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ORIGINAL ARTICLE

Generation of neutrophil extracellular traps in patients with acute liver failure is associated with poor outcome

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Abstract

Background and Aims: Acute liver failure (ALF) is characterized by significant changes in the hemostatic system and by systemic inflammation. The formation of neutrophil extracellular traps (NETs), in which an activated neutrophil expels its DNA, histones, and granular enzymes, such as myeloperoxidase (MPO), has been associated with immune-mediated and thrombotic diseases. We hypothesized that formation of NETs in patients with ALF contributes to progression of disease.

Approach and Results: A total of 676 patients with ALF (international normalized ratio [INR], ≥ 1.5) or severe acute liver injury (ALI; INR, ≥ 2.0) were recruited from the U.S. ALF Study Group Registry between 2011 and 2018, of whom 308 patients (45.6%) had acetaminophen-induced ALF. Up to 21 days after admission, 483 patients (71.5%) survived without liver transplantation (LT). Levels of cell-free DNA (cfDNA) and the specific NET marker MPO-DNA complexes were measured in plasma samples obtained on admission and compared to levels in healthy controls. In addition, liver tissue obtained at transplantation of 20 ALF patients was stained for NETs. Levels of cfDNA were 7.1-fold, and MPO-DNA complexes 2.5-fold, higher in patients with ALF compared to healthy controls. cfDNA levels were not associated with 21-day transplant-free survival, but were higher in those patients with more-severe disease on admission, as reflected by various laboratory and clinical parameters. MPO-DNA levels were 30% higher in patients with ALF who died or required urgent LT. Liver tissue of ALF patients stained positive for NETs in 12 of 18 evaluable patients.

Conclusions: Here, we provide evidence for NET formation in patients with ALF. Elevated plasma levels of MPO-DNA complexes in patients with ALF

Abbreviations: ACLF, acute-on-chronic liver failure; ALF, acute liver failure; ALI, acute liver injury; APAP, acetaminophen; AU, arbitrary units; cfDNA, cell-free DNA; INR, international normalized ratio; LT, liver transplantation; NETs, neutrophil extracellular traps; MPO, myeloperoxidase; RRT, renal replacement therapy; SIRS, systemic inflammatory response syndrome; TFS, transplant-free survival.

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were associated with poor outcome, which suggests that NET formation contributes to disease progression.

INTRODUCTION

Acute liver failure (ALF) is a critical condition associated with high mortality that requires highly specialized clinical care. Although survival rates have improved significantly over the past decades, ~30% of patients with ALF currently die from their illness, and another 25% require liver transplantation (LT).^[1,2] Optimal treatment strategies for patients with ALF remain poorly defined, given that the condition is rare, and therefore rely on expert opinion. In addition, it remains difficult to distinguish which patients will die, spontaneously survive, or require (urgent) LT. This stresses the need for a better understanding of the mechanisms that contribute to disease progression and better tools for the assessment of prognosis in ALF.

ALF is characterized by a profound inflammatory response with a substantial release of proinflammatory cytokines, resulting in systemic inflammation and, often, multiple organ failure.^[1,3] This systemic inflammatory response contributes to the profound alterations in the hemostatic system we have previously identified.^[4] Notably, experimental studies suggest that intrahepatic activation of coagulation contributes to progression of liver injury in ALF.^[5,6] In addition, experimental studies and observations in humans suggest a role for intrahepatic platelet accumulation in the progression of ALF.^[7,8] Mechanisms underlying intrahepatic activation of hemostasis have not been fully elucidated, but likely include inflammatory responses leading to endothelial cell activation and activation of the natural activator of coagulation, tissue factor, within the liver.^[9]

Neutrophil extracellular traps (NETs) provide a link between inflammation and coagulation, both of which play a central role in the pathophysiology of ALF. Upon activation, neutrophils can form web-like structures, referred to as NETs, consisting of DNA, nuclear, and granular proteins. The process in which a neutrophil expels its cellular content was first described as an important feature of the innate immune system, given that these traps could both physically and chemically capture and kill pathogens.^[10] NETs, however, were subsequently identified to play crucial roles in various diseases characterized by sterile inflammation. For example, elevated levels of markers of NETs have been reported in autoimmune diseases, cancer, and ischemia-reperfusion injury and have been associated with severity of disease.^[11–13] In addition, in experimental models, interventions that decrease NET formation or dismantle NETs have been demonstrated to lead to decreased disease severity and/or progression.^[14–16]

NETs have also been shown to activate the hemostatic system by various mechanisms.^[17] Experimental

studies have shown that NETs contribute to macrovascular thrombosis, and that blocking of NETs inhibited thrombosis.^[18] In humans, elevated markers of NETs have been shown to be associated with thrombotic disease, and there is histological evidence for the presence of NETs in human thrombi.^[19–21] NETs may also be involved in microvascular thrombotic complications, which are frequent in organ injury, and associated with conditions such as ischemia-reperfusion injury or sepsis.^[22–24] Liver diseases are also thought to be accompanied by microvascular (intrahepatic) thrombotic events that may drive disease progression.^[25] Indeed, a recent study showed that NETs promote progression of liver disease by the formation of (micro)thrombi in a mouse model of portal hypertension.^[26] NETs have been implicated in various types of liver disease,^[27] but whether NET-mediated activation of coagulation also contributes to liver disease progression has not yet been established.

