Research Article

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

Proteomic analysis reveals that apolipoprotein A1 levels are decreased in patients with Budd–Chiari syndrome

Simone Talens[1,a], Jildou Hoekstra[2,a], Steffen P.G. Dirkx[1], Sarwa Darwish Murad[2], Jonel Trebicka[3], Elwyn Elias[4], Massimo Primignani[5], Juan-Carlos García-Pagán[6], Dominique C. Valla[7], Harry L.A. Janssen[2], Frank W.G. Leebeek[1], Dingeman C. Rijken[1,*], for the EN-Vie Study Group [b]

[1]*Department of Hematology, Erasmus University Medical Center, Rotterdam, The Netherlands;* [2]*Department of Gastroenterology and Hepatology, Erasmus University Medical Center, Rotterdam, The Netherlands;* [3]*Department of Internal Medicine I, University Hospital of Bonn, Bonn, Germany;* [4]*Liver Unit, Queen Elizabeth University Hospital, Birmingham, United Kingdom;* [5]*Gastroenterology and Gastrointestinal Endoscopy Unit, Ospedale Policlinico, Mangiagalli and Regina Elena Foundation, Milan, Italy;* [6]*Hepatic Hemodynamic Laboratory, Liver Unit, Hospital Clinic, IDIBAPS and Ciberehd, Barcelona, Spain;* [7]*Department of Hepatology, Hôpital Beaujon, AP-HP, INSERM-U773 & University Paris-7, Clichy, France*

Background & Aims: Budd–Chiari syndrome (BCS) is a rare vascular liver disorder caused by thrombosis of the hepatic veins. In some patients, no known thrombophilic factor can be identified. This study aimed to identify novel factors that might play a role in thrombosis in BCS-patients by using a proteomic approach.

Methods: The abundance of plasma clot-bound proteins was compared between nine BCS-patients and nine controls by using two-dimensional difference gel electrophoresis. The protein with the most significant decrease in patients was identified by mass spectrometry. Plasma levels of this protein were measured and the results were validated in a large cohort of BCS-patients.

Results: A total of 26 protein spots significantly differed (p <0.001). The spot that decreased with the highest statistical significance in patients was identified by mass spectrometry as apolipoprotein A1 (apo A1). The mean level of apo A1 in the plasma of these BCS-patients (0.74 g/L) was also significantly lower than in controls (1.45 g/L, p = 0.002). This finding was validated in a large cohort of 101 BCS-patients and 101 controls (0.97 g/L vs. 1.32 g/L, p <0.0001). There was no major correlation between plasma levels of apo A1 and various liver function tests.

Conclusions: BCS-patients show decreased clot-bound protein abundance and plasma levels of apo A1. Decreased levels of apo A1 may play a role in the etiology of thrombosis in BCS-patients and possibly in other patients with venous thrombosis.

Keywords: 2D-DIGE; Apolipoprotein A1; Budd–Chiari syndrome; Proteomics; Venous thrombosis.

Received 16 April 2010; received in revised form 29 July 2010; accepted 17 August 2010; available online 14 January 2011

*Corresponding author. Address: Department of Hematology, Erasmus University Medical Center Rotterdam, Room Ee 1393, Dr. Molewaterplein 50, 3015 GE Rotterdam, The Netherlands. Tel.: +31 10 70 44 723; fax: +31 10 70 44 745.

E-mail address: d.rijken@erasmusmc.nl (D.C. Rijken).

[a] These authors contributed equally to this paper.

[b] All the members of the European network for vascular disorders of the liver (En-Vie) are listed in the appendix.

Abbreviations: BCS, Budd–Chiari syndrome; 2D-DIGE, two-dimensional difference gel electrophoresis; DIA, differential in-gel analysis; BVA, biological variation analysis; ACN, acetonitrile; MALDI-TOF, Matrix Assisted Laser Desorption/Ionization – Time of Flight; Apo A1, apolipoprotein A1; AST, aspartate aminotransferase; ALT, alanine aminotransferase; HDL, high density lipoprotein.

Introduction

Venous thrombosis is a frequent cause of morbidity and mortality in the Western world, with an incidence rate of approximately one per 1000 patient-year [1,2]. The most common manifestations of venous thrombosis are deep venous thrombosis of the lower extremities and pulmonary embolism. Other localizations, such as thrombosis of the cerebral or abdominal veins, are seen infrequently. When thrombosis involves the hepatic veins or the inferior vena cava, blocking the outflow of blood from the liver, it is referred to as Budd–Chiari syndrome (BCS) [3]. BCS is a rare vascular liver disorder but if left untreated, liver failure or death may ensue [4]. As is the case with thrombosis at other locations, various inherited and acquired factors have been identified that are associated with BCS [5–8]. Moreover, current evidence suggests that BCS is a multifactorial disease that often develops in the presence of more than one risk factor [9]. Although one or more underlying causes can be found in the majority of patients, there are still cases in which none of the known risk factors are present.

Fibrin clot formation is the final step of a complex cascade of reactions that represents the coagulation system. Dysregulation of any of the numerous components of the clotting cascade and the fibrinolytic system can potentially disrupt the hemostatic balance, leading to an increased tendency of either bleeding or thrombosis. Alterations in the plasma concentration of certain proteins influencing blood coagulation (e.g. elevated levels of factor VIII or a protein C deficiency) can, therefore, be involved in the onset of venous thrombosis [10,11]. Several proteins bind to fibrin as an essential step in their mechanism of action (e.g.

thrombin, factor XIII, plasminogen, and tissue-type plasminogen activator) [12]. Consequently, some prothrombotic abnormalities may also be reflected by changes in the concentration of proteins that bind to a plasma clot.

