Elevated Plasma Levels of Cell-Free DNA During Liver Transplantation Are Associated With Activation of Coagulation

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Patients undergoing liver transplantation have complex changes in their hemostatic system, and the net effect of these changes appears to be a "rebalanced" hemostatic profile. Recently, a process called NETosis in which a neutrophil expels DNA and proteins that form a weblike structure, has been described as a mechanism of pathogen entrapment. Increasing evidence suggests a pivotal role for neutrophil extracellular traps (NETs) and their main component, cell-free DNA (cfDNA), in activation of coagulation. Because liver transplantation is associated with substantial (hepatocyte) cell death and intrahepatic neutrophil accumulation, NETs might play an important role in the hemostatic balance during liver transplantation. Here, we determined markers for NETs in the plasma of patients undergoing a liver transplantation and examined their association with activation of coagulation. Markers for NETs and markers for activation of coagulation were determined in serial plasma samples taken from patients undergoing a liver transplantation (n = 21) and compared with plasma levels in healthy controls. We found perioperative increases of markers for NETs with levels of cfDNA and nucleosomes that peaked after reperfusion and myeloperoxidase (MPO)–DNA complexes that peaked during the anhepatic phase. CfDNA and nucleosome levels, but not MPO-DNA levels, correlated with prothrombin fragment 1+2 and thrombin-antithrombin complex levels, which are established markers for activation of coagulation. Neutrophils undergoing NETosis were observed by immunostainings in postreperfusion biopsies. In conclusion, although NETosis occurs during liver transplantation, the majority of circulating DNA appears to be derived from cell death within the graft. The perioperative increases in cfDNA and nucleosomes might contribute to the complex hemostatic rebalance during liver transplantation.

Patients with end-stage liver disease have complex changes in their hemostatic system, which includes decreased platelet count and function, low plasma

Abbreviations: ALD, alcoholic liver disease; BSA, bovine serum albumin; cfDNA, cell-free DNA; DBD, donation after brain death; DCD, donation after circulatory death; DNase, deoxyribonuclease; EDTA, ethylene diamine tetraacetic acid; ELISA, enzyme-linked immunosorbent assay; F1+2, prothrombin fragment 1+2; INR, international normalized ratio; IRI, ischemia/reperfusion injury; MELD, Model for End-Stage Liver Disease; MPO, myeloperoxidase; NASH, nonalcoholic steatohepatitis; NE, neutrophil elastase; NET, neutrophil extracellular trap; PBS, phosphate-buffered saline; PTC, posttransplant cholangiopathy; TAT, thrombin/ antithrombin III complex; tPA, tissue plasminogen activator; Tris, trishydroxymethylaminomethane; UMCG, University Medical Center Groningen; VWF, von Willebrand factor.

levels of procoagulant and anticoagulant proteins, and a decrease in profibrinolytic and antifibrinolytic proteins.[1] During liver transplantation, due to consumption, hemodilution, and the lack of clearance and synthesis of hemostatic proteins in the anhepatic phase, the abnormalities in the hemostatic profile further increase. Previous studies by our group have described the following 4 major changes and their consequences for the hemostatic system during liver transplantation. First, the decreased platelet count and function seem to be compensated by highly elevated plasma levels of von Willebrand factor (VWF) that are found throughout the liver transplant procedure.[2] Moreover, low plasma levels of the VWF-cleaving protease ADAMTS13 may also compensate for the platelet deficit.[2,3] Second, the concomitant decrease in procoagulant and anticoagulant factors ensures intact thrombin

generation. Even though routine coagulation tests such as the prothrombin time test suggest a hypocoagulable state, state-of-the-art hemostatic tests show normal to enhanced thrombin generating capacity in samples taken during liver transplantation.[4] Third, fibrinogen concentrations are low during liver transplantation, leading to impaired clot structure and function.[5,6] Lastly, patients undergoing a liver transplantation may become hyperfibrinolytic during the anhepatic and reperfusion phases, which could be partly explained by the loss of tissue plasminogen activator (tPA) clearance during the anhepatic phase and tPA release from the graft after reperfusion.[7,8] The net effects of the hemostatic changes during liver transplantation appear to result in a "rebalanced" hemostasis. Clinical evidence for maintenance of a hemostatic balance, at least in a proportion of patients, is provided by reports on liver transplantation without the requirement for blood product transfusion, despite profoundly abnormal conventional tests of coagulation throughout surgery.[9]

