Preserved hemostatic status in patients with non-alcoholic fatty liver disease

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Abstract

Background & Aims: Non-alcoholic fatty liver disease (NAFLD) is associated with an increased risk of thrombosis. However, it remains unclear if hypercoagulability contributes to this risk. We, therefore, determined an in-depth hemostatic profile in a cohort of well-defined patients with NAFLD.

Methods: We drew blood samples from 68 patients with biopsy-proven NAFLD (simple steatosis n = 24, NASH n = 22, and NASH cirrhosis n = 22), 30 lean controls, 30 overweight controls (body mass index (BMI) >25 kg/m2 ), and 15 patients with alcoholic (ASH) cirrhosis, and performed in-depth hemostatic profiling.

Results: Basal and agonist-induced platelet activation, plasma levels of markers of platelet activation, and plasma levels of the platelet adhesion regulators von Willebrand factor and ADAMTS13 were comparable between patients with non-cirrhotic NAFLD and controls. Agonist-induced platelet activation was decreased in patients with cirrhosis. Thrombomodulin-modified thrombin generation was comparable between all patients and controls, although patients with cirrhosis had a reduced anticoagulant response to thrombomodulin. Thromboelastography test results were comparable between controls and non-cirrhotic NAFLD patients, but revealed moderate hypocoagulability in cirrhosis. Plasma fibrinolytic potential was decreased in overweight controls and non-cirrhotic NAFLD, but accelerated fibrinolysis was observed in ASH cirrhosis. Clot permeability was decreased in overweight controls and patients with NAFLD.

Conclusions: The overall hemostatic profile is comparable between patients with non-cirrhotic NAFLD and controls. Additionally, pro-thrombotic features (hypofibrinolysis and a prothrombotic structure of fibrin clot) in patients with NAFLD are likely driven by obesity. Our study suggests a limited role for hyperactive hemostasis in the increased thrombotic risk in NAFLD.
Graphical abstract
Introduction
Non-alcoholic fatty liver disease (NAFLD) is associated with an increased risk of cardiovascular disease (CVD). Increasing evidence suggests that the higher incidence of cardiovascular disease (CVD) morbidity and mortality in patients with NAFLD is independent of conventional cardiometabolic risk factors (such as obesity, insulin resistance, and diabetes mellitus) [1–3]. However, the exact mechanisms linking NAFLD to increased risk of CVD are incompletely understood and likely reflect multiple coexisting pathways [3]. Furthermore, rates of venous thromboembolism (VTE) and portal vein thrombosis (PVT) appear also increased in patients with NAFLD [4,5]. Recent studies have suggested a role for a hypercoagulable state in the increased risk of thrombosis in patients with NAFLD. Increased plasma levels of various pro-thrombotic factors (e.g., fibrinogen, factor VIII, and plasminogen activator inhibitor 1 (PAI-1)) have been described in patients with NAFLD [6–9]. Furthermore, studies have shown hypercoagulable features in patients with NAFLD detected with either thromboelastography (TEG) [10] or thrombin generation testing [11]. Platelet hyperactivity has also been implicated as a contributor of the increased risk of cardiovascular disease in patients with the metabolic syndrome [12–14], but its role in NAFLD remains unclear [3].

Nevertheless, results on the hemostatic status in NAFLD are inconsistent [3]. Furthermore, most studies have reported plasma levels of individual hemostatic proteins rather than functional tests of hemostasis. In addition, no study has profiled all components of the hemostatic system simultaneously, and patients with cirrhosis were excluded from most of these studies. The hemostatic status across the spectrum of NAFLD stages thus remains unclear. Whether the hemostatic status might explain the increased risk of thrombosis in these patients also remains to be firmly established. We, therefore, determined an in-depth hemostatic profile by performing functional hemostatic tests of platelets, coagulation, fibrinolysis, and fibrin clot structure in a cohort of well-defined patients with NAFLD. Furthermore, we compared the hemostatic status of patients with NASH-related cirrhosis to that of patients with alcoholic (ASH) related cirrhosis. This is the first study that comprehensively investigated all components of the hemostatic system (i.e., platelets, coagulation, and fibrinolysis) using both biomarkers and functional tests in patients with various histological severities of NAFLD.

