PRESENCE OF PORTAL VEIN THROMBOSIS IN LIVER CIRRHOSIS IS STRONGLY ASSOCIATED WITH LOW LEVELS OF ADAMTS-13: A PILOT STUDY

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Abstract

Portal vein thrombosis (PVT) dramatically changes the prognosis of cirrhotic patients, especially those waiting for liver transplantation. However, the possible contribution to PVT of von Willebrand factor (VWF) and ADAMTS-13 is poorly documented. The aim of our study was to assess the presence of alterations of VWF and ADAMTS-13 serum levels in cirrhotic patients with PVT. Twenty-four patients with PVT (group A) and 60 without PVT (group B) were enrolled. Comprehensive analysis of biochemical and haemostatic parameters was performed. ADAMTS-13 activity was significantly lower in group A (median=16.8% vs. 69.1%, p=0.0047). Group A, compared to group B, showed a significantly higher VWF:act, (median:308.4% vs 203.3%, p=0.032), whereas no difference was observed for VWF:Ag, FVIII level and presence of risk factors for venous thromboembolism. No correlation was found between the Child-Pugh score and ADAMTS-13 activity. In multivariable logistic regression analysis performed on data concerning both group A and B only the ADAMTS-13 activity (p=0.007) resulted to be independently associated with PVT.

In conclusion, ADAMTS-13 activity is independently associated with PVT in cirrhotic patients. Prospective investigations are needed to clarify whether ADAMTS-13 level could be considered a predictive biomarker of PVT development in cirrhosis.

List of abbreviations in the order of appearance:
PVT: Portal Vein Thrombosis; VWF: von Willebrand factor; ADAMTS-13: A Disintegrin And Metalloprotease with Thrombospondin 1 repeats Nr. 13; VTE: Venous Thromboembolism
VWF:act: VWF activity; VWF:Ag: VWF antigen; UL-VWF: Ultra large VWF; PT: Prothrombin Time; APTT: Activated Partial Thromboplastin Time; US: Ultrasound.

Introduction

Von Willebrand factor (VWF), a multimeric glycoprotein mostly secreted by activated endothelial cells, supports platelet adhesion and aggregation in a high shear stress environment [1]. The hemostatic and thrombogenic potential of VWF depends on its multimer size, which is regulated by ADAMTS-13 (A Disintegrin And Metalloprotease with Thrombospondin 1 repeats Nr. 13), which cleaves VWF multimers to smaller forms having lower thrombogenic activity [2]. After secretion, VWF is indeed cleaved into the smaller multimers by ADAMTS-13 at the peptide bond between Tyr1605 and Met1606 within the VWF-A2 domain [3,4]. The lack of ADAMTS-13 induces excess expression and activity of ultra-large (UL)-VWF multimers and may result in microcirculatory disturbance due to formation of platelet/VWF-rich thrombi in microvasculature. Early northern blotting studies indicated that ADAMTS-13 mRNA is expressed only in the liver [5], specifically in hepatic stellate cells (Itoh cells) [6]. However, ADAMTS-13 was also identified in vascular endothelial cell [7], platelets [8], and kidney podocytes [9]. Severe decrease of ADAMTS-13 level is a feature of thrombotic thrombocytopenic purpura (TTP), an inherited or acquired disorder characterized by the massive formation of platelet/VWF-rich thrombi in the microcirculation, causing severe thrombocythopenia, microangiopathic hemolytic anemia, renal failure and neurological disturbances [2,10]. A partial deficiency of ADAMTS-13 has also been reported during pregnancy, severe sepsis, multiple organ failure and chronic liver diseases [11-15]. The role of ADAMTS-13 in chronic liver disease is debated [15-18], although in most clinical studies the severity of liver fibrosis and function decline was associated with decreased ADAMTS-13 levels [15,17-19]. In addition, systemic inflammatory processes may directly contribute to reduce ADAMTS-13 expression and activity, as found in clinical and experimental studies [14,20,21]. In contrast with ADAMTS-13, VWF levels in cirrhosis are generally elevated [22,23] and this may account for a prothrombotic state, especially in advanced disease. Portal vein thrombosis (PVT) is a pathological event that dramatically changes the course and prognosis of cirrhotic patients, especially if they are waiting for liver transplantation. The prevalence of PVT in cirrhotic patients is often associated with worsening of liver function and has been shown to be as high as 15% at pathological analysis of the explanted liver in patients undergone to liver transplantation [24]. Several studies in the past few years have been published about the association of risk factors for venous thromboembolism (VTE) with occurrence of PVT in cirrhosis and chronic liver diseases [25-30]. Only the reduction of blood flow in the portal vein was found significantly associated with development of PVT [31,32]. Furthermore, most of the studies concerning the possible association of PVT with thrombophilic factors in liver cirrhosis were not performed in series of consecutive patients and a systematic investigation of the possible contribution of VWF and ADAMTS-13 to the pathogenesis of PVT in this setting is scarcely documented. Thus, we investigated the possible contribution of both classical risk factors for VTE and thrombotic microangiopathies (VWF/ADAMTS-13 system) to the pathogenesis of PVT in a series of cirrhotic patients.