Recent studies have described procoagulant properties of cell-free DNA (cfDNA) in vitro. CfDNA promotes thrombin generation in a dose-dependent manner and induces activation of coagulation via factor XI and XII of the intrinsic pathway of coagulation.[10-13] Furthermore, it has been shown that cfDNA impairs fibrinolysis via several mechanisms, including alterations in clot structure, inhibition of plasminogen activation, and inhibition of plasmin.[10,14] Notably, the in vitro procoagulant properties of cfDNA might be overestimated, as a recent study has demonstrated that silica particles in commercial kits used for the isolation of DNA are procoagulant and contribute variably to the procoagulant activity.[15]

CfDNA could originate from damaged, necrotic, or apoptotic cells or from a recently described process called NETosis, in which a neutrophil expels DNA filaments and proteins including elastase and myeloperoxidase (MPO). These constituents together form a weblike structure, referred to as neutrophil extracellular traps (NETs), which was first described as a mechanism of pathogen entrapment and clearance.[16] Clinical studies have shown that increased plasma levels of cfDNA and other (more specific) markers for NETs are found in patients with thrombotic diseases, such as deep vein thrombosis, myocardial infarction, and stroke, and it has been suggested that NETs form a risk factor for development of thrombotic disease.[17-19] Experimental animal models have established that cfDNA and NETs are important contributors to the formation of thrombi.[20,21] Thrombi contain cfDNA, often colocalized with VWF, histones, and other markers for NETs,[22,23] and occlusive thrombi can even be formed exclusively by NETs when endogenous mechanisms to clear NETs are impaired.[24] Administration of deoxyribonuclease (DNase) protected mice from developing deep vein thrombosis, strengthening the hypothesis that cfDNA contributes to thrombus formation.[22]

NETs have also been implicated in ischemia/reperfusion injury (IRI). It has been well established that neutrophils contribute to hepatic IRI. Interestingly, it has been demonstrated that platelet neutrophil crosstalk, which is key in the formation of NETs, contributes to liver IRI.[25] It may be that NETs drive IRI by supporting intrahepatic thrombus formation because inhibition of coagulation also decreases hepatic IRI.[26] The key role of neutrophils in IRI and the substantial cell death during liver transplantation support the hypothesis that cfDNA and NETs may contribute to the hemostatic balance during liver transplantation. In this study, we aimed to determine markers of NETs in serial plasma samples taken from patients undergoing a liver transplantation and to examine their association with activation of coagulation.

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Patients and Methods

STUDY POPULATION

Between December 2015 and April 2017, 10 adult patients undergoing a liver transplantation for cirrhosis at

the University Medical Center Groningen (UMCG) were included in this study. In addition, 11 adult patients undergoing a liver transplantation at the UMCG between September 2012 and December 2015 were included in this study, but from these 11 patients only intraoperative blood samples were taken. All of these patients gave informed consent. The medical ethical committee of UMCG approved the study protocol. Exclusion criteria for this study were acute liver failure, known hereditary thrombophilia, the use of vitamin K antagonists, blood transfusion up to 7 days prior to transplantation, and deep vein thrombosis within 30 days prior to the transplantation. Twenty healthy volunteers recruited at the UMCG served as controls to establish the reference values for the various tests performed. Exclusion criteria for the controls were known hereditary thrombophilia, the use of vitamin K antagonists, blood transfusion up to 7 days prior to transplantation, and a history of deep vein thrombosis. The medical ethical committee of UMCG approved the study protocol.

BLOOD SAMPLES

Blood samples were collected at the following time points: 30 minutes after induction of anesthesia; 30 minutes after the start of the anhepatic phase; 30 minutes after portal reperfusion; at the end of surgery; and (for 10 of the 21 patients) at postoperative days 1, 3, and 6. During liver transplantation, blood was drawn from a dedicated nonheparinized arterial line into a 3.2% sodium citrate (9:1 vol/vol) tube. Postoperatively, blood was drawn via venipuncture. Within 2 hours after the blood draw, the sample was centrifuged at 2000g and 10,000g, respectively, for 10 minutes at ambient temperature. Plasma was stored at −80°C until it was used for analyses.