NETs may also be useful as markers of prognosis. For example, high levels of cell-free DNA (cfDNA), a major component of NETs, have been documented in the plasma of patients with sepsis in proportion to poor survival rates.^[28,29] Furthermore, a recent study reported that high levels of cfDNA were associated with increased 30-day mortality in patients with acute-on-chronic liver failure (ACLF).^[30] Although cfDNA is often used as a marker of NETs, it is not specific for NETs and could also originate from various other cell types. A more specific marker for NETs are myeloperoxidase (MPO)/DNA complexes, which are neutrophil granular enzymes bound to DNA, and levels were also elevated in patients with ACLF, but not associated with mortality.^[30] Notably, NETs have not been previously studied in patients with ALF.

The potential role for NETs in liver disease, combined with the central role of disturbances in inflammation and coagulation in ALF, led us to study the formation of NETs in a large cohort of patients with severe acute liver injury (ALI) and ALF. We hypothesized that NETs are formed in patients with ALF, contribute to disease progression, and could have prognostic value in ALF.

PATIENTS AND METHODS

Study population

We studied patients with severe ALI or ALF who were recruited from the U.S. Acute Liver Failure Study Group Registry between 2011 and 2018. Details of this cohort were described elsewhere.^[31] In short,

severe ALI was defined as international normalized ratio (INR) ≥ 2.0 on admission in patients without previously known liver disease, in the absence of HE, and a maximum duration of disease of 26 weeks. ALF was defined as ALI, in the presence of HE, and with INR ≥ 1.5 on admission. All patients, or in the case of incapacity, their consultee, gave informed consent for participation in this study. Ethical approval was obtained from the study-wide Institutional Review Board (IRB) of the University of Texas Southwestern Medical Center and by the IRB of each participating center. In addition, 40 healthy volunteers were included to establish reference values for the various tests performed.

Blood sampling procedures

Blood samples were collected on day 1 or 2 of admission to the study-site hospital. Whole blood was collected into vacuum tubes containing 3.2% trisodium citrate at a blood-to-anticoagulant ratio of 9:1. The citrated blood was processed to platelet-poor plasma by centrifugation at 1,500g for 15 minutes within 2 hours after collection, separated into 0.5-mL aliquots, and stored at -80°C until used for analyses.

Assessment of cfDNA and MPO-DNA complexes in plasma

Levels of cfDNA and MPO-DNA complexes were determined in plasma as markers for cellular injury and NET formation, respectively. cfDNA was quantified using the Quant-iT PicoGreen double-stranded DNA (dsDNA) assay kit (Fisher Scientific, Landsmeer, The Netherlands) as described.^[13] Concentration of MPO-DNA complexes in plasma was determined by ELISA, based on described methods.^[11] We made the following adjustments to the described protocol; we used a commercially available monoclonal antibody to MPO (5 $\mu\text{g}/\text{mL}$; Sanbio, Uden, The Netherlands), the detection antibody derived from the cell-death detection ELISA (Sigma-Aldrich, Zwijndrecht, The Netherlands), and tetramethylbenzidine (Sigma-Aldrich, Zwijndrecht, The Netherlands) as a substrate. After 15 minutes of incubation in the dark at room temperature, absorbance at a 405-nm wavelength was measured with the use of a VersaMax reader (Molecular Devices, San Jose, CA).

Histological analyses of NETs in the liver

To study whether NET formation occurs in the liver parenchyma of patients with ALF, we performed histological analyses on liver tissue from 20 patients in our

cohort. We specifically studied 10 patients with acetaminophen (APAP) and 10 patients with non-APAP etiology. Liver tissue was obtained at transplantation, and after fixation in formalin, embedded in paraffin for histological assessment. Liver tissue was stained for citrullinated histones (H3citB) to assess NET formation on an automated staining machine (Bond Max autostainer; Leica, Wetzlar, Germany). We specifically used the commercially available antibody, antihistone H3 (diluted 1:100; ab5103; Abcam, Cambridge, UK). Cut tissue slides were pretreated in citrate buffer for 20 minutes. We included five liver-tissue samples taken from otherwise discarded human donor livers obtained from another experiment (institutional ethical approval code UMCG: M14.152454) to serve as controls. Images were reviewed by an experienced liver pathologist (Y.Z.) and classified based on degree of expression of H3citB, ranging from no expression (score 0), focal expression (positive in $<10\%$ of neutrophils; score 1), moderate expression (10%-50% of neutrophils; score 2), to extensive expression ($>50\%$ of neutrophils; score 3).