Patients and methods

Patients
All subjects (healthy controls, patients with various severity of NAFLD, and patients with ASH cirrhosis) were enrolled through the NASH Clinic at the Virginia Commonwealth University (Richmond, VA). The study protocol was IRB approved and written informed consent was obtained from each subject before inclusion in the study. NAFLD was defined by the evidence of hepatic steatosis on liver biopsy and the absence of causes for secondary hepatic fat accumulation (such as significant alcohol consumption) [15]. The liver biopsy was graded according to the NASH Clinical Research Network (NASH-CRN) scoring system, and the NAFLD activity score (NAS) was based on the unweighted sum of steatosis, lobular inflammation, and hepatocellular ballooning scores [16]. The NAFLD cohort was further subdivided into patients with simple hepatic steatosis (defined as the presence of hepatic steatosis on liver biopsy and the absence of causes for secondary hepatic fat accumulation (such as significant alcohol consumption) [15]. The liver biopsy was graded according to the NASH Clinical Research Network (NASH-CRN) scoring system, and the NAFLD activity score (NAS) was based on the unweighted sum of steatosis, lobular inflammation, and hepatocellular ballooning scores [16]. The NAFLD cohort was further subdivided into patients with simple hepatic steatosis (defined as the presence of hepatic steatosis with no evidence of hepatocellular injury (ballooning), n = 24), patients with non-alcoholic steatohepatitis (NASH; defined as the presence of hepatic steatosis and inflammation with hepatocyte injury with or without fibrosis, n = 22), and patients with NASH-related cirrhosis (defined as the presence of...
cirrhosis with current or previous histological evidence of steatosis or steatohepatitis, n = 22) [15]. Two control groups of lean (BMI <25 kg/m2; n = 30) and overweight (BMI >25 kg/m2; n = 30) subjects with no evidence of chronic liver disease were included to establish reference values for the various tests performed. The absence of liver disease was established by normal liver enzymes and a normal liver sonogram. Furthermore, fifteen patients with alcoholic (ASH) cirrhosis were included as a control group for the patients with NASH-related cirrhosis. Exclusion criteria were documented history of congenital coagulation disorders, presence of active infection (<2 weeks), use of anticoagulant or anti-platelet drugs, pregnancy, human immunodeficiency virus positivity, and recent (<7 days) transfusion with blood products.

Figure 1. Schematic representation of the hemostatic system. Upon vessel wall injury, subendothelial collagen is exposed (A). Plasma VWF, which is a globular protein in circulation, unwinds over the collagen surface and becomes adhesive to platelets. The multimeric size of VWF is regulated by the protease ADAMTS13. Platelets lose velocity by rolling over collagen-bound VWF (B), and eventually stably adhere by multiple receptors (C). Platelets become activated by multiple triggers including collagen and thrombin, which results in a conformational change in αIIbβ3. This conformational change allows platelet-platelet interactions mediated by VWF and fibrinogen. During platelet activation, proteins within platelet α-granules (among which PF4) are excreted. In addition, the α-granule transmembrane protein P-selectin is translocated to the outer membrane during α-granule secretion. Part of this P-selectin is shed from the platelet surface and soluble P-selectin can be found in plasma. Simultaneously, vessel wall injury exposes tissue factor (TF), which initiates coagulation (D, left section). Thrombin (coagulation factor IIa) is the terminal enzyme of the coagulation system, and is responsible for cleavage of fibrinogen to fibrin. Thrombin generation is the net result of the work of pro- and anticoagulant systems. The anticoagulant systems are indicated by the interrupted line. The fibrin clot is degraded by the fibrinolytic system, which is activated by release of tPA from endothelial cells, resulting in conversion of plasminogen to plasmin (D, right section). Multiple regulatory steps in fibrinolysis are depicted with interrupted lines. Importantly, thrombin not only cleaves fibrinogen to fibrin, but also activates two major inhibitors of fibrinolysis (FXIII and thrombin activatable fibrinolysis inhibitor; TAFI).
**Blood samples**

Blood was drawn by venapuncture in 3.8% citrate tubes. A sample was processed directly for flow cytometry and thromboelastography. The remainder of the blood was processed to platelet-poor plasma (PPP) by double centrifugation at 2000g and 10,000g respectively for 10 min. Plasma was snap-frozen and stored at -80°C until use.

Figure 1 shows an overview of hemostasis and all tests performed are indicated in this schematic.

**Platelet activation status**

The platelet activation status was assessed using flow cytometry in whole blood. Details of sample processing have been described previously [17]. Platelets were kept in a resting state or activated by either adenosine diphosphate (ADP, 15 μM, Sigma-Aldrich, St. Louis, USA) or thrombin receptor activating peptide (TRAP6, 15 μM, Bachem, Bubendorf, Switzerland). Samples were analyzed within six hours after processing using a BD LSRFortessa™ X-20 cell analyzer (BD Biosciences, Franklin Lakes, NJ, USA). Samples were gated on the basis of their forward and sideward scatter properties. The percentage of platelets expressing P-selectin and the geometric mean fluorescence intensity (MFI) of the platelet population were recorded. The percentage of platelets positive for P-selectin after activation was corrected for the percentage of platelets positive for P-selectin in the non-activated sample of each patient and control. The increase in the percentage of P-selectin positive platelets obtained in this manner represents the extent to which platelets in a given sample can be activated by a given activator (i.e., the platelet activatability).