ASSESSMENT OF FREE DNA AND COAGULATION ACTIVATION

The concentration of cfDNA was measured using the Quant-iT PicoGreen double-stranded DNA assay kit (Fisher Scientific, Landsmeer, the Netherlands). In short, 10 μL of plasma was added to 90 μL of trishydroxymethylaminomethane (Tris)–ethylene diamine tetraacetic acid (EDTA) 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 Tris-EDTA buffer) was added to the wells. After 10 minutes of incubation in the dark at room temperature, fluorescence was measured

at 480-nm excitation and 520-nm emission using a Victor (PerkinElmer, Groningen, the Netherlands) photometer. The concentration of nucleosomes in the plasma samples was determined using the cell death detection enzyme-linked immunosorbent assay (ELISA; Sigma-Aldrich, Zwijndrecht, the Netherlands). The test was performed according to the manufacturer's instructions. Plasma levels of MPO-DNA complexes, which are a selective marker for NETs, were quantified by ELISA as previously described,[27] using a commercially available monoclonal antibody to MPO (Sanbio, Uden, the Netherlands) and the detection antibody derived from the cell death detection ELISA (Sigma-Aldrich, Zwijndrecht, the Netherlands). To assess the activation of coagulation during liver transplantation, the concentration of thrombin/antithrombin III complex (TAT) and prothrombin fragment 1+2 (F1+2), which are established markers for in vivo thrombin generation, were determined. Quantification of TAT and F1+2 was done with the use of Enzygnost TAT micro (Siemens Healthcare Diagnostics, the Hague, the Netherlands) and the Enzygnost F1+2 ELISA kit (Siemens Healthcare Diagnostics), respectively. Both ELISAs were carried out according to the manufacturers' protocol.

IMMUNOHISTOCHEMICAL ANALYSES OF NET FORMATION

Frozen sections (4 μM) of 5 postreperfusion human liver needle biopsies, which were randomly taken from a series of biopsies collected and described previously,[28] were generated with a Reichert-Jung 2055 microtome (Leica, Wetzlar, Germany) and immunostained for the neutrophil marker CD66b and for neutrophil elastase (NE). Immunostaining was performed using the CD66b antibody (Ab197678, Abcam, Cambridge, United Kingdom) and the NE antibody (NP57, BioConnect, Huissen, the Netherlands). Shortly, slides were fixed with acetone for 10 minutes and, thereafter, blocked with endogenous peroxidase and alkaline phosphatase solution for 10 minutes (BLOXALL, Vector laboratories, Peterborough, United Kingdom). After washing with phosphate-buffered saline (PBS)/0.1% Tween 20, slides were blocked with 10% goat serum for CD66b and 10% normal horse serum for NE (both diluted in 1% bovine serum albumin [BSA] in PBS). Slides were incubated overnight at 4°C with a rabbit anti-human CD66b antibody (diluted 1:100 in 1% BSA/PBS solution) or with a mouse anti-human NE antibody (diluted 1:100 in 1% BSA/PBS solution). Slides

were blocked using the commercial avidin/biotin block (X0590, Dako, Glostrup, Denmark) for 20 minutes. Next, an anti-rabbit-biotinylated (diluted 1:100 in 1% BSA/PBS solution) or an anti-mouse-biotinylated antibody (diluted 1:100 in 1% BSA/PBS solution) was added for 30 minutes, followed by an ABC-AP kit and Vector RED substrate for 30 and 20 minutes, respectively. The latter and the secondary antibodies were purchased from Vector Laboratories, Peterborough, United Kingdom. Lastly, sections were counterstained with hematoxylin. Stained slides were scanned with the use of the Hamamatsu nanozoomer (Almere, the Netherlands).

STATISTICAL ANALYSES

Statistical analyses were performed using SPSS statistics, version 23 (IBM Inc., Chicago, IL) and GraphPad Prism (GraphPad Software, Inc., San Diego, CA). The results were presented as medians and interquartile ranges for continuous variables and as percentages for categorical variables. To compare patient values with baseline values (control group), we used 1-way analysis of variance or the Kruskal-Wallis H test as appropriate. Post hoc analyses were performed using the Dunnett's or Dunn's posttest, as appropriate. Correlations between the different measurements were made using the Pearson correlation coefficient analysis. Differences between test results of patients who did or did not develop complications were assessed by student t test or Mann-Whitney U test as appropriate. A P value <0.05 was considered significant.

Results

PATIENT CHARACTERISTICS

We studied levels of DNA and markers for activation of coagulation in the plasma of 21 patients undergoing liver transplantation at the UMCG and compared these levels with plasma concentrations in 20 healthy controls. The baseline characteristics of the patients and the transplant procedures are summarized in Table 1.