Statistical analyses

Levels of cfDNA and MPO-DNA complexes are shown as means with 95% CIs. Mean marker levels of various groups were compared using the Student *t* test. Multivariable regression analyses were carried out with a logistic regression model. Potential confounders, such as high-risk etiology and admission values of INR, bilirubin, pressor use, and HE grade 3-4, were included in all multivariable models. All analyses were performed using SAS software (version 9.4; SAS Institute, Inc. Cary, NC) with a two-sided significance level of 0.05.

RESULTS

Patient characteristics

A total of 676 patients with severe ALI or ALF were included in this study, of which 308 had APAP-induced liver injury (ALILI). Up to 21 days after admission, 483 patients survived without LT. Patient characteristics are summarized in Table 1.

Elevated levels of cfDNA are associated with severity of disease on admission

Levels of cfDNA were 7.1-fold higher in patients with severe ALI or ALF compared to levels found in healthy controls (Figure 1). We initially assessed whether levels of cfDNA were related to severity of disease, as reflected by various laboratory values and clinical parameters, as

TABLE 1 Patient characteristics

Variable	ALI/ALF Patients (n = 676)	ALI/ALF Patients for Histological Study (n = 18)
Age	40.8 ± 15.8	40.2 ± 14.1
Female	418 (61.8)	7 (38.9)
Body mass index (kg/m ²)	28.7 ± 7.9	28.8 ± 5.9
Etiology		
APAP	308 (45.6)	10 (55.6)
Autoimmune	39 (5.8)	1 (5.6)
Drug-induced	63 (9.3)	3 (16.7)
Ischemia	79 (11.7)	0 (0.0)
Other ^a	114 (16.9)	2 (11.1)
Viral	73 (10.8)	2 (11.1)
HE (grade 3-4)	170 (25.2)	8 (44.4)
SIRS (≥2) ^b on admission	333 (49.3)	
Infection on admission	84 (12.4)	
Laboratory values on admission		
MELD score	29.1 ± 8.8	35.4 ± 8.6
Albumin (g/dL)	2.8 ± 0.6	3.1 ± 0.8
Arterial pH	7.4 ± 0.1	7.4 ± 0.1
Aspartate aminotransferase (IU/L)	3,624.6 ± 4,041.6	4,762.0 ± 5,547.0
Bicarbonate (mmol/L)	22.1 ± 5.0	21.1 ± 7.2
Creatinine (mg/dL)	1.7 ± 1.6	2.1 ± 1.5
INR	3.2 ± 1.7	3.9 ± 1.8
Lactate (mmol/L)	4.0 ± 3.5	6.1 ± 6.7
Phosphate (mg/dL)	3.2 ± 1.8	3.7 ± 1.3
Platelet count (×10 ⁹ /L)	145 ± 87	135 ± 106
Total bilirubin (mg/dL)	9.0 ± 9.1	16.8 ± 15.3
Interventions during admission		
Red blood cell transfusion	92 (13.6)	7 (38.9)
RRT	171 (25.3)	8 (44.4)
Use of vasopressors	172 (25.4)	7 (38.9)
Outcome (up to 21 days after admission)		
Bleeding	50 (7.4)	2 (11.1)
Death	127 (18.8)	1 (5.6)
LT	71 (10.5) ^c	16 (88.9)
TFS	483 (71.5)	2 (11.1)

Note: Results are presented as mean ± SD for continuous variables, and number (percentage) for categorical variables.

^aOther etiologies of ALF include Budd-Chiari syndrome, Wilson's disease, mushroom intoxication, and unknown.

^bNumber of positive SIRS criteria.

^cFive patients who received an LT died within 21 days after admission.

Abbreviation: MELD, Model for End-Stage Liver Disease.

shown in Table 2. Significantly higher levels of cfDNA were found in patients with abnormal serum lactate, bicarbonate, phosphate, and creatinine levels measured on admission, compared to patients with normal admission levels. In contrast, significantly lower levels of cfDNA were found in patients with abnormal total bilirubin levels compared to patients with normal levels. In addition, patients with platelet counts $\leq 100 \times 10^9/L$ had significantly higher levels of cfDNA compared to

patients with platelet counts $>100 \times 10^9/L$, and levels of cfDNA were higher in patients with INR >2.5 . Moreover, renal replacement therapy (RRT) and the use of vasopressors were associated with high levels of cfDNA. High-grade HE (grade 3-4) was not associated with a difference in plasma levels of cfDNA. Patients with systemic inflammatory response syndrome (SIRS) on admission had higher levels of cfDNA compared to patients without SIRS, and higher cfDNA levels were found