In this study, we aimed to identify novel factors that may play a role in venous thrombosis observed in patients with BCS by using a proteomic approach. To this end, we prepared plasma clots *in vitro* by addition of thrombin to freshly frozen plasma samples and compared the plasma clot composition of BCS-patients and healthy controls using two-dimensional fluorescence-based difference gel electrophoresis (2D-DIGE) [13]. Furthermore, the specific protein found to have the most significant decrease in abundance in cases versus controls was identified with mass-spectrometry. Subsequently, this finding was validated in a large case-control study using plasma samples of BCS-patients and controls.

Patients and methods

Materials

Urea, thiourea, CHAPS, DTT, and iodoacetamide were obtained from Fluka (St. Louis, MO, USA). Aprotinin (Trasylol) was obtained from Bayer (Leverkusen, Germany). Tris (PlusOne), CyDyes, DeStreak, IPG buffer pH 3–10, and immobiline strips were obtained from GE Healthcare (Uppsala, Sweden). The anchorchip plate, α-cyano-4-hydroxycinnamic acid matrix, and the Ultraflex-II apparatus were from Bruker Daltonics (Bremen, Germany). Thrombin was obtained from Sigma–Aldrich (St. Louis, MO, USA). Colloidal blue staining kit was obtained from Invitrogen (Paisley, UK) and Trypsin Gold was obtained from Promega Corporation (Madison, WI, USA).

Plasma samples from cases and controls used for in vitro clot formation

To examine the plasma clot composition of patients with BCS and healthy controls, blood samples were collected from nine consecutive patients with BCS admitted to the Department of Gastroenterology and Hepatology of the Erasmus Medical Center in Rotterdam, the Netherlands. For each patient, a healthy, non-related control person was recruited from department personnel. Controls were of the same sex, ethnicity, and age (with a range of 5 years) as the patient. Furthermore, controls did not have a previous history of thrombosis or malignancy and were not using oral contraceptives. Peripheral blood samples were obtained from both patients and controls by means of venapuncture and collected in tubes containing 0.11 M trisodium citrate. Platelet-free plasma was acquired by a two-step centrifugation method (10 min at 2,000*g* and 10 min at 20,000*g*) at 4 °C and subsequently stored at −70 °C until further analysis.

This study was conducted with approval from the ethics committee of the Erasmus University Medical Center. All patients and controls agreed to participate by means of a written informed consent.

Sample preparation for 2D-DIGE

Clots of 500 μl citrated plasma were prepared by adding calcium chloride (20 mM) and thrombin (1 NIH U/ml) for the initiation of coagulation and aprotinin (100 KIU/ml) to prevent proteolytic degradation [14]. After an incubation period of 2 h at room temperature, the clots were extensively washed by permeating them with 10 ml Tris-buffered saline (50 mM Tris–HCl, 100 mM NaCl, pH 7.4) containing aprotinin (100 KIU/ml) overnight at 4 °C. The clots were compacted by centrifugation, washed with deionized water and noncovalently clot-bound proteins were extracted with 150 μl rehydration buffer (7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 30 mM Tris–HCl, and pH 8.5) for 1 h at room temperature. Samples of 50 μl were labeled with the *N*-hydroxysuccinimide esters of Cy3 or Cy5 minimal fluorescent cyanine dyes. Five patient samples were labeled with Cy3 minimal dye and the other four patients with Cy5 minimal dye. The matched controls were labeled with Cy5 and Cy3 minimal dye, respectively. The samples were randomized to Cy3 and Cy5 labeling to minimize dye-based artifacts. Labeling was performed using 400 pmol dye per 50 μl sample, containing about 1.5 μg protein. Samples were labeled on ice for 30 min and quenched with 0.2 mM lysine. A pool of all nine patient and nine control samples was prepared and 50 μl was labeled with Cy2 minimal dye. This sample was used as an internal standard. For 2D-gel electrophoresis, each labeled patient sample was pooled with the labeled matched control sample and the labeled internal standard and analyzed simultaneously to reduce gel-to-gel variations. The total volume of sample was adjusted to 185 μl with rehydration buffer, 0.9% IPG-buffer 3–10 pH range, and 1.2% (v/v) DeStreak.

2D-DIGE

The nine pools containing the labeled samples of the BCS-patients, their matched controls, and the internal standard were run on nine different gels. The presence of the internal standard in each pool facilitated gel-to-gel matching. The proteins were separated in the first dimension with a 17 cm immobiline drystrip with a 3–10NL pH range. The isoelectric focusing was carried out at 20 °C on the IPGphore II with the following running protocol: 12 h at 30 V (rehydration), 1 h at 500 V, 1 h at 1000 V, and 8 h at 8000 V, with a 50 μA limit per gel.

The strips were equilibrated in equilibration buffer (6 M urea, 50 mM Tris–HCl pH 8.8, 20% (v/v) glycerol, and 2% (w/v) SDS) with 1% (w/v) DTT for 15 min followed by a second equilibration step with equilibration buffer with 1% (w/v) iodoacetamide for 15 min. For the second dimension the strips were run on 10.5% polyacrylamide gels (20 × 22.4 cm) at 12 mA/2 gels constant current for 18.5 h.