DNA LEVELS IN SYSTEMIC CIRCULATION DURING LIVER TRANSPLANTATION

We quantified plasma levels of total cfDNA, nucleosomes, and MPO-DNA complexes in samples taken during and after liver transplantation (Fig. 1). Plasma

TABLE 1. Patient Characteristics and Procedural Variables

	Values (n = 21)
Recipients	
Age, years	58.0 (42.5-63.0)
Sex, male	42.9
Indication for liver transplantation, n	
ALD*	5
Alpha-1-antitrypsin deficiency	1
Cryptogenic	4
Glycogen storage disease type 1A	1
NASH	3
Other†	2
Primary biliary cirrhosis	1
Primary sclerosing cholangitis	3
Wilson's disease	1
Body mass index, kg/m^2	25.8 (23.3-29.6)
Ascites	62.0
Hepatic encephalopathy (mild)	43.0
Preoperative laboratory values	
MELD score	16.0 (8.1-23.5)
Platelet count, 10^9/L	100 (69.0-147)
Fibrinogen, mg/mL	2.4 (1.9-4.1)
INR	1.3 (1.1-1.6)
Prothrombin time, seconds	13.3 (12.0-15.7)
Procedural variables	
Type of donor (DBD:DCD)	57.1:42.9
Cold ischemia time, minutes‡	405 (378-427)
First warm ischemia time, minutes§	14.0 (10.0-17.0)
Second warm ischemia time, minutes‖	40.0 (35.0-47.0)

NOTE: The results are presented as medians (interquartile ranges) for continuous variables and percentages for categorical variables unless otherwise noted.
*Indication for liver transplantation was ALD combined with hepatitis C for 1 patient.
†Other indications for liver transplantation were primary hyperoxaluria type 1, secondary biliary cirrhosis.
‡Cold ischemia time is defined as the time from aortic (cold) flush in the donor until (portal) reperfusion in the recipient.
§First warm ischemia time is defined as the time from circulatory arrest until aortic (cold) flush in the donor, thus only in DCD (n = 9).
‖Second warm ischemia time is defined as the time from when the liver is taken out of the ice until the blood flow to the liver is restored in the recipient.

levels of cfDNA were comparable between controls and samples taken at the start of the liver transplantation and during the anhepatic phase. The concentration of cfDNA increased significantly during liver transplantation with the highest levels observed at the end of surgery. Postoperatively, cfDNA levels rapidly decreased to levels comparable to controls.
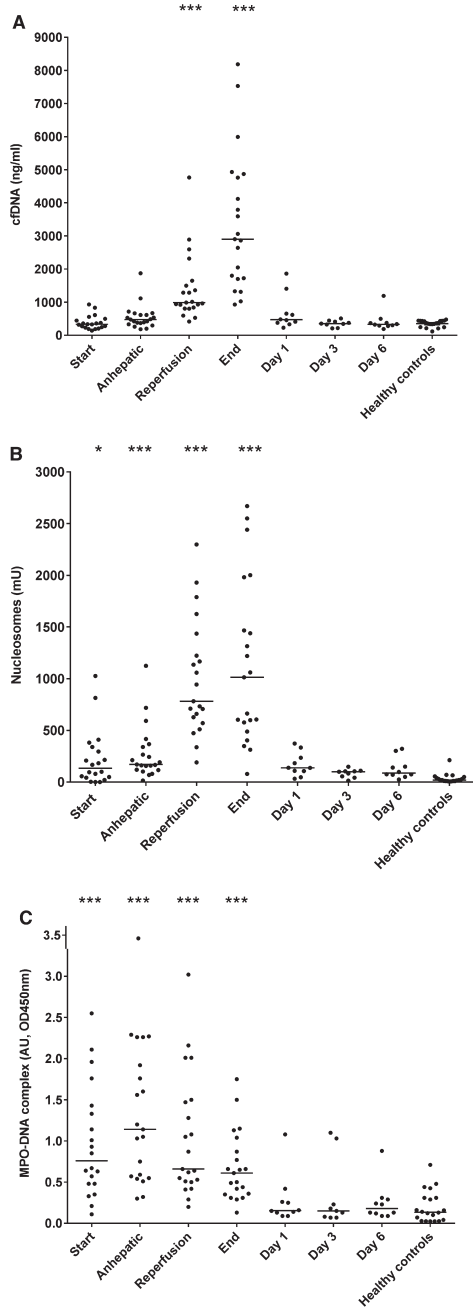
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FIG. 1. Plasma levels of DNA in patients during and after liver transplantation. Levels of (A) cfDNA, (B) nucleosomes, and (C) MPO-DNA complexes were determined in plasma samples using ELISAs. Samples were taken at 4 time points during liver transplantation (n = 21) and at postoperative day 1, 3, and 6 (n = 10). Horizontal lines represent medians. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ compared with controls.

Plasma nucleosome levels were slightly higher in patients at the start of surgery compared with controls.

Nucleosome levels increased during liver transplantation with substantially elevated levels after reperfusion and at the end of surgery. On postoperative day 1, nucleosome plasma levels returned to baseline levels and remained so up until day 6.