Materials and Methods

Patients

Twenty-four Caucasian patients with PVT and liver cirrhosis of any etiology, diagnosed using ultrasound and/or histological criteria and observed in the Liver Unit of the "A. Gemelli" University Hospital from January to December 2013, were included in this study (Group A). Sixty cirrhotic patients without PVT observed during the same period and agreed to participate to the study served as control group (Group B) (Table 1). All patients signed an informed consent; the study protocol was approved by the local Ethics Board Committee and conforms to the ethical guidelines of the 1975 Declaration of Helsinki. The severity of liver function failure was assessed using the Child-Pugh score [33]. All patients had not actual or previous hepatocellular carcinoma or any other malignancy, were not treated with anticoagulant/antiplatelet drugs and did not suffer from congenital haemostatic disorders (haemophilia A/B, Von Willebrand disease, other inherited coagulation factor deficiency) or severe thrombocythopenia (<30000 plt/µl). Upper

gastrointestinal endoscopy was performed in all patients; the size of esophageal varices was recorded as well as the presence of previous episodes of gastrointestinal bleeding. The diagnosis of PVT was established using Color Doppler US. The US criteria for the diagnosis of PVT were the detection of echogenic material within one or more of the following vessels: portal trunk, right portal branch, left portal branch. Color and pulsed Doppler analysis allowed to confirm the diagnosis and to distinguish partial from complete obstructive thrombosis and in all cases the US Doppler diagnosis of PVT was confirmed by contrast enhanced abdomen computed tomography. The presence and/or extension of the thrombosis to the other major vessels of the portal venous system (mesenteric or splenic vein) were registered in all patients. PVT was classified according to the extension of thrombosis, as previously reported (Table 1) [34].

Coagulation and thrombophilia factors

Determinations of PT, APTT, fibrinogen, antithrombin, protein C, protein S, lupus anticoagulant (performed by diluted Russel viper venom clotting time), FVIII (two-stage chromogenic assay, Coamatic FVIII, Werfen Group, Milano, Italy) and D-dimer were carried out using commercial kits from Instrumentation Laboratory S.p.A. (Milan, Italy) on an automatic coagulometer (Top, Instrumentation Laboratory). VWF-antigen (VWF:Ag) and VWF:activity (VWF:act) were measured by chemiluminescence assays (Instrumentation Laboratory, Milan, Italy) [35] with an automatic chemiluminometer (Acustar, Instrumentation Laboratory). The inter-assay coefficients of variation of these assays were 5-7%. VWF:act, was highly correlated with the traditional platelet aggregation test (Ristocetin Cofactor, $\rho=0.78$, $n=50$, $p<0.001$), and highly sensitive to low levels of VWF:act [36]. VWF multimer (VWFm) pattern was measured as previously reported [37]. FV Leiden R506Q and prothrombin G20210A genotyping of these polymorphisms was performed by using commercial kits based on PCR-RT-based method (from EliTechGroup S.p.A., Torino, Italy) on an automatic instrument (Model 7300, Applied Biosystem, Foster City, CA, USA).