Image acquisition and analysis

After 2D-DIGE separation, gels were scanned using Typhoon 9410 (Amersham Pharmacia Biotech) at 100-micron resolution. 2-D images from the Cy2-, Cy3-, and Cy5-labeled protein fractions were scanned using a 488, 532, and 633 nm laser, respectively. Gel images were cropped using ImageQuant TL software and image analysis was performed with Decyder V6.5 software (Amersham Pharmacia Biotech). Spot detection was performed using the differential in-gel analysis (DIA) module by setting 7500 as the estimated number of spots. The Cy2, Cy3, and Cy5 images of each gel were merged and spot boundaries were detected. Spots resulting from a non-protein source, like dust particles, were filtered out by removing spots with a slope greater than 1.3. The gel with the highest spot-count was assigned as the master gel, which was used as a template. Gel-to-gel matching of the standard spot maps of each gel was performed using the biological variation analysis (BVA) software module to ensure that the same protein spots were compared between gels. Normalized Cy3 and Cy5 spot volumes were compared to the corresponding Cy2 standard spot volume within each gel, which gave a standardized abundance.

Mass spectrometry

Preparative gels were run with 185 μl unlabeled internal standard following the same procedure as described above. The gels were stained with Colloidal blue staining kit for 3 h and destained with deionized water overnight, as recommended by the manufacturer.

For mass spectrometry, protein spots were manually excised from the preparative gel, washed in deionized water, and destained in 30% (v/v) acetonitrile (ACN)/50 mM NH_4HCO_3. Destained gel pieces were washed briefly with deionized water, vacuum dried and rehydrated in 4 μl trypsin digest solution (75 μg/ml trypsin gold in 20 mM NH_4HCO_3, pH 8.0) for digestion overnight at room temperature. Peptide extraction was performed with 5 μl of 50% ACN/0.1% trifluoroacetic acid. The extracted sample was spotted on an anchorchip plate with saturated α-cyano-4-hydroxycinnamic acid matrix solution in 100% ACN (1:1). Digested peptide fragments were analyzed in a Matrix Assisted Laser Desorption/Ionization – Time of Flight (MALDI-ToF) mass spectrometer using an Ultraflex-II apparatus. Flexanalysis 2.4 and Biotools 3.1 software were used for data processing. The obtained mass spectra were analyzed using peptide mass finger print spectra with the online Matrix Science Database with MASCOT software (www.matrixscience.com). The MSDB database 20060831 (3239079 sequences; 1079594700 residues) was searched with the Mascot parameters set as follows: Taxonomy, homo sapiens; mass tolerance, 100 ppm; maximum one missed cleavage per peptide; fixed modification of carboxymethylation of cysteine residues; variable modification of partial oxidation of methionine residues. Scores above MSDB database threshold of 64 were considered significant (p <0.05).

Plasma samples from a large case-control study

To validate findings from the proteomic study in a larger case-control population, we used plasma samples from the EN-Vie Study. The EN-Vie Study, as described previously, is a prospective multicenter observational study of patients with BCS [8,15]. During the study period of two years, a total of 163 newly diagnosed patients with BCS were included from nine European countries. Apart from

recorded data on clinical parameters, underlying etiology, and treatment outcome, blood samples were also collected. Furthermore, each enrolled patient was asked to provide a sex- and age-matched (with a range of five years) control person. Controls had to be of the same race as their matched cases and had to have no previous history of thrombosis. If patients were unable to provide a control person themselves, the national study coordinating centers attempted to find equally matched controls from their own resources. Blood samples were obtained from all control subjects. As for patients, blood samples were collected through venapuncture in tubes containing 0.11 M trisodium citrate. From all blood samples, plasma was acquired by centrifugation at 2000*g* for 10 min. Plasma samples were stored at −70 °C at one central facility until analysis.

The EN-Vie Study was conducted with approval from all national and, if necessary, local ethical committees, in accordance with the nation-specific rules. All patients and controls agreed to participate in the study by means of a written informed consent. For this case-control study, only patients for whom stored plasma samples and a matched control person were available were considered eligible.

Measurement of apolipoprotein A1 and HDL cholesterol levels in plasma

In all plasma samples, those from the cases and controls used for *in vitro* clot formation and 2D-DIGE and those from the EN-Vie study, the concentration of apolipoprotein A1 (apo A1) was determined with a Beckman Coulter nephelometer using commercially available monoclonal antibodies. Levels of HDL cholesterol were measured in the samples from the EN-Vie study with an enzymatic colorimetric test (HDL-C plus 3rd generation, Roche Diagnostics, Mannheim, Germany) and expressed as percentage of the mean level of the control group. This level amounted to 1.0 mM, but might be somewhat underestimated because of the use of citrate in the samples.

Miscellaneous methods

Data on plasma levels of albumin, total bilirubin, aspartate aminotransferase (AST), alanine aminotransferase (ALT), and factor V were obtained from the EN-Vie Study database. All these assays were performed locally. To correct for possible variation between different laboratory assessments, values of AST and ALT were analyzed as a fraction of the upper limit of normal, which was calculated using the local cut-off values.

Statistical analyses

The protein abundance in plasma clots of patients with BCS and controls was compared using the Student's *T*-test within the Decyder software. Differences in apo A1 levels in plasma were tested using the non-parametric Mann–Whitney *U* Test for the nine BCS patients or the Student's *T*-test for the larger cohort of BCS-patients from the EN-Vie study. Spearman's Rho correlation coefficients were calculated as a non-parametric measure of correlation between apo A1 levels and liver function tests. A *p*-value of <0.05 was considered statistically significant. All statistical analyses were performed with the Statistical Package for Social Sciences for Windows, version 15.0 (SPSS, Chicago, IL).