**Thromboelastography**

Thromboelastography (TEG) was performed using the Thrombelastograph Hemostasis Analyzer 5000 (Haemonetics Corp., Haemoscope Division, Niles, IL, USA) as described previously [18]. Briefly, 1 ml of citrated whole blood (3.2% citrate) was subjected to kaolin-activated TEG within two hours of the blood draw. Kinetic changes in clot formation and clot dissolution were measured for 30 minutes after reaching maximal clot firmness. The following parameters were recorded: reaction time (R-time: the time taken for a clot to begin forming), kinetics time (K-time: the time from initial clot formation required to reach a specific clot firmness, α-Angle (the kinetics of clot formation), Maximum amplitude (MA: the maximal clot strength), and lysis at 30 minutes (Lysis-30 the percentage of clot lysis 30 minutes after MA is established).

**Thrombin generation, PT, APTT**

Thrombin generation testing was performed in platelet-poor plasma (PPP) with the fluorimetric method described by Hemker, Calibrated Automated Thrombography® (CAT) [19]. Reagents and protocols were purchased from Thrombinoscope BV, Maastricht, the Netherlands. Details of thrombin generation testing have been described previously [20]. The following parameters were recorded: endogenous thrombin potential (ETP; which represents the total enzymatic work performed by thrombin during the time that it was active), peak, velocity index (slope between the end of lag time and peak thrombin), and lag time (time needed for thrombin concentration to reach 1/6th of the peak concentration). In addition, a normalized thrombomodulin sensitivity ratio (TM-SR) was determined by dividing the ETP in the presence
of TM divided by the ETP in the absence of TM of an individual, by the ETP in the presence of TM divided by the ETP in the absence of TM of pooled normal plasma. A TM-SR > 1 reflects a decreased anticoagulant response to TM in comparison to pooled normal plasma.

The prothrombin time (PT) and activated partial thrombin time (APTT) were assessed on an automated coagulation analyzer (ACL 300 TOP) with reagents (Recombiplastin 2G for PT and SynthaSil for APTT) and protocols from the manufacturer (Instrumentation Laboratory, Breda, the Netherlands).

**Clot lysis**

Lysis of a tissue factor-induced clot by exogenous tPA was studied by monitoring changes in turbidity during clot formation and subsequent lysis as described previously [21]. Clot lysis time was determined as the time from the midpoint of the clear to maximum turbid transition, which characterizes clot formation, to the midpoint of the maximum turbid to clear transition, which represents clot lysis.

**Fibrin structure**

The average pore size of the fibrin clot (expressed as the Darcy constant Ks) was determined in permeation studies as described previously [22,23].

Fibrin density was assessed by laser-scanning confocal microscopy of clots supplemented with fluorescently labeled fibrinogen. Plasma clots were generated as described previously [24]. Clots were visualized with a Leica TCS SP8 confocal laser scanning microscope using a 63X/1.40 NA oil objective (Leica Microsystems, Eindhoven, The Netherlands). Alexa Fluor-488 fibrinogen was excited at 488 nm with an argon laser. Optical sections were taken at three randomly chosen areas throughout the clot to visualize the fibrin network. Fiber density was determined by counting the number of fibers crossing 100 µm lines placed in the image using the Image J plug-in grid (Fiji, National Institute of Health, Bethesda, Maryland, USA).

Fibrinogen was isolated from plasma samples as described previously [25]. Carbonylation of purified fibrinogen samples was quantified using a commercially available ELISA kit (Enzo Life Sciences, Farmingdale, NY) following the manufacturer’s instructions.

**Plasma markers of primary hemostasis**

Soluble P-selectin (sP-selectin) and platelet factor 4 (PF4) levels were determined using a commercially available sP-selectin or PF4 enzyme-linked immunosorbent assay (ELISA) (R&D Systems, Abingdon, UK). Levels of von Willebrand Factor antigen (VWF) were assessed with an in-house ELISA using commercially available polyclonal antibodies against VWF (DAKO, Glostrup, Denmark). Plasma a disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13 (ADAMTS13) activity was measured using the FRETS-VWF73 assay (Peptanova, Sandhausen, Germany). Levels of VWF and ADAMTS13 in pooled normal plasma were set at 100%, and values obtained in test plasmas were expressed as a percentage of pooled normal plasma.
Plasma markers of coagulation
Levels of fibrinogen, factor (F) VII, FVIII, antithrombin, protein C, and D-dimer were assessed on an automated coagulation analyzer (ACL 300 TOP) with reagents (QFA thrombin (Hemosil) for fibrinogen, Factor VII deficient plasma and Recombiplastin for FVII, Factor VIII deficient plasma and Hemosil (R) SynthASil for FVIII, Liquid Antithrombin reagent for antithrombin, IL reagent for protein C, and Ddimer 500 HS for D-dimer) and protocols from the manufacturer (Instrumentation Laboratory, Breda, the Netherlands).

