Research Article

EASL EUROPEAN ASSOCIATION FOR THE STUDY OF THE LIVER | JOURNAL OF HEPATOLOGY

Procoagulant imbalance in patients with non-alcoholic fatty liver disease

Armando Tripodi[1,5,*], Anna L. Fracanzani[2,5], Massimo Primignani[3,5], Veena Chantarangkul[1,5], Marigrazia Clerici[1,5], Pier Mannuccio Mannucci[4,5], Flora Peyvandi[1,5], Cristina Bertelli[2,5], Luca Valenti[2,5], Silvia Fargion[2,5]

[1]*Angelo Bianchi Bonomi Hemophilia and Thrombosis Center, Department of Clinical Sciences and Community Health, Università degli Studi di Milano, Milano, Italy;* [2]*Metabolic Liver Diseases Center, Department of Pathophysiology and Transplantation, Section of Internal Medicine, Università degli Studi di Milano, Italy;* [3]*First Division of Gastroenterology;* [4]*Scientific Direction;* [5]*IRCCS Cà Granda Maggiore Hospital Foundation, Milano, Italy*

Background & Aims: Non-alcoholic fatty liver disease (NAFLD) is characterized by increased risk of cardiovascular events and liver-fibrosis. Both could be explained by a procoagulant-imbalance that was surmised but never directly demonstrated. We investigated 113 patients with varying histological liver damage [steatosis (n = 32), steatohepatitis (n = 51), metabolic-cirrhosis (n = 30)], 54 with alcoholic/viral-cirrhosis and 179 controls.

Methods: Plasma was evaluated for levels of pro- and anti-coagulants, and for thrombin-generation assessed as endogenous-thrombin-potential (ETP) with and without thrombomodulin or Protac® as protein C activators. The procoagulant-imbalance was defined as ETP-ratio (with-to-without thrombomodulin) or as Protac®-induced-coagulation-inhibition (PICI%). High ETP-ratios or low PICI% indicate resistance to thrombomodulin or Protac® and hence a procoagulant-imbalance.

Results: ETP-ratio increased from controls [0.57 (0.11–0.89)] to steatosis [0.72 (0.33–0.86)] and metabolic-cirrhosis [0.80 (0.57–0.95)], (p <0.001), the latter being comparable to that for alcoholic/viral-cirrhosis [0.80 (0.57–0.95) *vs.* 0.80 (0.44–0.96)]. Factor VIII (a potent procoagulant for thrombin-generation) increased from steatosis [99% (71–150)] to metabolic-cirrhosis [157% (64–232)], p <0.001. Protein C (a powerful anticoagulant) decreased from steatosis [103% (77–228)] to metabolic-cirrhosis [77 (17–146)], p <0.001. As a consequence, factor VIII-to-protein C ratio increased from steatosis [0.96 (0.36–1.60)] to metabolic-cirrhosis [2.05 (0.81–12.1)], p <0.001 and was correlated with the ETP-ratio (rho = 0.543, p <0.001). Similar results were obtained for PICI%. Patients with procoagulant-imbalance detected as ETP-ratio greater or PICI% lower than the median value of controls tended to have a higher risk of metabolic-syndrome, higher intima-media thickness, fibrosis, steatosis or lobular inflammation, all considered clinical manifestations of NAFLD.

Conclusion: NAFLD is characterized by a procoagulant-imbalance progressing from the less severe (steatosis) to the most severe form of the disease (metabolic-cirrhosis). This imbalance appears to result from increased factor VIII and reduced protein C and might play a role in the risk of cardiovascular events and liver-fibrosis commonly observed in NAFLD.

Introduction

Non-alcoholic fatty liver disease (NAFLD) defines a group of liver diseases progressing from simple steatosis to non-alcoholic steatohepatitis (NASH), fibrosis, and cirrhosis [1,2] The prevalence of NAFLD in industrialized countries ranges from 35% to 58% [3] and is expected to increase in parallel with the increased incidence of such metabolic disorders as obesity, diabetes, and dyslipidemia that are associated with insulin-resistance and are risk factors for the occurrence/recurrence of cardiovascular events including micro- and macro-vascular thrombosis related diseases observed in NAFLD [4–6]. Furthermore, NAFLD has been associated with accelerated atherogenesis independently from other common risk factors [6,7] and metabolic cirrhosis is expected to be the leading indication for liver transplantation within the next fifteen years in the US [8].