Results

Plasma samples used for *in vitro* clot formation were collected from nine patients with BCS, seven females and two males, with a median age at diagnosis of 35 years (range 16–54 years). Underlying etiologic factors were myeloproliferative disorders ($n = 4$), homozygous Factor V Leiden mutation ($n = 1$), antiphospholipid antibodies ($n = 3$), and oral contraceptives ($n = 2$). In two patients, no risk factors could be identified and in three patients two prothrombotic factors were present. The median age of the controls (seven females and two males) was 31 years (range 22–49 years).

Differences in plasma clot composition between BCS-patients and controls

After *in vitro* plasma clot formation, the protein composition of clots from patients with BCS was compared to that of plasma clots from controls. On the master gel, 1369 different protein spots were detected after 2D-DIGE analysis. All other gels of case-control pairs were matched and compared with the master gel and this resulted in the detection of 26 protein spots that differed statistically significantly ($p < 0.001$) in abundance between patients and controls (Fig. 1). The protein spot with the most significant decrease ($p = 6.6 \times 10^{-5}$) was spot number 2 (Table 1). The mean value for the standardized abundance of patient samples for this spot was 0.70 as compared to 1.65 for the controls, corresponding to a 2.4-fold lower abundance in patient samples (Fig. 2). This protein spot was excised from preparative gels and analyzed using MALDI-TOF-MS. The protein was identified as apolipoprotein A1 (apo A1) with the following identification details; NCBI gi|90108664, mass 28061 Da, Mascot score 76-237, number of peptides matched 18–24, sequence coverage (%) 54–81, with a *p*-value between 3e-19 and 0.0036 ($n = 4$). The other 25 spots with a statistically significant difference in protein abundance between patients and controls have not yet been identified.

Plasma levels of apo A1 and HDL cholesterol

The low abundance of apo A1 in plasma clots of BCS-patients was also seen in plasma levels. Mean (±SD) plasma level of apo A1 in these nine cases was significantly lower than in the controls, 0.74 (±0.21) g/L vs. 1.45 (±0.31) g/L, respectively ($p = 0.002$), corresponding to a 2.0-fold difference in plasma levels.

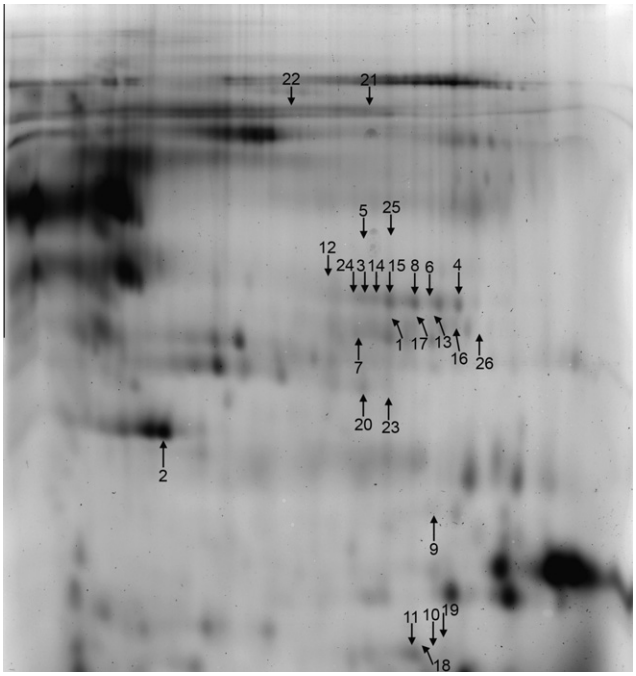

Fig. 1. Image of the master gel of 2D-DIGE, displaying proteins that significantly differed in abundance between BCS-patients and controls, as analyzed with Decyder software. The arrows on the 10.5% polyacrylamide gel indicate the 26 protein spots that display a statistically significant difference ($p < 0.001$) between patients and controls. Some arrows point to a protein spot that is not visible on this scan of the master gel, however, with Decyder software a protein spot was detected and shown to be different between patients and controls. Spot number 2 was identified with mass spectrometry as apolipoprotein A1 with a mass of 28 kDa. The other 25 protein spots have not yet been identified.

Table 1. Protein spots with a statistically significant difference in abundance between controls and patients with BCS as analyzed with Decyder software.

Spot number	Average ratio (controls/patients)	*p* value
1	- 4.31	2.70E-05
2	2.35	6.60E-05
3	- 6.94	0.00013
4	- 13.19	0.00013
5	- 4.34	0.00014
6	- 12.49	0.00016
7	- 8.15	0.00016
8	- 7.27	0.00018
9	- 8.10	0.00019
10	- 3.64	0.00024
11	- 2.63	0.00030
12	- 5.10	0.00032
13	- 4.22	0.00034
14	- 9.67	0.00037
15	- 5.01	0.00037
16	- 4.96	0.00047
17	- 4.34	0.00050
18	- 3.70	0.00055
19	- 2.45	0.00061
20	- 2.73	0.00073
21	2.35	0.00076
22	3.09	0.00077
23	- 4.08	0.00079
24	- 5.51	0.00081
25	- 3.21	0.00090
26	- 3.03	0.00097

The 26 protein spots that significantly (p <0.001) differed between patients with BCS-syndrome and controls are displayed. The average ratio is the ratio of the standardized abundance of controls and patients. Positive ratios indicate that the standardized abundance in controls is higher than in patients. Negative ratios indicate that the protein abundance in patients is higher than in controls. The p-value is calculated using the Student's T-test in the BVA module of the DeCyder software.

