Research Article

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

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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/m^2), 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 pro-thrombotic 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.

Lay summary: The combined results of this study show that the overall hemostatic status is comparable between healthy individuals and patients with a fatty liver disease.

Keywords: Non-alcoholic fatty liver disease; Hemostasis; Cirrhosis; Thrombosis; Obesity; Platelets; Coagulation; Fibrin structure.

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Abbreviations: NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; ASH, alcoholic steatohepatitis; ADAMTS13, a disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13; CVD, cardiovascular disease; VT, venous thromboembolism; PVT, portal vein thrombosis; PAI-1, plasminogen activator inhibitor 1; TEG, thromboelastography; NASH-CRN, NASH Clinical Research Network; NAS, NAFLD activity score; ADP, adenosine diphosphate; TRAP, thrombin receptor activating peptide; MFI, mean fluorescence intensity; sP-selectin, Soluble P-selectin; PF4, platelet factor 4; ELISA, enzyme-linked immunosorbent assay; VWF, von Willebrand Factor; R-time, reaction time; K-time, kinetics time; MA, maximum amplitude; Lysis-30, lysis at 30 min; PT, prothrombin time; APTT, activated partial thrombin time; F, factor; tPA, tissue plasminogen activator; PPP, platelet-poor plasma; CAT, Calibrated Automated Thrombography; ETP, endogenous thrombin potential; TM-SR, normalized thrombomodulin sensitivity ratio; SD, standard deviation; MELD, model for end-stage liver disease; HDL-C, high density lipoprotein; LDL-C, low density lipoprotein; TGs, triglycerides; AST, aspartate aminotransferase; ALT, alanine aminotransferase; CRP, c-reactive protein.

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 co-existing 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 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 consisting of lean (BMI <25 kg/m^2; n = 30) and overweight (BMI >25 kg/m^2; 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.

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 2000 g and 10,000 g respectively for 10 min. Plasma was snap-frozen and stored at −80 °C until use.

Assays

Detailed methodology of the assays performed can be found in the Supplementary data. Supplementary Fig. 1 shows an overview of hemostasis and all tests performed are indicated in this schematic. In short, we performed functional tests of hemostasis:

1) The platelet activation status using flow cytometry in whole blood, as described previously [17]. Platelets were kept in a resting state or activated by either adenosine diphosphate (ADP) or thrombin receptor activating peptide (TRAP). The percentage of platelets expressing P-selectin and the geometric mean fluorescence intensity (MFI) of the platelet population were recorded.
2) TEG, a whole blood study of clot formation, was performed using the Thromboelastography Hemostasis Analyzer 5000 (Haemonetics Corp., Haemoscope Division, Niles, IL, USA) as described previously [18].
3) Thrombin generation testing (performed in platelet-poor plasma (PPP) as described previously [19]) and the prothrombin time (PT) and activated partial thrombin time (APTT).
4) A plasma-based clot lysis time indicating fibrinolytic potential was determined as described previously [20].
5) Structural features of *in vitro* formed fibrin clots. The average pore size of the fibrin clot (expressed as the Darcy constant Ks) was determined in permeation studies as described previously [21,22]. Fibrin density was assessed by laser-scanning confocal microscopy of clots supplemented with fluorescently labeled fibrinogen as described previously [23]. Carbonylation of purified fibrinogen samples was quantified as described previously [24].

Furthermore we assessed plasma levels of soluble P-selectin (sP-selectin), platelet factor 4 (PF4), von Willebrand Factor antigen (VWF), and a disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13 (ADAMTS13) (all proteins involved in platelet function), levels of fibrinogen, factor (F) VII, FVIII, antithrombin, protein C, and D-dimer (proteins involved in coagulation), and levels of PAI-1 and tissue plasminogen activator (tPA) (proteins involved in fibrinolysis).

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/m^2), thirty overweight controls (BMI >25 kg/m^2), 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 Supplementary Table 1; parts of these data are also graphically represented herein.

Platelet activation status

The basal platelet activation status and the agonist-induced platelet activatability in patients and controls are reported in Fig. 1. 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 significance compared to