Plasma levels of MPO-DNA complexes were higher in patients at the start of surgery compared with controls. Levels slightly increased during the anhepatic phase and decreased thereafter. From postoperative day 1 onward, levels were similar to those found in controls.

In addition, we compared postreperfusion plasma levels of total cfDNA, nucleosomes, and MPO-DNA complexes in recipients receiving a liver from donation after brain death (DBD) with recipients of a liver from donation after circulatory death (DCD). Plasma levels of all markers were similar between DBD and DCD liver recipients (data not shown).

ACTIVATION OF COAGULATION DURING LIVER TRANSPLANTATION

Activation of coagulation during liver transplantation was assessed by plasma levels of TAT and F1+2 (Fig. 2). At the start of surgery, TAT and F1+2 levels were comparable to levels found in the controls. The plasma concentration of TAT and F1+2 increased during transplantation with peak values observed after reperfusion. On postoperative day 1, TAT and F1+2 levels returned to baseline levels. On postoperative day 3, both TAT and F1+2 levels were slightly higher compared with controls. F1+2 levels remained elevated up until postoperative day 6.

ARE INCREASED PLASMA LEVELS OF CIRCULATING DNA ASSOCIATED WITH COAGULATION ACTIVATION?

Multiple studies have demonstrated procoagulant properties of circulating DNA.[10-12] To test whether plasma levels of circulating DNA are associated with the activation of coagulation in patients undergoing a liver transplantation, we studied correlations between markers for activation of coagulation and circulating DNA levels. Scatterplots and the associated correlation coefficients of the different analyses are presented in Fig. 3. Significant correlations were found between concentrations of cfDNA and TAT
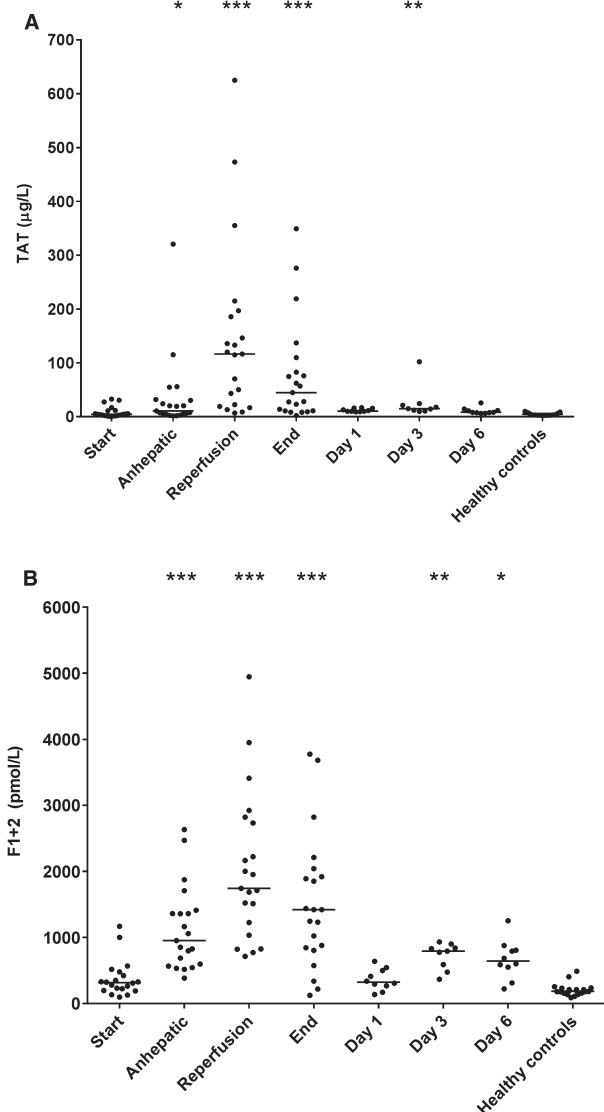
FIG. 2. Plasma levels of markers for activation of coagulation during and after liver transplantation. Levels of (A) TAT and (B) F1+2 were determined in plasma samples using ELISAs. Samples were taken at 4 time points during liver transplantation (n = 21) and at postoperative day 1, 3, and 6 (n = 10). Horizontal lines represent medians. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ compared with controls.

(Fig. 3A) and also cfDNA and F1+2 (Fig. 3B). In accordance with these results, the concentrations of TAT and F1+2 were significantly associated with the concentrations of nucleosomes in a patient's plasma, as shown in Fig. 3C,D. In contrast, we did not observe an association between MPO-DNA complex concentrations and the concentrations of TAT and F1+2 in liver transplant patients (Fig. 3E,F).