To validate these findings we used plasma samples from the EN-Vie Study cohort. From this study, plasma samples for measurement of apo A1 levels were available for 107 patients. However, for six patients there was no control person and these patients were excluded from the analysis, leaving 101 eligible case-control pairs. Mean age of the patient population was 38 years (range 16–83) and 42% were males. Results from the apo A1 assay are shown in Fig. 3A. Patients with BCS had significantly lower apo A1 levels in plasma as compared to the controls (mean ± SD 0.97 ± 0.36 g/L vs. 1.32 ± 0.24 g/L, p <0.0001). The pattern of the patient values might suggest the existence of a subgroup with low levels of apo A1. When the group of patients with apo A1 levels below 0.85 g/L were compared to those with higher apo A1 levels, it became clear that the subgroup of cases with lower apo A1 levels included relatively more patients with a myeloproliferative disorder (20 out of 36 (56%) vs. 15 out of 63 (24%), respectively, p = 0.001). No other differences in underlying etiology of thrombosis or characteristics at diagnosis were found between these two apparent subgroups.

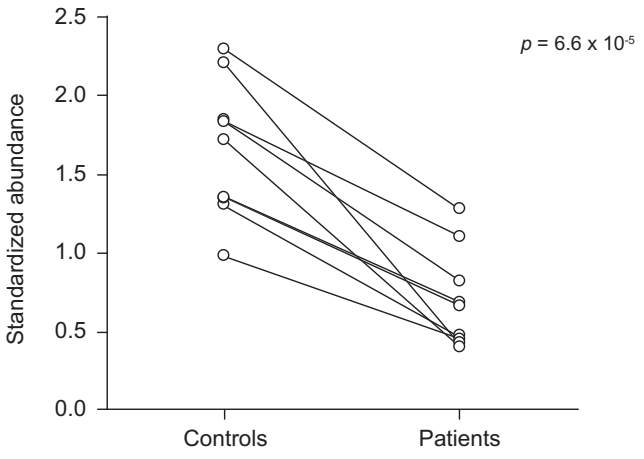

Fig. 2. Protein abundance of apolipoprotein A1 of BCS-patients and their matched controls. Statistical analysis was performed using the BVA module of DeCyder software and shows on average a 2.4-fold lower abundance for apolipoprotein A1 in the patient plasma clots (mean abundance of 0.70) compared to control plasma clots (mean abundance of 1.65). Individual data points are indicated and lines connect the standardized abundance data of patients with their matched controls that were analyzed on a single gel.

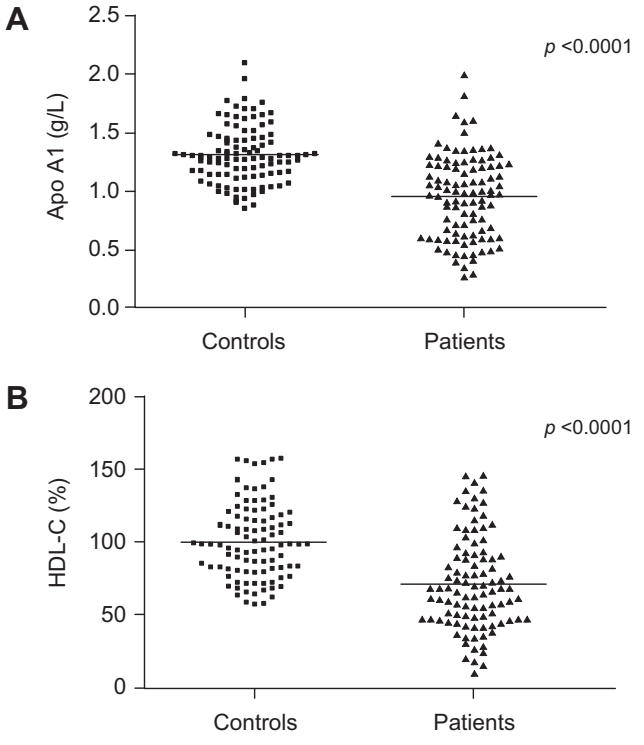

Fig. 3. Apolipoprotein A1 levels (A) and HDL cholesterol levels (B) in plasma of patients with Budd–Chiari syndrome and healthy controls. Individual data points and mean values are given.

The plasma levels of apo A1 correlated with plasma levels of HDL cholesterol (HDL-C) in BCS-patients from the EN-Vie study cohort (Pearson's correlation coefficient of 0.863, p <0.0001). Mean (±SD) HDL-C levels were 71% (±32) in patients as compared to 100% (±26) in controls (p <0.0001) (Fig. 3B).

Table 2. Correlation between apo A1 levels and parameters of liver function in patients with BCS.

	Apo A1		
	n	Spearman's Rho	*p*
Albumin	99	0.319	0.005
Total bilirubin	97	-0.211	0.038
ALT	87	-0.197	0.067
AST	86	-0.217	0.044
Factor V	28	0.327	0.089

Apo A1, apolipoprotein A1; BCS, Budd–Chiari syndrome; ALT, alanine aminotransferase; AST, aspartate aminotransferase.

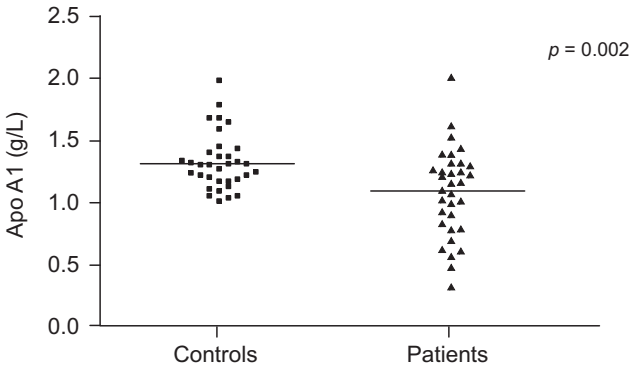

Fig. 4. Apolipoprotein A1 levels in plasma of cases with normal albumin levels (>35 g/L; *n* = 34) compared to their healthy controls. Individual data points and mean values are given.