Measurement of ADAMTS-13 activity

ADAMTS-13 activity was measured by a fluorescence resonance energy transfer (FRET)-based assay using a VWF86 amino-acid peptide substrate (Instrumentation Laboratory) in a Varian Eclipse spectrofluorometer, as previously detailed [38,39] and expressed as % of normal plasma. The samples were diluted tenfold in the appropriate buffer for the determination of ADAMTS-13 activity. Moreover, due to the presence in cirrhotic patients of abnormal levels of bilirubin, a correction factor was introduced to calculate the real ADAMTS-13 activity, which is negatively affected by high bilirubin concentrations [40] (see Supplementary Material, Fig. S1). The presence of anti-ADAMTS-13 antibodies in the patients' plasma was measured by an ELISA technique, using the IMUBIND ® ADAMTS13 ELISA kit from American Diagnostica (Instrumentation Laboratory, Werfen Group, Milano, Italy). The capture and detection antibodies contained in this kit are highly specific for human ADAMTS13. The test is characterized by an intra- and inter-assay coefficient of variations (CV) of 4.0% and 7.3% respectively.

Clinical Chemistry assays

All clinical chemistry assays (plasma albumin, creatinine, alanine-leucine-amino-transferase (ALT) and total bilirubin) were performed as a part of routine diagnostic work-up within 24 hours of clinical observation in the central laboratory of the "A. Gemelli" Hospital, using commercial reagents from Roche and automated immunoassays carried out on a Hitachi 704 instrument.

Statistical analysis

Bonferroni's correction of data was applied to avoid biases from unequal variance and type 1 error. As first step, data pertaining to cirrhotic patients with and without PVT were compared by Mann-Whitney U test. The parameters correlated in univariate analysis with $p<0.05$ were used as independent variables predicting PVT in multivariable logistic regression analysis. In the latter we decided to exclude some single parameters such as prothrombin time (INR), serum albumin and total bilirubin, and to use the Child-Pugh score only, which includes all of them, thus avoiding multi-collinearity problems. A two-sided $p<0.05$ was defined as statistically significant. Analyses were performed using SPSS software (version 21, Chicago, IL, USA) and GraphPad Prism software (GraphPad Software, Inc, La Jolla, CA, USA).

Results

Coagulation factors, cirrhosis severity score and PVT

The main clinical characteristics of patients enrolled in the study are listed in Table 1. The groups A and B did not differ significantly apart from the severity of the Child-Pugh score that was higher in patients with PVT (p=0.011). Of interest, ADAMTS-13 activity was significantly lower in group A than in group B, as shown in Fig. 1 and Table 2 (Group A: median 16.8% [5.7-87.0]; Group B: median: 69.1% [IQR=43.9-98.8], p=0.0047). No autoantibody against ADAMTS-13 was found in all cases with deficiency of the metalloprotease. Notably, although in the entire cohort of patients there was a tendency of lower ADAMTS-13 levels as a function of Child-Pugh score, univariate analysis did not show a significant inverse relationship between these two parameters (Spearman: b= -4.27, R square=0.004, p=0.57). Platelet count, prothrombin time expressed as % of normal plasma, protein C level and albumin concentration were significantly lower in group A (Table 2). VWF:act was globally higher in all cirrhotic patients than in 100 normal control subjects of the central coagulation laboratory, matched for age, sex and blood group (cirrhosis: median= 231%, IQR: 172-327%; normal controls: median: 92%, IQR (73-129%, p<0.001). In Group A, VWF activity was significantly higher than in Group B (median: 308.4% vs 203.3, p=0.032, Table 2). Likewise, D-dimer level (median= 912 vs 238.5 ng/ml, p=0.02) was significantly higher in Group A (Fig.2). In the entire cohort of cirrhotic patients, the median value of the VWF:act/VWF:Ag ratio was significantly lower than in normal control subjects matched for age and sex (cirrhosis: 0.78, [IQR: 0.69-0.95] vs controls: 0.94 [IQR: 0.91-0.97], p<0.001). However, concerning the VWF:act/VWF:Ag ratio and the correlated pattern of VWF multimers [41], VWF:Ag, FVIII level and presence of risk factors for VTE (protein C, protein S, AT, fibrinogen, activated protein C resistance, lupus anticoagulant) no difference was observed between Group A and B (Table 2 and , Fig. 3). Only one case of G20210A mutation of prothrombin in groupA and two cases of heterozygous FV Leiden in group B were found. Hence, due to the paucity of data, these congenital thrombophilia factors were excluded from further statistical analysis.