Plasma markers of fibrinolysis
PAI-1 levels were determined with an ELISA kit from R&D systems (Abingdon, UK). Levels of tissue plasminogen activator (tPA) were measured using an ELISA kit from Sekisui (Lexington, USA).

Statistical analysis
Data are expressed as means (with standard deviations (SDs)), medians (with ranges), or numbers (with percentages) as appropriate. Means of two groups were compared by Student’s t-test or distributions in the two groups by Mann-Whitney U test as appropriate. Multiple groups were compared using one-way ANOVA (with the Bonferroni posttest) or Kruskal-Wallis H test (with Dunn’s posttest) as appropriate. Spearman’s correlation coefficient was used to assess correlation between continuous variables. P values of 0.05 or less were considered statistically significant. GraphPad Prism (San Diego, USA) and IBM SPSS Statistics 20 (New York, USA) were used for analyses.
Results

Patient characteristics

Patient characteristics are reported in Table 1. Sixty-eighth patients with biopsy-proven NAFLD (simple steatosis n = 24, NASH n = 22, and NASH cirrhosis n = 22), thirty lean controls (BMI 25 kg/m²), and fifteen patients with alcoholic (ASH) cirrhosis were included. None of the patients were diagnosed with another form of liver disease (e.g., hepatitis B, hepatitis C, autoimmune hepatitis, hereditary haemochromatosis etc.). The NAS score increased from patients with simple steatosis to patients with NASH and patients with NASH-related cirrhosis.

Data from all hemostasis tests performed are summarized in Table 2; parts of these data are also graphically represented herein.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Lean controls n=30</th>
<th>Overweight controls n=30</th>
<th>Steatosis n=24</th>
<th>NASH n=22</th>
<th>NASH cirrhosis n=22</th>
<th>ASH cirrhosis n=15</th>
<th>P-value</th>
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<td>5 (17)</td>
<td>10 (42)</td>
<td>8 (36)</td>
<td>9 (41)</td>
<td>12 (80)</td>
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<td>BMI (kg/m²)</td>
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<td>30.4 [3.6]**</td>
<td>33.6 [6.5]**</td>
<td>34.6 [9.2]**</td>
<td>35.2 [5.4]**</td>
<td>27.2 [5.7]</td>
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<td>Alcohol (units/week)</td>
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<td>1 [0-42]</td>
<td>0 [0-7]</td>
<td>0 [0-3]</td>
<td>0 [0-130]</td>
<td>0.032</td>
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<td>Caucasian</td>
<td>18 (60)</td>
<td>23 (77)</td>
<td>19 (79)</td>
<td>22 (100)*</td>
<td>21 (95.5)</td>
<td>10 (67)</td>
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<td>African American</td>
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<td>4 (17)</td>
<td>0</td>
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<tr>
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<td>1 [13]</td>
<td>1 (4)</td>
<td>0</td>
<td>0</td>
<td>0.076</td>
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<tr>
<td>OAC use</td>
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<td>3 (10)</td>
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<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
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<tr>
<td>Diabetes mellitus</td>
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<td>6 (25)</td>
<td>12 (54.5)**</td>
<td>15 (68.2)**</td>
<td>1 (6.7)</td>
<td>&lt;0.001</td>
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<td>Insulin dependent</td>
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<td>0</td>
<td>1 (4.2)</td>
<td>4 (18.2)</td>
<td>3 (13.6)</td>
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<td>Hemoglobin (g/dL)</td>
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<td>13.3 [1.2]</td>
<td>12.2 [2.2]</td>
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<tr>
<td>Leukocytes (10⁹/L)</td>
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<td>7.5</td>
<td>5.3</td>
<td>5.5</td>
<td>0.060</td>
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<td>[3.1-15.4]</td>
<td>[3.9-9.2]</td>
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<td>Platelets (10⁹/L)</td>
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<td>243 [64]</td>
<td>131 [69]</td>
<td>134 [64]</td>
<td>&lt;0.0001</td>
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<tr>
<td>AST (U/L)</td>
<td>17 [7-66]</td>
<td>18 [12-35]</td>
<td>32**[<strong>n</strong>es**]</td>
<td>44***[n<strong>es</strong>]</td>
<td>52***[n<strong>es</strong>]</td>
<td>36***[n<strong>es</strong>]</td>
<td>&lt;0.0001</td>
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<td>[18-128]</td>
<td>[20-571]</td>
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<td></td>
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<tr>
<td>ALT (U/L)</td>
<td>12 [5-65]</td>
<td>15 [4-31]</td>
<td>40***[n<strong>es</strong>]</td>
<td>56***[n<strong>es</strong>]</td>
<td>35***[n<strong>es</strong>]</td>
<td>28**</td>
<td>&lt;0.0001</td>
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<tr>
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<td>[17-217]</td>
<td>[8-169]</td>
<td>[13-230]</td>
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<tr>
<td>Alk phos (U/L)</td>
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<td>81</td>
<td>81</td>
<td>90</td>
<td>108</td>
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<td>[29-437]</td>
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<tr>
<td>Bilirubin (mg/dL)</td>
<td>0.3</td>
<td>0.2</td>
<td>0.6**[n<strong>es</strong>]</td>
<td>0.6**[n<strong>es</strong>]</td>
<td>0.9**[n<strong>es</strong>]</td>
<td>1.1**[n<strong>es</strong>]</td>
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<td>[0.2-4.6]</td>
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<tr>
<td></td>
<td>Triglycerides (mg/dL)</td>
<td>91 [64]</td>
<td>136 [95]</td>
<td>121 [47]**</td>
<td>154 [70]</td>
<td>129 [54]</td>
<td>72 [38]**</td>
</tr>
<tr>
<td></td>
<td>NAS score</td>
<td>3 [1-4]</td>
<td>5 [4-6]</td>
<td>5 [4-8]</td>
<td>5 [4-8]</td>
<td>&lt;0.0001</td>
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</table>

