 (2019): 193–209.e9.
46. M. Stoimenou, G. Tzoros, P. Skendros, and A. Chrysanthopoulou, "Methods for the Assessment of NET Formation: From Neutrophil Biology to Translational Research," *International Journal of Molecular Sciences* 23, no. 24 (2022): 15823.
47. F. A. von Meijenfeldt, R. T. Stravitz, J. Zhang, et al., "Generation of Neutrophil Extracellular Traps in Patients With Acute Liver Failure Is Associated With Poor Outcome," *Hepatology* 75, no. 3 (2022): 623–633.

Supporting Information

Additional supporting information can be found online in the Supporting Information section. **Data S1:** liv70323-sup-0001-DataS1.docx.