Apo A1 levels and liver function tests

Because apo A1 is synthesized by the liver, an impaired liver function may influence the plasma levels. To estimate the effect of decreased hepatic synthetic function on apo A1 levels in patients with BCS, we determined the correlation between apo A1 and different parameters of liver function in the EN-Vie study cohort. As shown in Table 2, there was an association between apo A1 levels and several parameters of liver function. However, the correlations were only weak, with correlation coefficients lower than 0.35. Furthermore, when we compared the apo A1 levels of cases with normal albumin levels (>35 g/L, $n = 34$) to their matched controls, BCS-patients still had significantly lower apo A1 levels than their healthy controls (1.08 ± 0.36 g/L vs. 1.31 ± 0.23 g/L, $p = 0.002$) (Fig. 4). To assure that albumin levels were not artificially elevated, we assessed whether patients had received prior albumin infusion. None of the cases with albumin levels in the normal range had been treated with albumin.

Discussion

In this study, we have used a proteomic approach to investigate differences in proteins bound to a plasma clot in order to detect novel players in the regulation of hemostasis that may be associated with the development of venous thrombosis in BCS. To our knowledge this is the first report on the analysis of plasma clot proteins in patient samples using proteomics as a detection method. Previously, we have shown that this technique could be used to identify new fibrin-binding proteins in plasma [14]. In 2D-DIGE, an internal standard is run on each gel together with a patient sample and a control sample. This allows a direct comparison of protein abundance between a series of samples without interference by gel-to-gel variation. Using 2D-DIGE, nine samples of patients with BCS were compared with samples of their matched controls. A total of 26 protein spots significantly differed ($p < 0.001$) in abundance between cases and controls and were either increased or decreased in the patient samples. The protein spot displaying one of the most significant differences between both groups was identified with mass spectrometry as apo A1. The other 25 protein spots, with a different abundance in cases and controls, still need to be identified. The standardized protein abundance of apo A1 was reduced by a factor 2.4 in BCS-patient samples. A similar reduction was found in the plasma concentration of apo A1 in these nine patients. The findings of the first part of this study were confirmed in a validation study by measuring the concentration of apo A1 in plasma in a large cohort of 101 BCS-patients. Apo A1 was found to be significantly decreased compared to the healthy, matched controls. HDL cholesterol levels were also found to be decreased in patients compared to controls, and correlated well with apo A1 plasma levels, as seen before [16].

Information on specific lifestyle factors was not recorded, however, patients and controls were of the same age and race and were recruited from the same geographical area. In some patients with BCS, hepatic synthetic function may be impaired as a result of venous thrombosis, which can result in venous congestion and hepatocyte necrosis. Because apo A1, like albumin, is one of the main proteins synthesized by the liver, the observed differences in plasma levels of apo A1 between BCS-patients and healthy controls could have been caused by the liver disease in the former group. However, in our study group there was only a weak correlation between apo A1 levels in plasma and different liver function tests. Moreover, in a subgroup of BCS-patients with normal albumin levels, the concentration of apo A1 was still significantly lower as compared to their healthy matched controls. Due to the marked clinical heterogeneity of BCS and the finding that many patients have a more or less acute-on-chronic form of disease presentation [15,17], it is difficult to clearly distinguish between acute and chronic forms of BCS. Still, when we compared patients with an acute onset of symptoms to those with a more chronic development of symptoms, apo A1 levels were comparable between both groups (data not shown). Overall, the decreased plasma concentration of apo A1 and also the decrease in standardized protein abundance of apo A1 found in plasma clots of BCS-patients cannot entirely be explained by an impaired liver synthetic function in these patients.

A low apo A1 level has previously been reported as a marker of liver fibrosis [18,19]. Therefore, we cannot exclude that fibrosis might partially explain the low levels of apo A1 found in BCS-patients. Nevertheless, we found no association between apo A1 levels and the presence of fibrosis and/or cirrhosis in a subgroup of 26 BCS-patients of whom a liver biopsy sample was available (data not shown). Hence, we believe that low levels of apo A1, next to other risk factors, might play a causal role in the development of venous thrombosis in BCS, in addition to being a consequence of venous thrombosis. It is of interest that apo A1 levels appeared to be particularly low in BCS-patients with an underlying myeloproliferative disorder. It has been

shown that in myeloproliferative disorders, which represent a major risk factor for BCS, HDL levels can be markedly decreased due to an increased catabolism of apo A1 [20]. Further studies are needed to clarify this association and its potential role in the development of venous thrombosis.