Table 1. Patient characteristics

NASH: Non-alcoholic steatohepatitis, ASH cirrhosis: alcoholic cirrhosis, OAC: oral contraceptive, AST: aspartate transaminase, ALT: alanine transaminase, Alk phos: alkaline phosphatase, LDL: low-density lipoprotein, HDL: high-density lipoprotein, NAS: NAFLD activity score, MELD: model for end-stage liver disease. *P<0.05, **P<0.01, ***P<0.001 compared to lean controls. #P<0.05, #P<0.01, #P<0.001 compared to overweight controls. Data are expressed as number (%), mean [SD], or median [range].
Platelet activation status

The basal platelet activation status and the agonist-induced platelet activatability in patients and controls are reported in Figure 2. There was no statistically significant difference in the number of P-selectin positive platelets at baseline between patients with NAFLD, patients with ASH cirrhosis, and lean controls, although few individual patients appeared to have a slightly increased basal platelet activation status. Also the MFI of the P-selectin signal was comparable between all patients and controls. When platelets were activated in vitro using either TRAP or ADP, the percentage of platelets expressing P-selectin were decreased in patients with cirrhosis compared to controls when corrected for baseline values, although the difference did not reach statistical significance. Furthermore, the MFI after activation with TRAP or ADP was also decreased in patients with cirrhosis, although the difference did not reach statistical

Figure 2. Basal and agonist-induced platelet activation.
The basal platelet activation status (A) and the agonist-induced platelet activatability using TRAP (B) and ADP (C) as assessed by flow cytometry for P-selectin in lean controls, overweight controls, patients with simple steatosis, patients with non-alcoholic steatohepatitis (NASH), patients with NASH cirrhosis, and patients with alcoholic (ASH) cirrhosis. Shown are the percentage of P-selectin positive platelets (left panels) and the mean fluorescent intensity (MFI) of the same flow cytometry experiments (right panels). Agonist-induced platelet activatability values were corrected for baseline values. Horizontal lines represent medians. Bars indicate medians with the error bars representing ranges.
significance compared to controls. Neither basal platelet activation status nor platelet activatability in patients with NAFLD correlated with BMI, lipid levels (high density lipoprotein (HDL)-C, low density lipoprotein (LDL)-C, total cholesterol, and triglycerides (TGs)), NAS score or individual histological parameters (steatosis, inflammation, and ballooning), aspartate aminotransferase (AST), alanine aminotransferase (ALT), bilirubin, or c-reactive protein (CRP).

**Thromboelastography**

TEG test results were comparable between lean and overweight controls, patients with simple steatosis, and patients with NASH. However, TEG revealed moderate hypo-coagulability in both NASH and ASH cirrhosis as demonstrated by a prolonged K-time (NASH cirrhosis vs. overweight controls $p <0.01$) and a decreased a-angle (NASH cirrhosis vs. overweight controls $p <0.01$) and MA (ASH cirrhosis vs. overweight controls $p <0.05$).

**Thrombin generation, PT, APTT**

Compared to lean controls, there was a trend towards increased thrombin generation in overweight controls but this did not reach statistical significance. In the absence of TM, thrombin generation was decreased in patients with cirrhosis, as demonstrated by a decrease in ETP and peak. However, in the presence of TM, thrombin generation was comparable between all patient groups and controls (Fig. 3). When these data were recalculated to a normalized thrombomodulin sensitivity ratio (TM-SR), it became evident that TM was less effective at regulating thrombin generation in patients with cirrhosis compared to controls (Fig. 3). There was a strong negative correlation between TM-SR and levels of protein C ($r = -0.60; p <0.0001$) and levels of antithrombin ($r = -0.57; p <0.0001$). In addition, the TM-SR correlated with the ratio of factor VIII to protein C ($r = 0.50; p <0.0001$). However, TM-SR values did not correlate with BMI, lipid levels, or NAS score.