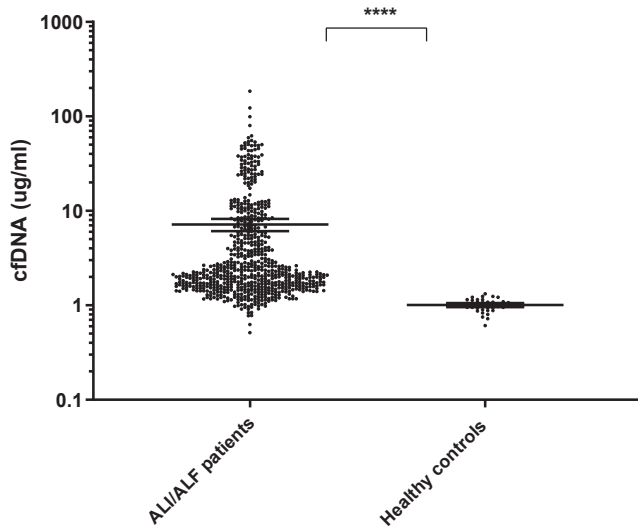


FIGURE 1 Plasma levels of cfDNA were determined in patients with severe ALI or ALF ($n = 675$) and compared to levels of cfDNA in healthy controls ($n = 40$). **** $p < 0.0001$

in patients with three or four positive SIRS criteria compared to patients with zero to two positive criteria. There was no difference in levels of cfDNA between patients where infection was present on admission compared to patients without infection on admission. Patients with severe ALI had similar levels of cfDNA to patients with ALF. Interestingly, higher levels of cfDNA were found in patients with APAP etiology in comparison to patients with non-APAP-induced ALI/ALF.

No association between plasma levels of cfDNA and transplant-free survival

Transplant-free survival (TFS) was defined as patients with severe ALI or ALF who did not die or undergo LT up to 21 days after admission to a participating medical center. There was no association between plasma levels of cfDNA and TFS. More specifically, levels in transplant-free survivors were 7.0 (6.0-8.0) $\mu\text{g/mL}$ in comparison to 7.6 (4.8-10.3) $\mu\text{g/mL}$ in patients who died or required (urgent) LT. In addition, we compared cfDNA levels in patients who died with levels in transplant-free survivors and found no difference (9.6 [5.5-13.7] $\mu\text{g/mL}$ and 7.0 [6.0-8.0] $\mu\text{g/mL}$, respectively; $p = 0.226$).

No association between plasma levels of MPO-DNA complexes and markers of disease severity

Patients with severe ALI or ALF had plasma levels of MPO-DNA complexes that were 2.5-fold higher than levels found in healthy controls, as shown in Figure 2. MPO-DNA complex levels were not associated with severity of disease on admission (Table 3). More

specifically, MPO-DNA was not associated with serum levels of lactate, phosphate, or creatinine. However, elevated plasma levels of MPO-DNA complexes were associated with elevated total bilirubin levels and decreased serum bicarbonate. In addition, platelet count was not related to MPO-DNA complex levels. Patients with $\text{INR} > 2.5$ on admission had significantly higher plasma levels of MPO-DNA complexes compared to patients with $\text{INR} \leq 2.5$ on admission. Use of RRT or vasopressors was not related to levels of MPO-DNA complexes. Furthermore, HE grade 3-4 was not associated with MPO-DNA complex levels. Complex levels were higher in patients who were admitted with SIRS compared to patients without SIRS. Moreover, patients with three or four positive SIRS criteria on admission had higher levels of MPO-DNA complexes in comparison to patients with zero to two positive criteria. There was no difference in complex levels between patients with infection on admission compared to patients without. Patients admitted with severe ALI had lower MPO-DNA complex levels compared to patients admitted with ALF. Last, patients with APAP-induced ALI/ALF had significantly lower plasma levels of MPO-DNA complexes than patients with other etiologies of ALI/ALF.

High levels of MPO-DNA complexes are associated with death or LT

We next assessed the relationship between MPO-DNA complex levels and TFS in patients with severe ALI or ALF. Plasma levels of MPO-DNA complexes were significantly higher in patients who died or underwent LT compared to transplant-free survivors during the first 21 days after hospital admission (0.59 [0.52-0.66] arbitrary units [AU] versus 0.43 [0.39-0.47] AU, respectively; $p < 0.0001$). When solely death and TFS were compared, patients who died had significantly higher levels of MPO-DNA complexes compared to transplant-free survivors (0.60 [0.51-0.70] AU and 0.43 [0.39-0.47] AU, respectively; $p < 0.001$). Multivariable regression analyses showed that MPO-DNA complexes were independent predictors of TFS with an OR of 0.547 (0.323-0.926; Table 4). The AUC of the receiver operating curve was 0.625 (0.579-0.671) for MPO-DNA complexes alone. The AUC was 0.884 (0.856-0.913) for a prognostic model, including HE, etiology, vasopressor use, bilirubin, and INR. Addition of MPO-DNA complexes to this prognostic model increased the AUC to 0.888 (0.861-0.916).