Univariate and multivariable analysis

In univariate analysis (Spearman method), the presence of PVT in the entire cohort of cirrhotic patients (n=84) was analyzed as a function of biochemical, clinical and coagulation parameters. Among these, the Child-Pugh severity score (ρ_s= 0.332, p=0.002), protein C (ρ_s=-0.247, p=0.027), D-dimer (ρ_s=0.26, p=0.019), VWF:act (ρ_s=0.24, p=0.031), platelet count (ρ_s=-0.34, p=0.002), ALT level (ρ_s=-0.30, p=0.007) and ADAMTS-13 activity (ρ_s=-0.32, p=0.004) were found to be significantly associated with PVT. However, in a multivariable logistic regression analysis between the presence of PVT (dependent variable) and the parameters significantly associated with PVT in the univariate analysis (ALT and protein C were excluded to avoid collinearity problems between themselves [Spearman, p=0.015, r= -0.27] and between protein C and

the Child-Pugh score [Spearman, p<0.001, r= -0.69]), only the ADAMTS-13 activity (n=84, p=0.007) was found to be significantly and inversely associated with the presence of PVT (see Fig. 1 and Table 3). Notably, the worsening of the Child-Pugh score resulted only weakly associated with PVT (p=0.05, Table 3). For ADAMTS-13 level, ROC analysis revealed an optimum cut-off to predict the risk of being diagnosed with PVT of 18% (AUC 0.709, [SE=0.077, p=0.005], asymptotic 95% confidence interval equal to 0.559-0.85999), as shown in Fig. 4.

Discussion

The results of this investigation provide some new elements to reconstruct the puzzle of the pathogenesis of PVT in cirrhosis. In patients candidates to liver transplantation, grade I and II PVT (confined to the portal vein beyond the confluence of the splenic vein or extended to the superior mesenteric vein, but with patent mesenteric vessels) increase the risk of surgical complications, whereas grade III and IV (extended to the whole splanchnic venous system, but with large collaterals or with only fine collaterals) may represent a contraindication to the transplant procedure [42,32]. Several points emerged from the present study: 1) the Child-Pugh score was positively and significantly associated with development of PVT in univariate analysis but at the limit of statistical significance in multivariable analysis; 2) the Child-Pugh score was not significantly associated with levels of circulating ADAMTS-13, suggesting that the reduction of circulating enzyme level is not directly related to the loss of hepatocytes mass and subsequent worsening of liver function. Concerning the classical risk factors for VTE, such as antithrombin, protein C and protein S deficiency as well as the increase of fibrinogen and FVIII level or the presence of lupus anticoagulant, the association with PVT is more problematic. These thrombophilic factors, though likely contributing to increase the thrombotic risk [43], were found in this study to not assume *per se* a central role in the pathogenesis of PVT, according to previous findings [31]; 3) in multivariable analysis, ADAMTS-13 alone was independently and inversely correlated with the presence of PVT. One previous study [17] showed that, at variance with the finding concerning the above point 2, ADAMTS-13 level decreases with increasing severity of liver disease. This discrepancy could arise from different inclusion and exclusion criteria used in that study, being our patients prevalently under outpatient clinic follow up. Further supportive evidence that the loss of hepatocytes *per se* does not correlate with ADAMTS-13 decrease derives from the finding that acute liver failure does not cause a decrease of ADAMTS-13 levels [44]. Notably and in part unexpectedly, the level of circulating VWF, although much higher than in matched control subjects, was not found significantly associated with PVT in multivariable analysis. Some hypotheses may be put forward to explain the latter finding. In our cohort of patients, both VWF:Ag and VWF:act showed strongly elevated levels compared to normal controls. These findings are in agreement with previously published data showing increased levels of VWF:Ag with possible prognostic value in cirrhotic subjects [23]. Likewise, the level of FVIII, which circulates in association with VWF, was higher in cirrhotic patients compared to normal subjects, although no difference was found between Group A and B. The similar pattern of circulating VWF multimers in cirrhotic patients with and without PVT (see Fig. 3) suggests that the perturbation of VWF proteolysis due to decrease of ADAMTS-13 levels, would mostly occur locally in the sinusoidal microcirculation as a consequence of the altered activity of the stellate cells responsible for the ADAMTS-13 deficiency. A similar scenario was previously reported for glomerular microcirculation, where defective secretion of ADAMTS-13 by endothelial cells causes microcirculatory thrombosis in the glomerulus, without altering the pattern of circulating VWF multimers [45,46]. Thus, the level of ADAMTS-13 seems to be a highly sensitive parameter for the presence of PVT in cirrhosis. This result must be prospectively confirmed in a larger series of patients but suggests for the first time that the ADAMTS-13 deficiency may play a causative role for this thrombotic complication. We can speculate that the ADAMTS-13 deficiency, caused by activation of stellate cells by capillarization of liver sinusoidal endothelial cells [47], may cause in sinusoids a local elevation of ultra-large VWF multimers. This phenomenon may support the formation of