The presence of a hypercoagulable state leading to a higher cardiovascular risk in NAFLD has been suggested on the basis of the inflammatory state associated with this condition and on epidemiological studies [1]. However, a direct link between blood coagulation and NAFLD has not yet been established and this may in part be due to the lack of appropriate laboratory tests exploring coagulation under conditions mimicking those occurring *in vivo*. Historically, coagulation has been evaluated by such traditional global tests as the prothrombin and activated partial

Keywords: Factor VIII; Protein C; Protac®; Thrombomodulin; Thrombin generation.
Received 21 August 2013; received in revised form 9 February 2014; accepted 10 March 2014; available online 18 March 2014
* Corresponding author. Address: Via Pace 9, 20122 Milano, Italy. Tel.: +39 02 503 20725; fax: +39 02 503 20723.
E-mail address: armando.tripodi@unimi.it (A. Tripodi).
Abbreviations: NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; ETP, endogenous thrombin potential; PICI, Protac® induced coagulation inhibition.

thromboplastin times (PT/APTT) or through the measurement of the individual pro- or anti-coagulants. However, neither of these two approaches are suitable to mimic the complex interplay between the procoagulants (i.e., thrombin generation drivers) and their naturally occurring anticoagulant counterparts (i.e., thrombin neutralization drivers) operating *in vivo*. Global tests that monitor the time course of thrombin from generation to decay [9], represent much better than the PT/APTT or the measurement of the individual pro- or anti-coagulants what occurs *in vivo*. We therefore sought to investigate plasma from patients with NAFLD with two thrombin generation assays and report data showing that these patients display a procoagulant imbalance progressing from simple steatosis to metabolic cirrhosis. These results help to understand the link between NAFLD and coagulation, may contribute to evaluate the severity of liver damage and the relative cardiovascular risk, and perhaps may open new therapeutic avenues.

Materials and methods

Patients and controls

The study enrolled 113 patients with NAFLD including 32 with simple steatosis [(men/women), 27/5; age range, 56 (35–67)y]; 51 with NASH [(42/9); 49 (16–75)y] and 30 with metabolic cirrhosis [(17/13), 61 (43–72)y], who were seen consecutively from January 2010 to January 2012. Sample size was chosen based on the previous experience investigating hypercoagulability in patients with alcoholic/viral cirrhosis [10]. All, but 19 patients with metabolic cirrhosis in whom the diagnosis was based on clinical features, had biopsy proven diagnosis of NAFLD, all other causes of liver disease including excessive alcohol intake (>20 g daily) being excluded. The diagnosis of NASH and the NAFLD activity (NAS) score was based on Brunt and Kleiner criteria, respectively [11,12]; in particular for steatosis 0 = <5%, 1 = 5–33%, 2 = 34–66%, 3 = >66%; for lobular inflammation 0 = none, 1 = <2, 2 = 3–4, 3 = >4; NAS was based on the unweighted sum of the scores for steatosis (0–3), lobular inflammation (0–3), and ballooning degeneration (0–2). The stage of fibrosis was scored based on the 5-point scale: stage 0, no fibrosis; stage 1, perisinusoidal or portal; stage 2, perisinusoidal and portal/periportal; stage 3, septal or bridging fibrosis; stage 4, cirrhosis. The metabolic syndrome was diagnosed according to the presence of three or more of the revised ATPIII (Adult Treatment Panel III) criteria [13]. B-mode ultrasound was used to evaluate the carotid intima-media thickness using 0.65 mm as cut-off value [7]. The distribution of patients with metabolic cirrhosis according to Child-Turcotte-Pugh classes was A (n = 27) and B (n = 3). Fifty-four patients with alcoholic/viral cirrhosis of graded severity (Child A/B/C, n = 28/17/6) [40 males, median age (range) 63 (41–80)y, 14 females, 64 (52–73)y] and 179 healthy subjects were taken as controls. Patients and controls were comparable for age, gender, blood drawing, plasma preparation and storage, and were not taking anticoagulants nor drugs interfering with coagulation at the time of blood sampling. Inclusion criteria for controls were no evidence of liver disease as shown by liver enzyme plasma levels within the normal reference range, no evidence of diabetes and body mass index (BMI) lower than 30 kg/m^2.

The study was approved by the institutional review board and the procedures carried out in accordance with the Helsinki declaration. Informed consent was obtained from patients and controls.