Histological evidence for NET formation in liver parenchyma

In order to study whether NET formation occurs within the liver, we performed histological analyses with the commonly used marker of NETs, H3citB, on

TABLE 2 Plasma levels of cfDNA in relation to laboratory and clinical data on admission in patients with severe ALI or ALF

cfDNA Levels ($\mu\text{g/mL}$)	Healthy Controls ($n = 40$)	1.01 (0.96-1.05)	p Value
Lactate (mmol/L)	Normal (≤ 2.2) $n = 115$ 5.9 (4.2-7.6)	Abnormal (> 2.2) $n = 244$ 10.6 (8.1-13.0)	0.002
Bicarbonate (mmol/L)	Normal (> 21) $n = 317$ 6.1 (4.5-7.6)	Abnormal (≤ 21) $n = 241$ 9.3 (7.3-11.2)	0.011
Phosphate (mg/dL)	Normal (≤ 4.6) $n = 446$ 6.9 (5.8-8.0)	Abnormal (> 4.6) $n = 80$ 13.5 (7.4-19.5)	0.035
Creatinine (mg/dL)	Normal (≤ 1.2) $n = 358$ 5.7 (4.7-6.8)	Abnormal (> 1.2) $n = 307$ 8.8 (6.8-10.7)	0.008
Total bilirubin (mg/dL)	Normal (≤ 1.9) $n = 107$ 10.3 (7.3-13.3)	Abnormal (> 1.9) $n = 555$ 6.7 (5.5-7.8)	0.016
Platelet count ($\times 10^9/\text{L}$)	> 100 ($n = 443$) 6.4 (5.2-7.6)	≤ 100 ($n = 208$) 9.2 (7.0-11.4)	0.031
INR	≤ 2.5 ($n = 289$) 5.2 (4.0-6.5)	> 2.5 ($n = 359$) 8.8 (7.1-10.5)	< 0.001
RRT	No ($n = 504$) 5.9 (5.0-6.9)	Yes ($n = 171$) 10.8 (7.7-13.9)	0.004
Vasopressor use	No ($n = 503$) 6.3 (5.2-7.4)	Yes ($n = 172$) 9.7 (7.1-12.2)	0.018
HE (grade 3-4)	No ($n = 505$) 6.9 (5.7-8.0)	Yes ($n = 170$) 8.1 (5.7-10.5)	0.373
SIRS (≥ 2) on admission	No ($n = 312$) 5.3 (4.2-6.3)	Yes ($n = 333$) 9.0 (7.2-10.8)	< 0.001
SIRS (3-4) on admission	No ($n = 482$) 6.1 (5.1-7.1)	Yes ($n = 163$) 10.5 (7.5-13.5)	0.007
Infection on admission	No ($n = 591$) 6.8 (5.8-7.9)	Yes ($n = 84$) 9.7 (5.5-14.0)	0.189
ALI/ALF diagnosis	ALI ($n = 296$) 6.5 (5.3-7.8)	ALF ($n = 379$) 7.7 (6.0-9.3)	0.280
APAP	No ($n = 367$) 5.7 (4.2-7.3)	Yes ($n = 308$) 8.9 (7.5-10.2)	0.003

Note: cfDNA levels are presented as mean estimates (95% confidence limits).

liver tissue of 20 ALF patients in this cohort. Of the 20 biopsies, 18 were suitable for histological analyses; two cases were excluded because there was almost no liver tissue present to assess. Detailed patient information is provided in Table 1. Of the 18 ALF patients, 12 (67%) liver specimens were positive for the NET marker, of which seven showed high expression levels (score 3). Figure 3 depicts representative examples of the different degrees (score 0-3) of H3cit-positive staining in liver tissue of ALF patients. H3cit was mainly expressed in infiltrating neutrophils, and H3cit-positive cells were present in both portal tracts and liver parenchyma. There was no difference in degree of expression between APAP and non-APAP etiology. The five cases of liver tissue obtained from

discarded human donor livers did not show any expression of H3cit (score 0).

DISCUSSION

In this study, we demonstrate that patients with severe ALI or ALF have 7.1-fold higher levels of cfDNA and 2.5-fold higher levels of MPO-DNA complexes in comparison to healthy controls. In addition, we provide histological evidence for NET formation in these patients. In contrast to a previous study in patients with ACLF,^[30] levels of cfDNA were associated with severity of disease on admission, but not with TFS or death. Interestingly, MPO-DNA

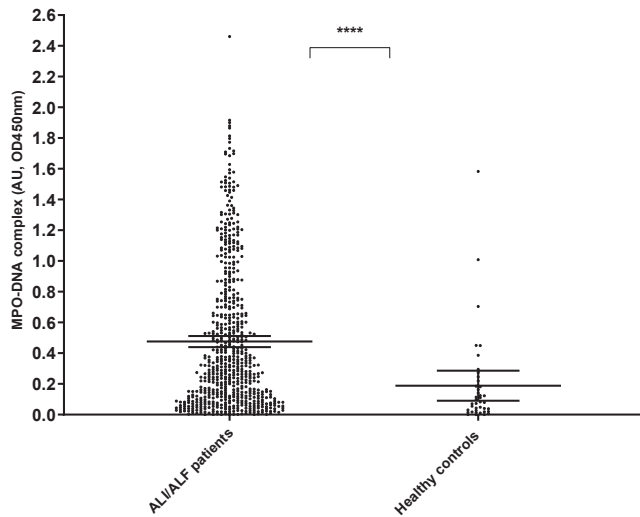


FIGURE 2 MPO-DNA complex levels were determined in plasma of patients with severe ALI or ALF ($n = 629$) and compared to levels of MPO-DNA complexes in healthy controls ($n = 40$). **** $p < 0.0001$, Abbreviation: OD450nm, optical density at 450 nm

complex levels were independent predictors of TFS, but were not associated with disease severity. The results of this study suggest that NET formation in patients with severe ALI or ALF may play a role in disease progression.