None of the thrombin generation parameters within the patients with NAFLD correlated with BMI, lipid levels (HDL-C, LDL-C, total cholesterol, and TGs), NAS score or individual histological parameters (steatosis, inflammation, and ballooning), AST, ALT, bilirubin, or CRP.

Both PT and APTT were comparable between patients with non-cirrhotic NAFLD and controls. However, in patients with cirrhosis the PT ($p <0.001$ compared to lean controls) and APTT ($p <0.01$ compared to lean controls) were prolonged.

**Figure 3. Results from the thrombin generation test.**

Endogenous thrombin potential (ETP) (A), ETP in the presence of thrombomodulin (TM+) (B), and the thrombomodulin sensitivity ratios (TM-SR) (C) in lean controls, overweight controls, patients with simple steatosis, patients with non-alcoholic steatohepatitis (NASH), patients with NASH cirrhosis, and patients with alcoholic (ASH) cirrhosis.

* $P <0.05$, ** $P <0.01$ compared to lean controls, # $P <0.01$, ## $P <0.01$ compared to overweight controls.
Fibrinolysis
The clot lysis time was higher in overweight controls and in patients with non-cirrhotic NAFLD compared to lean controls, although differences did not reach statistical significance. The clot lysis time was comparable between patients with NASH-related cirrhosis and lean controls, but was decreased in patients with ASH-related cirrhosis (Fig. 4).

Figure 4. Fibrinolytic potential.
Clot lysis time (A) and levels of plasminogen activator inhibitor-1 (PAI-1) (B) in lean controls, overweight controls, patients with simple steatosis, patients with non-alcoholic steatohepatitis (NASH), patients with NASH cirrhosis, and patients with alcoholic (ASH) cirrhosis. Horizontal lines represent medians.
* P <0.05, ** P <0.01 compared to lean controls. # P <0.01, ## P <0.01 compared to overweight controls.

Fibrin structure
Clot permeability was decreased in overweight controls and patients with NAFLD, indicating more dense clots compared to lean controls, although differences did not reach statistical significance (Fig. 5). Clot permeability was similar in patients with ASH-related cirrhosis compared to lean controls, whereas clots generated in NASH-related cirrhosis had substantially reduced permeability.

Confocal microscopy revealed a stepwise increase in fiber density from lean controls to overweight controls and patients with simple steatosis to patients with NASH and NASH cirrhosis (Fig. 5). Fiber density was also increased in patients with ASH-related cirrhosis compared to lean controls (p <0.001).
The protein carbonyl content of fibrinogen purified from patients with simple steatosis, NASH, and NASH cirrhosis was increased compared to lean controls, although differences did not reach statistical significance. However, the carbonyl content of fibrinogen purified from patients with ASH-related cirrhosis was significantly elevated (p <0.01 compared to lean controls).

We observed a negative correlation between fibrinogen levels and clot permeability (r = -0.66, p <0.0001). Furthermore, fiber density correlated with the BMI (r = 0.35, p <0.0001). However, within patients with NAFLD, values of clot permeability or fiber density did not correlate with lipid levels, NAS score, AST, ALT, bilirubin, or CRP.
Figure 5. Fibrin structure
The permeability coefficient ($K_s$, calculated following Darcy’s Law) (A) and fibrin fiber density assessed by laser-scanning confocal microscopy (expressed as the number of fibers per 100 µm) (B) in plasma clots of lean controls, overweight controls, patients with simple steatosis, patients with non-alcoholic steatohepatitis (NASH), patients with NASH cirrhosis, and patients with alcoholic (ASH) cirrhosis. Horizontal lines represent medians. * $P < 0.05$, ** $P < 0.01$ compared to lean controls.

Plasma levels of proteins involved in primary hemostasis
Levels of sP-selectin were slightly, but non-significantly increased in patients with NASH, NASH cirrhosis, and ASH cirrhosis compared to lean and overweight controls. In contrast, levels of PF4 were comparable between all patient groups and controls. Plasma levels of VWF were comparable between controls and patients with non-cirrhotic NAFLD. However, VWF levels were increased in patients with cirrhosis compared to patients with non-cirrhotic NAFLD and controls ($p < 0.001$). In contrast, ADAMTS13 activity was comparable between all cohorts.