LACK OF ASSOCIATION BETWEEN DURATION OF WARM ISCHEMIA OF THE LIVER AND PEAK PLASMA LEVELS OF CIRCULATING DNA

Warm ischemia has detrimental effects on the liver graft and is kept as short as possible by surgeons performing liver transplantations. We assessed the association between duration of warm ischemia and peak plasma concentrations of cfDNA, nucleosomes, and MPO-DNA complexes. We did not observe an association between the duration of warm ischemia and the peak plasma concentrations of DNA during liver transplantation (Supporting Fig. 1).

POSTTRANSPLANT CHOLANGIOPATHY AND DNA PLASMA LEVELS DURING LIVER TRANSPLANTATION

Of the 21 patients, 4 developed posttransplant cholangiopathy (PTC) according to our previously published definition.[29] Plasma levels of cfDNA, nucleosomes, and MPO-DNA complexes during liver transplantation were compared between patients who developed PTC and patients who did not. No differences in plasma levels were observed between the 2 groups (Supporting Fig. 2).

INTRAHEPATIC FORMATION OF NETS

Five human postreperfusion liver needle biopsies were stained for the established neutrophil marker CD66b and for NE in order to assess intrahepatic NET formation during liver transplantation. As shown in Fig. 4 in images from a single patient, neutrophils appear to have expelled NE, which is present outside of the neutrophil in weblike structures. These findings, which we consistently observed in the 5 biopsies examined, are suggestive of postreperfusion NET formation during liver transplantation.

Discussion

In this study, substantially elevated levels of markers for NETs in plasma samples of patients undergoing a liver transplantation were found. Levels of cfDNA and nucleosomes were especially high after reperfusion, and
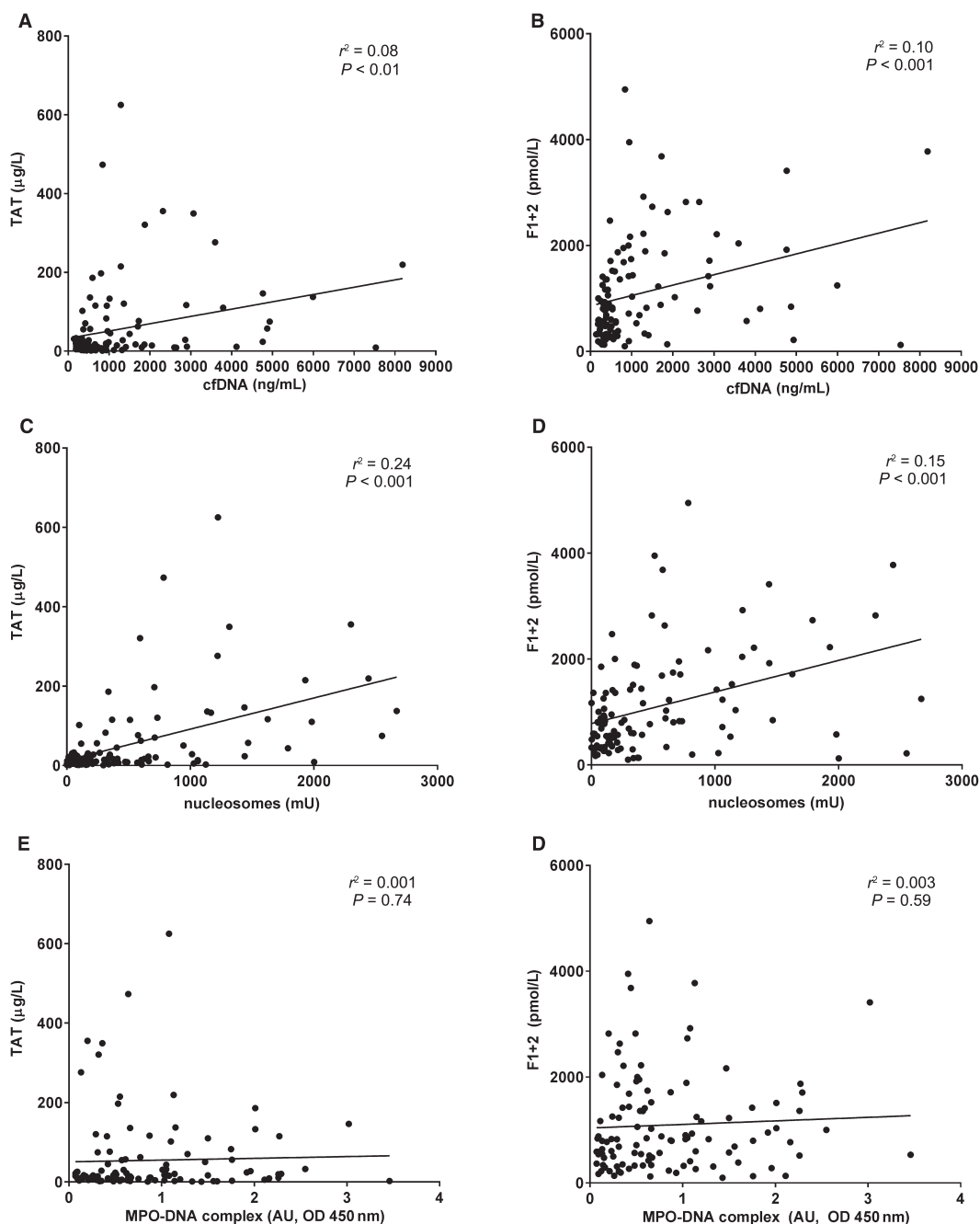
FIG. 3. Correlations between levels of DNA and markers for activation of coagulation during and after liver transplantation. Scatterplots displaying the correlations between levels of (A and B) cfDNA, (C and D) nucleosomes, and (E and F) MPO-DNA complexes and markers of activation of coagulation (TAT and F1+2) in serial plasma samples taken from patients undergoing liver transplantation. Correlation coefficients with corresponding *P* values are indicated.