Levels of cfDNA were higher in patients with more-severe disease on admission, as reflected by increased levels of lactate, phosphate, and creatinine, high INR, and low platelet counts. An apparent exception to this rule was the association of low total bilirubin with high cfDNA levels, which very likely reflects the contribution of APAP etiology, which is associated with low total bilirubin,^[1] and high levels of cfDNA (as we show here). In addition, high levels of cfDNA were associated with the use of RRT and vasopressors, suggesting a possible role in promoting multiple organ failure. Importantly, although cfDNA is commonly used as a marker for NETs, it can also originate from other types of injured cells and may be partially derived from degenerating hepatocytes and other failing organs, such as the kidneys. Increases in levels of cfDNA have been previously reported in patients during RRT, which normalized shortly after cessation of RRT.^[32] It has been hypothesized that increases in cfDNA during RRT are a result of increased cellular injury induced by the dialysis membranes. One study showed that the majority of cfDNA released during RRT was derived from apoptotic leukocytes,^[33] which may indicate that cfDNA in this setting originates from NETs, although this requires further study. One of the limitations of our study is that we could not establish the origin of the cfDNA with the assay

we used. The assay, which is selective for dsDNA, likely also measures circulating mitochondrial DNA (mtDNA).^[34] Interestingly, mtDNA could serve as a damage-associated molecular pattern that exacerbates NET formation,^[35] and this mechanism could partly explain our finding that high levels of circulating DNA are associated with severity of disease. High cfDNA has been proposed as a prognostic tool to estimate chances of survival in patients with sepsis,^[28,29] including patients with ACLF, in whom higher plasma levels of cfDNA were found in septic compared to nonseptic patients; in a further study, high cfDNA levels were associated with 30-day mortality.^[30] In agreement with these observations, we also found higher levels of cfDNA in patients with SIRS on admission, compared to those without SIRS. In contrast to the study in patients with ACLF, plasma levels of cfDNA were not associated with death or urgent LT within 21 days of admission in those with ALI or ALF. The discrepancy between these results might be explained by the difference in pathogenesis of ACLF and ALF: Whereas patients with ALF have normally functioning livers before the development of injury, patients with ACLF already have cirrhosis and some degree of portal hypertension and liver dysfunction before onset of the acute injury. Preexisting liver dysfunction, including portosystemic shunting, might thereby result in a decreased capacity to overcome the acute injury in patients with ACLF. Although conjectural, it may be the extent of cellular injury reflected by high cfDNA in ACLF, but the regenerative capacity of the liver in ALF, that is the more important predictor of outcome.

In contrast to cfDNA, plasma levels of the specific NET marker, MPO-DNA complexes, were not associated with severity of disease on admission, except for the presence of SIRS. The latter is in accordance with the notion that NET formation plays an important role in inflammation, and past research has shown increased levels of NET markers in sepsis.^[28,29] Of note, significantly higher levels of MPO-DNA complexes were found in patients with non-APAP-induced ALF, suggesting that NET formation particularly might play a role in the pathogenesis of other etiologies of ALF, such as ALF related to viral infection, ischemia, or autoimmune conditions. Indeed, NET formation has been suggested to contribute to sterile inflammation, autoimmune diseases, and ischemia in (experimental models of) liver disease.^[11,13,27] However, the timing of blood sampling might also explain the lower levels of MPO-DNA complexes in APAP-induced ALF patients, because of the hyperacute clinical course after APAP injury. In contrast to patients with non-APAP ALF, APAP-ALF patients are usually admitted to the hospital relatively shortly after the induction of injury, but peak NET formation might not occur until days later.

TABLE 3 Plasma levels of MPO-DNA complexes in relation to laboratory and clinical data on admission in patients with severe ALI or ALF