The mechanism through which decreased levels of apo A1 could result in venous thrombosis in patients with BCS is not yet clear. Apo A1 is the main protein component of the reverse cholesterol transporter HDL. For arterial thrombosis, plasma levels of HDL are known to be inversely related with risk [21]. For venous thrombosis, the role of HDL and apo A1 is less clear. However, there are strong indications that the same inverse relation between plasma levels and thrombosis risk in arterial thrombosis is seen with venous thrombosis [22–24]. Furthermore, it is thought that arterial thrombosis and venous thrombosis share common risk factors [24,25]. In a recent study by Eichinger et al., low levels of apo A1 in plasma were associated with a significantly higher risk of recurrent venous thrombosis [26]. These data are in conflict with the results of a population-based prospective study that did not find an association between HDL cholesterol and venous thromboembolism [27]. HDL can be involved in hemostasis in several ways [28]. One way, shown by Griffin et al., is that HDL can enhance the activated protein C pathway [29]. This pathway is part of the natural anticoagulant system and activation results in a prolongation of the prothrombin time, which is correlated with the plasma levels of apo A1 [29]. Consequently, when apo A1 concentration in plasma is decreased, this may potentially result in an impaired hemostatic balance and, thereby an increased tendency for thrombosis. Previous studies have shown that defects in the protein C pathway may, indeed, result in BCS, both in an experimental animal model [30] and in humans [5]. Another potential mode of action, recently published by Dahlbäck and colleagues, is that anionic phospholipids lose their procoagulant properties when incorporated into HDL [31]. Still, further studies are required to elucidate the exact mechanism through which apo A1 interacts with the hemostatic system, including the possible role of the clot binding of apo A1.

In conclusion, using 2D-DIGE as a detection method, we have shown that the protein composition of *in vitro* formed plasma clots differs between patients with BCS and healthy controls. Apo A1 is significantly less abundant in plasma clots of BCS-patients and this difference is caused by lower plasma levels of apo A1 in this group. Although the precise causative mechanism has not yet been elucidated, this is the first evidence that decreased apo A1 levels may play a role in the development of venous thrombosis in patients with BCS. Decreased apo A1 levels may also contribute to other manifestations of venous thrombosis.

Conflict of interest

The authors who have taken part in this study declared that they do not have anything to disclose regarding funding or conflict of interest with respect to this manuscript.

Acknowledgements

The EN-Vie project was financially supported by the Fifth Framework Program of the European Commission (contract number QLG1-CT-2002-01686). S. Darwish Murad is a member of the Mosaic Program of the Netherlands Organization for Scientific Research (NWO). Ciberehd is funded by Instituto de Salud Carlos III.

Appendix

The EN-Vie Study Group consists of the following members: Steering Committee – D.C. Valla, H.L.A. Janssen, J.C. Garcia-Pagan; Investigators: *Belgium* – P. Langlet, L. Lasser, Centre Hospitalier Universitaire Brugmann, Bruxelles; *France* – D.C. Valla, A. Plessier, B. Condat, Hopital Beaujon, AP-HP, INSERM-U773 & University Paris-7, Clichy; *Germany* – M. Bahr, Hannover Medical School, Hannover; J. Heller, J. Trebicka, University Hospital of Bonn, Bonn; M. Rössle, University of Freiburg, Freiburg; *Italy* – M. Primignani, F. Fabris, Ospedale Policlinico, Mangiagalli and Regina Elena Foundation, Milan; *the Netherlands* – H.L.A. Janssen, S. Darwish Murad, J. Hoekstra, F.W.G Leebeek, Erasmus University Medical Center, Rotterdam; F.R. Rosendaal, Leiden University Medical Center, Leiden; *Portugal* – H. Miranda, Hospital General Santo Antonio, Porto; *Spain* – J.C. Garcia-Pagan, M. Hernandez-Guerra, S. Raffa, R. Lozano, Hospital Clinic, Barcelona; *Switzerland* – A. Hadengue, I. Morard, G. Mentha, Geneva University Hospitals, Geneva; *United Kingdom* – E. Elias, C.E. Eapen, Queen Elisabeth Hospital Birmingham, Birmingham.

References

[1] Naess IA, Christiansen SC, Romundstad P, Cannegieter SC, Rosendaal FR, Hammerstrom J. Incidence and mortality of venous thrombosis: a population-based study. J Thromb Haemost 2007;5 (4):692–699.
[2] Anderson Jr FA, Wheeler HB, Goldberg RJ, Hosmer DW, Patwardhan NA, Jovanovic B, et al. A population-based perspective of the hospital incidence and case-fatality rates of deep vein thrombosis and pulmonary embolism. The Worcester DVT Study. Arch Intern Med 1991;151 (5):933–938.
[3] Janssen HL, Garcia-Pagan JC, Elias E, Mentha G, Hadengue A, Valla DC. Budd–Chiari syndrome: a review by an expert panel. J Hepatol 2003;38 (3):364–371.
[4] Valla DC. Hepatic vein thrombosis (Budd–Chiari syndrome). Semin Liver Dis 2002;22 (1):5–14.
[5] Janssen HL, Meinardi JR, Vleggaar FP, van Uum SH, Haagsma EB, van Der Meer FJ, et al. Factor V Leiden mutation, prothrombin gene mutation, and deficiencies in coagulation inhibitors associated with Budd–Chiari syndrome and portal vein thrombosis: results of a case-control study. Blood 2000;96 (7):2364–2368.
[6] De Stefano V, Teofili L, Leone G, Michiels JJ. Spontaneous erythroid colony formation as the clue to an underlying myeloproliferative disorder in patients with Budd–Chiari syndrome or portal vein thrombosis. Semin Thromb Hemost 1997;23 (5):411–418.
[7] Deltenre P, Denninger MH, Hillaire S, Guillin MC, Casadevall N, Briere J, et al. Factor V Leiden related Budd–Chiari syndrome. Gut 2001;48 (2): 264–268.
[8] Hoekstra J, Guimaraes AH, Leebeek FW, Darwish Murad S, Malfliet JJ, Plessier A, et al. Impaired fibrinolysis as a risk factor for Budd–Chiari syndrome. Blood 2010;115 (2):388–395.
[9] Denninger MH, Chait Y, Casadevall N, Hillaire S, Guillin MC, Bezeaud A, et al. Cause of portal or hepatic venous thrombosis in adults: the role of multiple concurrent factors. Hepatology 2000;31 (3):587–591.
[10] Koster T, Rosendaal FR, Briet E, van der Meer FJ, Colly LP, Trienekens PH, et al. Protein C deficiency in a controlled series of unselected outpatients: an infrequent but clear risk factor for venous thrombosis (Leiden Thrombophilia Study). Blood 1995;85 (10):2756–2761.
[11] Tsai AW, Cushman M, Rosamond WD, Heckbert SR, Tracy RP, Aleksic N, et al. Coagulation factors, inflammation markers, and venous thromboembolism: the longitudinal investigation of thromboembolism etiology (LITE). Am J Med 2002;113 (8):636–642.
[12] Rijken DC, Lijnen HR. New insights into the molecular mechanisms of the fibrinolytic system. J Thromb Haemost 2009;7 (1):4–13.
[13] Alban A, David SO, Bjorkesten L, Andersson C, Sloge E, Lewis S, et al. A novel experimental design for comparative two-dimensional gel analysis: two-dimensional difference gel electrophoresis incorporating a pooled internal standard. Proteomics 2003;3 (1):36–44.
[14] Rijken DC, Dirkx SP, Luider TM, Leebeek FW. Hepatocyte-derived fibrinogen-related protein-1 is associated with the fibrin matrix of a plasma clot. Biochem Biophys Res Commun 2006;350 (1):191–194.