Plasma levels of proteins involved in coagulation
Although fibrinogen levels were higher in overweight controls and patients with NAFLD compared to lean controls, this did not reach statistical significance. Patients with cirrhosis and lean controls had comparable fibrinogen levels. In NAFLD patients, levels of fibrinogen modestly correlated with BMI ($r = 0.21; p = 0.02$). Furthermore, fibrinogen levels increased with increasing severity of steatosis on liver biopsy (2.24 mg/ml [2.08–2.65] (median [range]) in steatosis <5%, 3.38 mg/ml [2.21–5.91] in steatosis 5–33%, 3.09 mg/ml [1.49–5.07] in steatosis 34–66%, and 3.80 mg/ml [3.44–4.43] in steatosis >66%; $p < 0.007$). However, fibrinogen levels did not correlate with other individual histological parameters (inflammation or ballooning) or with lipid levels. Plasma levels of FVII were slightly, but non-significantly increased in patients with non-cirrhotic NAFLD compared to lean controls. In contrast, in patients with cirrhosis levels were decreased (NASH cirrhosis $p < 0.05$; ASH cirrhosis $p < 0.001$ compared to lean controls). Levels of FVIII were increased in patients with NASH-related cirrhosis compared to lean controls ($p < 0.05$). In contrast, levels were similar between patients with ASH-related cirrhosis and controls. Both protein C and antithrombin levels were decreased in patients with NASH- and ASH-related cirrhosis ($p < 0.001$ for both protein C and antithrombin compared to lean controls). Levels of D-dimer were non-significantly increased in patients with non-cirrhotic NAFLD, but levels were significantly increased in patients with cirrhosis compared to lean controls ($p < 0.001$).
Table 2. Data from all hemostatic tests, NASH: Non-alcoholic steatohepatitis, AAS: alcoholic cirrhosis, MFI: mean fluorescence intensity, sP-selectin: soluble P-selectin, PF4: platelet factor 4, VWF: von Willebrand Factor, ADAMTS13: a disintegrin and metalloproteinase with a thrombospondin type 1 motif, R-time: thrombin potential, K-time: kinetic time, R -time: reaction time, K-time: kinetics time, R-value: maximum amplitude, LY30: lysis at 30 minutes, *P<0.05, **P<0.01, ***P<0.001 compared to lean controls. #P<0.05, ##P<0.01, ###P<0.001 compared to overweight controls. Data are expressed as number (%), mean [SD], or median [range].
**Plasma levels of proteins involved in fibrinolysis**

Levels of PAI-1 stepwise increased from lean controls to overweight controls to patients with simple steatosis, and were still increased in patients with NASH and NASH cirrhosis (Fig. 4), although levels were only significantly increased in patients with NASH compared to lean controls. Levels of tPA also stepwise increased from healthy controls to patients with NASH cirrhosis, although levels were only significantly increased in patients with NASH cirrhosis compared to lean controls.

The clot lysis time correlated with PAI-1 levels \( (r = 0.68; \ p < 0.0001) \) and fibrinogen levels \( (r = 0.25; \ p = 0.007) \) in patients with NAFLD. In addition, within the patients with NAFLD, clot lysis time slightly correlated with BMI \( (r = 0.19; \ p = 0.04) \), but not with lipid levels (HDL-C, LDL-C, total cholesterol, and TGs), NAS score or individual histological parameters (steatosis, inflammation, and ballooning). Levels of PAI-1 also correlated with BMI \( (r = 0.21; \ p = 0.02) \), but not with lipid levels. Furthermore, PAI-1 levels increased with increasing grade of NAS score \( (1.08 \text{ng/ml} [0.29-9.15] \text{ in patients with NAS } <3, \ 1.94 \text{ng/ml} [0.58-7.70] \text{ in patients with NAS } 3-4, \text{ and } 2.18 \text{ ng/ml} [0.19-9.12] \text{ in patients with NAS } \geq 5) \), although these differences were not significant. PAI-1 levels also appeared to increase with increasing severity of steatosis, inflammation, and ballooning on liver biopsy, although differences did not reach statistical significance (data not shown). Finally, within patients with NAFLD, none of the fibrinolysis parameters correlated with AST, ALT, bilirubin, or CRP.

**Discussion**

The combined results of this study show that the overall hemostatic status is comparable between patients with NAFLD and controls. Our study, therefore, suggests that the role for hyperactive hemostasis in the increased risk of thrombosis in patients with NAFLD is probably limited. Furthermore, since there were no differences in the coagulation status between patients with NASH- or ASH-related cirrhosis, there is probably also a limited role for hemostasis in the increased thrombotic risk in patients with NASH-related cirrhosis compared to patients with cirrhosis from other etiologies. However, our data show some pro-thrombotic features in patients with NAFLD, including hypofibrinolysis and a pro-thrombotic structure of the fibrin clot, which appear driven by obesity rather than the liver disease itself. We observed a hypofibrinolytic state in patients with non-cirrhotic NAFLD, which appeared to resolve in NASH-related cirrhosis. The hypofibrinolytic state in non-cirrhotic NAFLD may contribute to thrombosis risk in these patients as a hypofibrinolytic state as determined with our clot lysis assay has been demonstrated to form a risk factor for both venous and arterial thrombosis in the general population [26–28]. Furthermore, we observed decreased fibrin clot permeability and increased clot density in patients with NAFLD indicating a pro-thrombotic structure of the fibrin clot in these patients.