MPO-DNA Complex Levels (AU)	Healthy Controls (<i>n</i> = 40) 0.19 (0.09-0.29)		<i>p</i> Value
Lactate (mmol/L)	Normal (≤ 2.2) <i>n</i> = 04 0.40 (0.30-0.50)	Abnormal (> 2.2) <i>n</i> = 232 0.47 (0.41-0.52)	0.260
Bicarbonate (mmol/L)	Normal (> 21) <i>n</i> = 293 0.40 (0.36-0.45)	Abnormal (≤ 21) <i>n</i> = 229 0.51 (0.44-0.57)	0.009
Phosphate (mg/dL)	Normal (≤ 4.6) <i>n</i> = 410 0.45 (0.41-0.50)	Abnormal (> 4.6) <i>n</i> = 76 0.49 (0.39-0.59)	0.520
Creatinine (mg/dL)	Normal (≤ 1.2) <i>n</i> = 329 0.46 (0.41-0.51)	Abnormal (> 1.2) <i>n</i> = 290 0.49 (0.44-0.54)	0.402
Total bilirubin (mg/dL)	Normal (≤ 1.9) <i>n</i> = 100 0.32 (0.25-0.39)	Abnormal (> 1.9) <i>n</i> = 516 0.49 (0.45-0.53)	< 0.0001
Platelet count ($\times 10^9/L$)	> 100 (<i>n</i> = 407) 0.47 (0.43-0.52)	≤ 100 (<i>n</i> = 199) 0.47 (0.41-0.53)	0.844
INR	≤ 2.5 (<i>n</i> = 271) 0.42 (0.37-0.48)	> 2.5 (<i>n</i> = 332) 0.51 (0.46-0.56)	0.020
RRT	No (<i>n</i> = 466) 0.46 (0.42-0.51)	Yes (<i>n</i> = 163) 0.51 (0.44-0.58)	0.256
Vasopressor use	No (<i>n</i> = 460) 0.46 (0.41-0.50)	Yes (<i>n</i> = 169) 0.53 (0.46-0.60)	0.069
HE (grade 3-4)	No (<i>n</i> = 464) 0.46 (0.42-0.50)	Yes (<i>n</i> = 165) 0.53 (0.46-0.60)	0.087
SIRS (≥ 2) on admission	No (<i>n</i> = 284) 0.43 (0.38-0.48)	Yes (<i>n</i> = 316) 0.51 (0.46-0.56)	0.035
SIRS (3-4) on admission	No (<i>n</i> = 443) 0.45 (0.40-0.49)	Yes (<i>n</i> = 157) 0.55 (0.48-0.63)	0.014
Infection on admission	No (<i>n</i> = 549) 0.47 (0.43-0.50)	Yes (<i>n</i> = 80) 0.54 (0.43-0.66)	0.171
ALI/ALF diagnosis	ALI (<i>n</i> = 271) 0.43 (0.38-0.49)	ALF (<i>n</i> = 358) 0.51 (0.46-0.56)	0.054
APAP	No (<i>n</i> = 343) 0.51 (0.46-0.56)	Yes (<i>n</i> = 286) 0.43 (0.38-0.48)	0.026

Note: MPO-DNA complex levels are presented as mean estimates (95% confidence limits).

TABLE 4 Multivariable regression analyses on 21-day TFS for MPO-DNA complexes

	OR	Lower to Upper OR	<i>p</i> Value
MPO-DNA complexes (AU)	0.547	0.323-0.926	0.0246
HE grade 3-4 (on admission)	0.287	0.161-0.513	< 0.0001
High-risk etiology ^a	0.180	0.098-0.329	< 0.0001
Use of vasopressors	0.165	0.092-0.293	< 0.0001
Log_bilirubin	0.434	0.318-0.592	< 0.0001
Log_INR	0.296	0.171-0.510	< 0.0001

^aHigh-risk etiologies include autoimmune hepatitis, Budd-Chiari syndrome, drug-induced liver injury, hepatitis B (+/- delta), hepatitis C, hepatitis E, mushroom intoxication, Wilson's disease, indeterminate, other viruses, and other.

MPO-DNA complex levels were an independent predictor of TFS with an OR of 0.547 (0.323-0.926); the odds for a patient to reach 21-day TFS decreases ~45% for every unit increase in MPO-DNA complex levels. These results suggest that NETs contribute to progression of ALF in humans. Indeed, by histological examination of a small sample of liver tissue of patients with ALF within this cohort, we show that NET formation occurs in the liver parenchyma of these patients. However, the mechanism by which NET formation contributes to disease progression remains to be further explored. It is hypothesized that activation of coagulation by NETs results in the formation of (micro)thrombi that contribute to disease progression by inducing secondary ischemic injury. Although there is substantial