[15] Darwish Murad S, Plessier A, Hernandez-Guerra M, Fabris F, Eapen CE, Bahr MJ, et al. Etiology, management, and outcome of the Budd–Chiari syndrome. Ann Intern Med 2009;151 (3):167–175.
[16] Phillips NR, Havel RJ, Kane JP. Serum apolipoprotein A-I levels: relationship to lipoprotein lipid levels and selected demographic variables. Am J Epidemiol 1982;116 (2):302–313.
[17] Langlet P, Escolano S, Valla D, Coste-Zeitoun D, Denie C, Mallet A, et al. Clinicopathological forms and prognostic index in Budd–Chiari syndrome. J Hepatol 2003;39 (4):496–501.
[18] Bedossa P, Poynard T, Abella A, Aubert A, Pignon JP, Naveau S, et al. Apolipoprotein AI is a serum and tissue marker of liver fibrosis in alcoholic patients. Alcohol Clin Exp Res 1989;13 (6):829–833.
[19] Poynard T, Bedossa P, Mathurin P, Ratziu V, Paradis V. Apolipoprotein A1 and hepatic fibrosis. J Hepatol 1995;22 (2 Suppl):107–110.
[20] Ginsberg HN, Le NA, Gilbert HS. Altered high density lipoprotein metabolism in patients with myeloproliferative disorders and hypocholesterolemia. Metabolism 1986;35 (9):878–882.
[21] Eisenberg S. High density lipoprotein metabolism. J Lipid Res 1984;25 (10):1017–1058.
[22] Deguchi H, Pecheniuk NM, Elias DJ, Averell PM, Griffin JH. High-density lipoprotein deficiency and dyslipoproteinemia associated with venous thrombosis in men. Circulation 2005;112 (6):893–899.
[23] Doggen CJ, Smith NL, Lemaitre RN, Heckbert SR, Rosendaal FR, Psaty BM. Serum lipid levels and the risk of venous thrombosis. Arterioscler Thromb Vasc Biol 2004;24 (10):1970–1975.
[24] Ageno W, Becattini C, Brighton T, Selby R, Kamphuisen PW. Cardiovascular risk factors and venous thromboembolism: a meta-analysis. Circulation 2008;117 (1):93–102.
[25] Prandoni P, Bilora F, Marchiori A, Bernardi E, Petrobelli F, Lensing AW, et al. An association between atherosclerosis and venous thrombosis. N Engl J Med 2003;348 (15):1435–1441.
[26] Eichinger S, Pecheniuk NM, Hron G, Deguchi H, Schemper M, Kyrle PA, et al. High-density lipoprotein and the risk of recurrent venous thromboembolism. Circulation 2007;115 (12):1609–1614.
[27] Chamberlain AM, Folsom AR, Heckbert SR, Rosamond WD, Cushman M. High-density lipoprotein cholesterol and venous thromboembolism in the Longitudinal Investigation of Thromboembolism Etiology (LITE). Blood 2008;112 (7):2675–2680.
[28] Mineo C, Deguchi H, Griffin JH, Shaul PW. Endothelial and antithrombotic actions of HDL. Circ Res 2006;98 (11):1352–1364.
[29] Griffin JH, Kojima K, Banka CL, Curtiss LK, Fernandez JA. High-density lipoprotein enhancement of anticoagulant activities of plasma protein S and activated protein C. J Clin Invest 1999;103 (2):219–227.
[30] Isermann B, Hendrickson SB, Zogg M, Wing M, Cummiskey M, Kisanuki YY, et al. Endothelium-specific loss of murine thrombomodulin disrupts the protein C anticoagulant pathway and causes juvenile-onset thrombosis. J Clin Invest 2001;108 (4):537–546.
[31] Oslakovic C, Krisinger MJ, Andersson A, Jauhiainen M, Ehnholm C, Dahlback B. Anionic phospholipids lose their procoagulant properties when incorporated into high density lipoproteins. J Biol Chem 2009;284 (9):5896–5904.