they were correlated with markers for activation of coagulation. These results are in line with studies that have established the activation of coagulation by cfDNA in vitro[10-12,14] and suggest that cfDNA and NETs could contribute to the hemostatic rebalance during liver transplantation by activation of coagulation.

Plasma levels of cfDNA, nucleosomes, and MPO-DNA complexes were higher over the entire course of
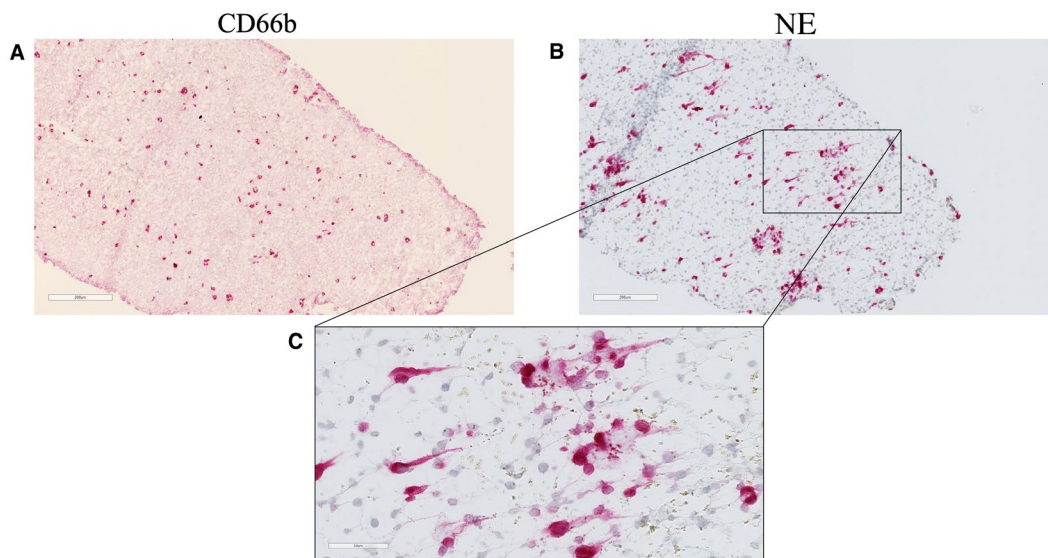
FIG. 4. Immunohistochemical analyses of human liver biopsies suggest intrahepatic formation of NETs. Representative image of a human postreperfusion liver needle biopsy stained for (A) CD66b and (B and C) NE. Scale bars indicate (A and B) 200 μm and (C) 50 μm.

liver transplantation compared with healthy controls and decreased toward levels found in healthy controls postoperatively. Levels of cfDNA and nucleosomes were especially high after reperfusion, which is thought to be the most damaging phase of transplantation, and is associated with substantial (hepatocellular) cell death. Plasma levels of MPO-DNA complexes, the most specific marker for NETs, were already substantially elevated at the start of surgery, which is in line with recent findings of elevated levels of platelet-neutrophil complexes in cirrhosis.[30] Whether activated neutrophils in cirrhosis actually undergo NETosis has to our knowledge not yet been established, but the demonstration of NETosis in specific liver pathologies such as nonalcoholic fatty liver disease[31] certainly suggest this to be a plausible scenario. MPO-DNA complexes peaked in the anhepatic phase, suggesting additional NETosis as a result of the transplant procedure. However, an alternative explanation for increased MPO-DNA levels at this time point is the decreased clearance of proteins by the liver in the anhepatic phase. Plasma levels of cfDNA and nucleosomes, and not MPO-DNA complexes, peaked after reperfusion, and cfDNA and nucleosome levels but not MPO-DNA levels correlated with plasma markers of activation of coagulation. These results suggest that the majority of cfDNA in patients undergoing

liver transplantation originates from cell damage and necrosis rather than from NETosis.