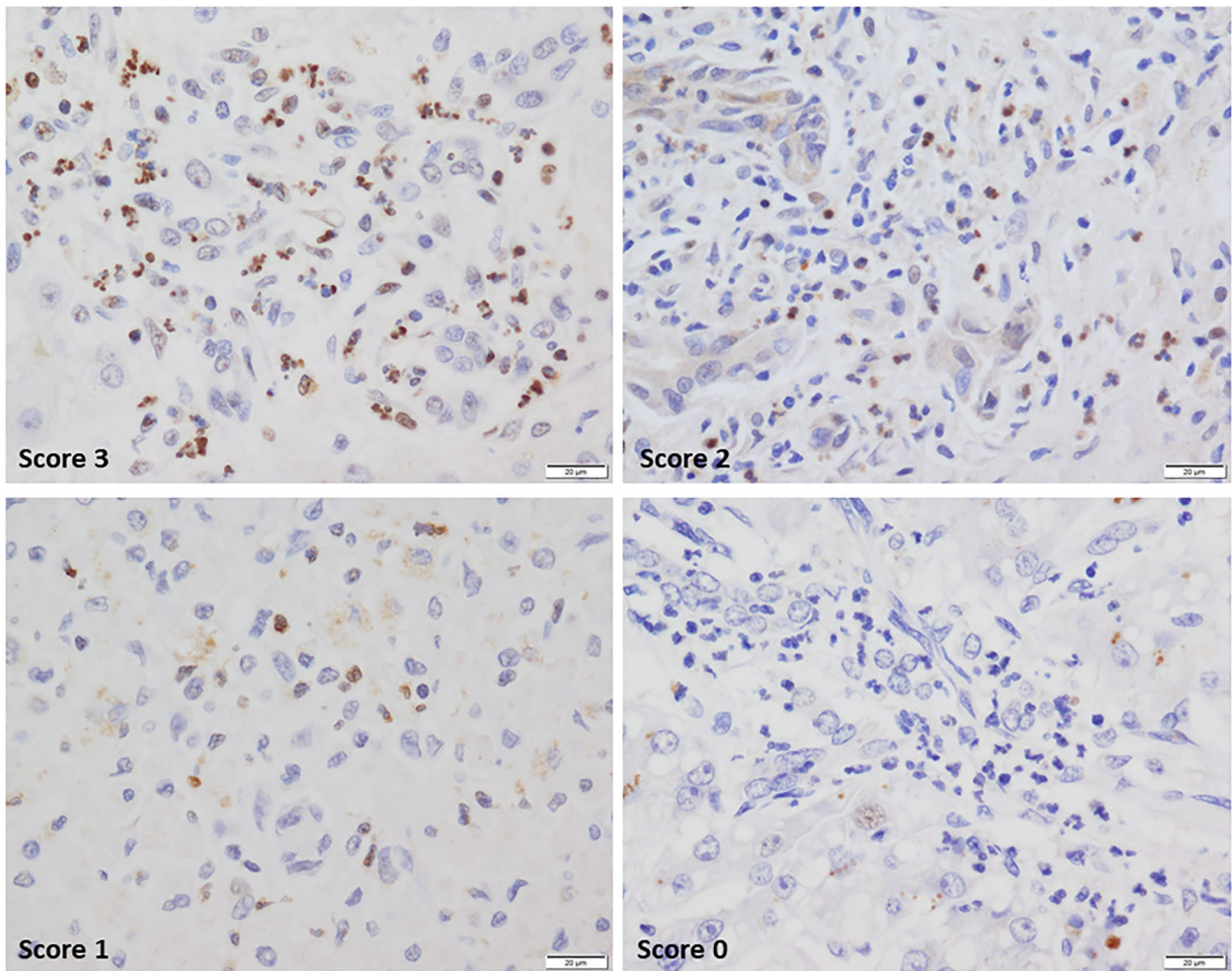


FIGURE 3 Liver tissue from ALF patients stained positive for NET marker. Representative images of liver tissue obtained from ALF patients with different degrees of citrullinated histone expression, which is a commonly used marker of NETs. Differences in expression, ranging from no expression (score 0), focal expression (positive in <10% of neutrophils; score 1), moderate expression (10%-50% of neutrophils; score 2), to extensive expression (>50% of neutrophils; score 3) are depicted

experimental evidence supporting this hypothesis,^[25,26] clinical evidence remains scarce. Interestingly, administration of DNase, which has been shown to dismantle NETs both in vitro and in vivo, reduced systemic inflammation, neutrophil migration, and liver injury in a mouse model of ALI.^[36] Hence, NETs might be explored as a therapeutic target that could potentially decrease the progression of severe ALI or ALF. DNA-degrading enzymes are in clinical use for patients with cystic fibrosis, and the vast experience in this context may facilitate studies of this drug in human ALF. Of note, NETs have an important function in the immune system, and NET-targeted therapy therefore requires careful evaluation of the risks (of infection) and benefits.

In conclusion, we demonstrate evidence for NET formation in patients with severe ALI or ALF. The NET marker, MPO-DNA complex, was an independent

predictor for TFS. The results of this study suggest that NETs contribute to progression of ALF, and that NETs may represent a viable therapeutic target in this difficult-to-treat population.

CONFLICT OF INTEREST

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Conception and design: Fien A. von Meijenfeldt, R. Todd Stravitz, and Ton Lisman. Data acquisition: R. Todd Stravitz, Jingwen Zhang, Yoh Zen, Valerie Durkalski, and William M. Lee. Laboratory analyses:

Jelle Adelmeijer, Fien A. von Meijenfeldt. Histological analyses: Yoh Zen. Analysis: Fien A. von Meijenfeldt, Jingwen Zhang, Valerie Durkalski, and Ton Lisman. Interpretation: Fien A. von Meijenfeldt, R. Todd Stravitz, Jingwen Zhang, Jelle Adelmeijer, Yoh Zen, Valerie Durkalski, William M. Lee, and Ton Lisman. Supervision: R. Todd Stravitz, Ton Lisman. Drafting of manuscript: Fien A. von Meijenfeldt, Ton Lisman. Revision of the manuscript: R. Todd Stravitz, Jingwen Zhang, Jelle Adelmeijer, Yoh Zen, Valerie Durkalski, and William M. Lee.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.
Fig S1

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