Venepunctures were performed with 21 gauge needles, the first tube was discarded or used for measurements other than coagulation. Blood for coagulation testing was collected into vacuum tubes at a proportion of 1:9 (citrate 0.109 M:blood), centrifuged soon after blood collection (no later than 30 min) for 20 min at 3000g. Plasma was immediately stored at −70 °C until testing.

To minimize analytical variability, the same number of patients and controls were analyzed within each working session.

Thrombin generation test in the presence/absence of thrombomodulin

This was assessed according to Hemker *et al.* [14] as described [15]. Testing was based on the activation of coagulation after addition to plasma of human recombinant tissue factor (1 pM) (Recombiplastin, Instrumentation Laboratory, Orangeburg, NY) and synthetic phospholipids (1.5 μM) (Avanti Polar, Alabaster, Alabama) as coagulation triggers. Testing was repeated in another plasma aliquot after addition of rabbit thrombomodulin (Haematologic Technologies, Inc, VT) (6 nM). To ensure consistency between experiments the concentration of thrombomodulin has been adjusted in such a way to drop thrombin generation in a pooled normal plasma by ~50%. The same lot and dilution of thrombomodulin was used throughout the study. Registration of thrombin generation was obtained with a fluorogenic substrate (Z-Gly-Gly-Arg-AMC HCl, Bachem, Bubendorf, Switzerland) (617 μM) by means of a fluorometer (Fluoroskan Ascent®, ThermoLabsystem, Helsinki, Finland). Readings were recorded and calculated with a dedicated software (Thrombinoscope™, Thrombinoscope BV, Maastricht, The Netherlands), which displays thrombin generation curves and calculates the area under the curve, defined as endogenous thrombin potential (ETP). Results for thrombin generation were expressed as ETP-ratio (i.e., the ratio of ETP measured in the presence of thrombomodulin to the ETP measured in its absence). This ratio represents the resistance to the anticoagulant action of thrombomodulin and can be taken as an index of procoagulant imbalance (the higher the ratio, the greater the procoagulant imbalance).

Thrombin generation test in the presence/absence of Protac®

This was assessed with a commercial chromogenic assay (HemosIL Thrombopath, Instrumentation Laboratory) designed to globally evaluate the protein C anticoagulant system [16]. It is based on the ability of endogenous activated protein C, generated upon addition to plasma of a snake venom extract (Protac®), to reduce tissue factor induced thrombin generation. This test, in addition to protein C is sensitive to high factor VIII [16]. The amount of thrombin was evaluated by recording changes in optical density in the presence (A) or absence (B) of Protac® after adding a thrombin specific chromogenic substrate. Results were expressed as Protac® Induced Coagulation Inhibition (PICI) percentage calculated as follows

$\text{PICI\%} = [(B - A)/B] \times 100$, the smaller the PICI% the greater being the procoagulant imbalance.

Other measurements

Pro- (factors II and VIII) and anti-coagulant (antithrombin and protein C) factors were measured as reported [10] with results expressed as percentage of a pooled normal plasma arbitrarily set at 100% of normal. PT and APTT results were expressed as ratio of patient-to-normal clotting times. The ratio of factor VIII-to-protein C activity was taken as an index of the procoagulant imbalance (the higher the ratio the greater the procoagulant imbalance).

Statistical analysis

Continuous variables were expressed as medians (ranges) and tested for statistical significance with the non-parametric Mann-Whitney *U* and Wilcoxon tests. Correlation was assessed with the Spearman rho correlation test. A p value of 0.05 or less was considered statistically significant. Odds ratios (95% confidence intervals) were calculated as a measure of the relative risk of having the metabolic syndrome, intima-media thickness or fibrosis in association with a procoagulant imbalance defined as ETP-ratio higher or PICI% lower than the median value of the distribution of results for the control population. Analyses were performed with the IBM SPSS software 20.0 (Chicago, IL).

Results

Main parameters of traditional coagulation

The PT-ratio was slightly higher than 1.0 (expressing prolongation of clotting times) in patients with NASH (p <0.05), metabolic cirrhosis (p <0.001) or alcoholic/viral cirrhosis (p <0.001) as compared to controls (Table 1). The APTT-ratio was higher in alcoholic/viral cirrhosis (p <0.001) as compared to controls (Table 1). Factor II was lower in metabolic (p <0.001) or alcoholic/viral cirrhosis (p <0.001) than in controls (Fig. 1, Table 1). Factor VIII was higher in NASH (p <0.001), metabolic (p <0.001) or alcoholic/viral cirrhosis (p <0.001) than in controls (Fig. 1, Table 1). Antithrombin was lower in metabolic