platelet thrombi within the hepatic micro- and macro-vasculature, including the portal vein [48]. Thus, in cirrhosis the progressive decrease of ADAMTS-13 production by activated stellate cells could elicit an elevated intra-sinusoidal presence of ultra-large VWF multimers that enable local adhesion and activation of platelets and plasma coagulation factors resulting in thrombus formation in portal circulation.

Several limitations of this study warrant some comments. Firstly, the observational design of this mono-centric study may hamper the accuracy of data collection, including information on additional risk factors, under-estimated at the time of the clinical observation. Secondly, we adopted a wide screening for common thrombotic risk factors, but we cannot exclude the presence of occult provoking factors for thrombosis. Thirdly, we cannot exclude that the ADAMTS-13 deficiency may be the consequence of PVT, triggered by different factors and not the cause of its occurrence. In conclusion, this study shows that low levels of ADAMTS-13 are significantly associated with occurrence of PVT. ROC analysis showed that an 18% cut-off level of ADAMTS-13 is associated with good sensitivity and specificity with the presence of PVT. These findings may potentially have translational implications in clinical practice. Whether measurement of plasma ADAMTS-13 level could be an adequate tool for early identification of high-risk patients for PVT during clinical course of cirrhosis needs to be prospectively investigated in larger cohorts of patients in a multi-centric and prospective trial.

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Figure legends

Figure 1. Mann-Whitney analysis of ADAMTS-13 levels of cirrhotic patients with PVT (Group A) and without PVT (Group B). Group A: median: 17% [IQR: 6-87%]: Group B: median=69% [IQR: 44-99%]. Notably, the apparent bimodal distribution of the ADAMTS-13 levels did not correlate with any clinical or laboratory parameter.

Figure 2. Mann-Whitney analysis of VWF:act and D-dimer levels of cirrhotic patients with PVT (Group A) and without PVT (Group B). A) Group A: median=308% [IQR: 203-406%]; Group B: median=203% [IQR: 170-315%]; B) Group A: median=912 ng/ml [IQR: 193-1463 ng/ml] Group B: median=239 ng/ml [IQR: 121-791 ng/ml].

Figure 3. SDS-agarose (0.8-1.2%) western blot gel of VWF samples from pooled normal plasma (NP) and cirrhotic patients with PVT (Group A, samples with asterisk, 3, 6, 8, 11) and without PVT (Group B, 1-2, 4-5, 7, 9-10). At the bottom, the corresponding values of the values of VWF:act/VWF:Ag and ADAMTS-13 activity are also listed in the Table.

Figure 4. Receiver operation characteristics (ROC) analysis of ADAMTS-13 level predicting the presence of PVT in cirrhotic patients. The ROC curve area is equal to 0.709 (SE=0.077, p=0.005) with asymptotic 95% confidence interval equal to 0.559-0.859. A cut-off value of ADAMTS-13 level equal to 18% can discriminate patients with and without the presence of PVT.