Previous studies have shown decreased fibrin clot permeability to be associated with thrombotic diseases [29–31]. We have recently shown that fibrin clot permeability in patients with cirrhosis of varying etiology is markedly reduced despite decreased fibrinogen plasma levels in these patients. The pro-coagulant properties of the fibrin clot in cirrhosis was attributed to increased oxidation of the fibrinogen molecule [25], which is known to result in a more thrombogenic fibrin clot. The thrombogenic nature of fibrin clots in patients with NAFLD, as identified in the present study, is likely attributable to multiple factors including increased oxidation of the fibrinogen molecule and elevated plasma fibrinogen levels.
addition, in those patients with diabetes, glycation of the fibrinogen molecule contributes to decreased fibrin clot permeability [32].

Previous studies have suggested an increased activity of platelets in patients with the metabolic syndrome [12,13] and in patients with NAFLD [14,33–35], which might contribute to their increased risk of thrombosis. However, results from these studies are inconsistent, most of these studies are limited by their relative small sample size, and use indirect markers of platelet activation (e.g., mean platelet volume). The results of our study show that NAFLD is not associated with platelet hyperactivity measured using a direct flow cytometric assay of platelet activation status. In addition, NAFLD was not associated with changes in pivotal proteins in primary hemostasis. We did observe decreased platelet activatability in patients with cirrhosis, which has been previously shown [17].

Although TEG and thrombin generation testing in the absence of thrombomodulin revealed a slight hypocoagulability in patients with cirrhosis, when tested in the presence of thrombomodulin thrombin generation was comparable to that of lean or overweight controls in patients with cirrhosis, which has been previously shown [36–38]. In extent, thrombin generation was also normal in patients with NAFLD. In contrast, Tripodi et al. concluded that NAFLD is characterized by a pro-coagulant-imbalance, as shown by an increase in ETP-ratio (with-to-without thrombomodulin) in these patients [11]. However, this ratio only represents the capacity of thrombomodulin to downregulate thrombin generation, and is by no means a direct indicator of hemostatic potential. An increase in the ETP-ratio in patients with NAFLD therefore does not explain their increased risk of thrombosis compared to patients with alcoholic/viral cirrhosis. We argue against the use of the ETP-ratio or TM-SR to classify if patient samples are normo- or hypercoagulable, but instead believe that the thrombin generation performed in the presence of thrombomodulin is the most accurate laboratory measure of the coagulant potential of a patients’ plasma.

Several studies have shown increased levels of various individual pro-thrombotic factors in patients with NAFLD [7–9,11,39–41]. Although results have been inconsistent, an increase in PAI-1, fibrinogen, and factor VIII and a decrease in antithrombin are most frequently reported. Levels of fibrinogen were also increased in patients with non-cirrhotic NAFLD in this study, although the difference with controls did not reach statistical significance. However, in patients with cirrhosis fibrinogen levels are mostly decreased [42], as also observed in patients with ASH-related cirrhosis in this study. Yet, patients with NASH-related cirrhosis in our study had fibrinogen levels comparable to that of the controls, which likely relates to a relative increase in fibrinogen production due to the fatty liver disease. Levels of factor VIII were increased in patients with NASH-related cirrhosis in this study. In contrast, levels of antithrombin were decreased in patients with cirrhosis, which is in agreement with previous studies [36,38,43]. Finally, we observed increased levels of PAI-1 in patients with NAFLD and levels increased with increasing severity of the disease and increasing severity of steatosis, which has been previously shown [9].

In our study, several hemostatic test results (such as platelet activity, clot lysis time, PAI-1 levels, and fiber density) showed a higher variability in patients than in controls, suggesting individual patients may have a more thrombogenic hemostatic profile. We did not identify any characteristics of individual patients to explain the more extreme values in individual
patients, although our study likely lacks power to identify such characteristics. We did observe relations between BMI and clot lysis time, PAI-1 levels, and fibrin fiber density, which in part have been previously described [27,44].

To conclude, the combined results of this study show that the overall hemostatic status is comparable between patients with NAFLD and controls, which contrasts with previously published results in similar populations. We did identify some pro-thrombotic features in patients with NAFLD, particularly a pro-thrombotic structure of the fibrin clots. Although the discrepancy between our study and previous ones is unclear, we included a well-defined cohort of patients and controls, included patients with established cirrhosis, and performed an exhaustive panel of hemostasis tests including functional assessment of platelets, coagulation, and fibrinolysis. The results of this study suggest that the role for hemostasis in the increased risk of thrombosis in patients with NAFLD and NASH-related cirrhosis is probably limited.

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