Several studies have described procoagulant properties of cfDNA in vitro. It has been shown that cfDNA induced activation of coagulation via factor XI and XII of the intrinsic pathway of coagulation, promoted thrombin generation, and impaired fibrinolysis via multiple mechanisms including inhibition of plasminogen activation, and inhibition of plasmin.[10-12,14] Recent studies have warranted careful interpretation of in vitro studies on the coagulation-activating properties of cfDNA. Specifically, studies have shown that the kits used for the isolation of DNA contain silica particles that can activate coagulation and that a substantial part of the procoagulant activity of isolated DNA preparation could be attributed to these silica particles. It has been recommended to study procoagulant activity of cfDNA with the use of different DNA-isolation techniques.[15,32] Our results suggest that the high levels of circulating cfDNA during liver transplantation contribute to in vivo activation of coagulation, which is in line with clinical studies that have described the association between elevated plasma levels of cfDNA (and nucleosomes) and thrombotic diseases.[17]

Although NETs do not seem to contribute to (systemic) activation of coagulation during liver

transplantation, it could be that the intrahepatic formation of NETs we have demonstrated contributes to local activation of coagulation. Because neutrophils, platelets, and activation of coagulation have been shown to be involved in the progression of hepatic IRI[25,26,33,34] and because renal IRI was diminished by DNase treatment,[35] it appears plausible that NET-mediated intrahepatic activation of coagulation would offer a unified mechanism explaining detrimental effects of platelets, coagulation, and neutrophils on hepatic IRI. Although we did not find an association between levels of our markers assessed and warm ischemia times or PTC, our study is obviously limited by small numbers.

The complex hemostatic changes during liver transplantation result in a hemostatic rebalance, as evidenced by clinical observations and laboratory studies.[4,7,9] The results of this study suggest that NETs and cfDNA might be a hitherto unrecognized player in this rebalance by facilitating activation of coagulation. However, this mechanism could also be prothrombotic and contribute to perioperative complications of liver transplantation.[36] In addition, cfDNA or intrahepatic formation of NETs in the liver might lead to local activation of coagulation and, consequently, the formation of (micro)thrombi in the liver microvasculature, thus aggravating hepatic IRI.

To minimize postoperative thrombotic complications, all patients who undergo a liver transplantation in our center receive venous thrombosis prophylaxis postoperatively, generally using the low-molecular-weight heparin Nadroparin (2850 IE daily, regardless of the patient's prothrombin time). Exceptions are patients with thrombocytopenia (<20 × 10⁹/L), patients with bleeding complications, or patients with preexisting bleeding disorders such as hemophilia. Indications for therapeutic dosages of low-molecular-weight heparin (5700-7600 IE twice-daily) include preexisting portal vein thrombosis, Budd-Chiari syndrome, metabolic disorders such a primary hyperoxaluria and familial amyloidotic polyneuropathy, intraoperative or early postoperative hepatic artery thrombosis requiring thrombectomy, and extensive arterial reconstructions or an arterial conduit. Patients who have increased formation of NETs and cfDNA, with accompanying increasing activation of coagulation, might also be at increased risk for thrombotic complications and may, therefore, also benefit from more aggressive (therapeutic) antithrombotic strategies. However, clinical studies

will be required to ascertain this. Interestingly, it has been suggested that direct targeting of NETs by DNA-degrading enzymes might be a safe and effective antithrombotic strategy in patients with thrombotic complications related to NET formation,[37] but although DNA degrading enzymes are clinically available for treatment of patients with cystic fibrosis, they have not yet been used in the context of thrombosis. The advantage of this strategy over traditional anticoagulants would be that they could be used already intraoperatively without a risk for excessive bleeding.

In conclusion, we have shown that increased plasma levels of markers for NETs are found in plasma of patients undergoing liver transplantation. The levels of cfDNA and nucleosomes were associated with activation of coagulation. These findings indicate that cfDNA and NETs might contribute to the complex reset of the hemostatic balance during liver transplantation by activation of coagulation. Whether circulating DNA or local NET formation contribute to IRI or thrombotic complications of liver disease and may be a novel therapeutic target should be assessed in future studies.

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