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**SITE-SPECIFIC RISK FACTORS FOR PORTAL VEIN THROMBOSIS AND
EVALUATION OF ANTICOAGULATION EFFICACY IN PATIENTS WITH
CIRRHOSIS**

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Abbreviations

aPTT	Activated Partial Thromboplastin Time
AT	Antithrombin
CAT	Calibrated Automated Thrombogram
CT	Computerized Tomography Scan
DVT	Deep Vein Thrombosis
ELISA	Enzyme-Linked Immunosorbent Assay
ETP	Endogenous Thrombin Potential
FII	Factor II
FITC	Fluorescein isothiocyanate
FV	Factor V
FVIII	Factor VIII
FXa	Activated Factor X
HIT	Heparin-induced thrombocytopenia
IgG	Immunoglobulin G
IL	Interleukin
INR	International Normalized Ratio
LMWH	Low Molecular Weight Heparin
MRI	Magnetic Resonance Imaging
RNA	Ribo-Nucleic Acid
OCT	Optical Cutting Temperature Compound
PBS	Phosphate buffered saline
PC	Protein C
PE	Pulmonary Embolism
PIVKA	Proteins Induced by Vitamin K Absence
PPP	Platelet-Poor Plasma
PRP	Platelet-Rich Plasma
PVT	Portal Vein Thrombosis
TG	Thrombin Generation

TM	Thrombomodulin
TNF	Tumor Necrosis Factor
VKA	Vitamin K Antagonists
VTE	Venous Thromboembolism
vWF	von Willebrand Factor

Summary

Advanced liver disease is characterized by profound hemostatic alterations that can lead both to bleeding or to thrombotic complications. While pro-hemorrhagic alterations are present including thrombocytopenia and reduced plasmatic levels of coagulation factors, pro-thrombotic abnormalities such as decrease in anti-coagulant proteins antithrombin, Proteins C and S, increase in prothrombotic Factor VIII, and von Willebrand factor are also present.

The most frequent site of thrombosis in cirrhotic patients is the portal vein, consequence of an interplay of factors including altered hemostasis and venous stasis. The endothelium, the third component of the thrombosis triad, however, probably plays an important role in the genesis of in situ thrombosis within the portal vein. In the present study, endothelium from portal and cava veins were analyzed in cirrhotic patients, and compared to that of non-cirrhotic subjects, in order to determine the possible role of local alterations in the development of thrombosis. The immunofluorescence study of the main endothelial anticoagulant protein, thrombomodulin, revealed decreased presence of this component in the endothelium of the portal vein with respect to the vena cava in cirrhosis patients. On the other hand, the immunohistochemical analysis of pro-coagulant Factor VIII revealed that this endothelial protein is present uninterruptedly lining the lumen of portal vein and vena cava of both cirrhosis patients and non-cirrhotic subjects, without showing any differences between them. Diminished thrombomodulin may hamper the endothelium's anticoagulant properties, which, in the presence of conserved Factor VIII, may lead to the development of thrombosis.

The thrombosis of the portal vein represents an important milestone in the natural history of patients with cirrhosis, often increasing morbidity before

and mortality after liver transplantation. Obtainment of recanalization through anticoagulation is therefore paramount, and in the present study, an analysis was performed regarding factors that may have an impact on efficacy of anticoagulation with low molecular weight heparin in cirrhotic patients with this complication. Anticoagulation with low molecular weight heparin was demonstrated to be a valid strategy for achieving portal vein recanalization, with a response rate of 65.2%, including complete recanalization in 24 of the 46 treated patients, after a mean of 4.5 months (± 3.1 months) of anticoagulation. Whereas the hemostatic status of patients did not correlate with the response to anticoagulation, the interval between thrombus onset and start of therapy was the only predictive factor of therapeutic efficacy. Specifically, thrombus age at diagnosis (1.9 ± 1.2 months vs 6.3 ± 4.5 months in the recanalization group and in the non-recanalization group, respectively, $p < .001$), and the interval between thrombus onset and start of anticoagulation (3.2 ± 1.7 months vs 7.78 ± 4.5 months in the recanalization group and in the non-recanalization group, respectively, $p < .002$) were the principal determinants of therapeutic efficacy. This underlines the importance of prompt diagnosis and start of therapy to increase the probability of successful anticoagulant therapy.

Although in cirrhosis the low levels of antithrombin, which is necessary for the action of heparins, could theoretically hamper the anticoagulant effect, the clinical efficacy of anticoagulant therapy with low molecular weight heparin has been herein demonstrated.

The anticoagulant effect of low molecular weight heparin was then tested *in vitro* using the thrombin generation assay, and concentrations within the therapeutic range achieved reduction of endogenous thrombin potential notwithstanding the marked reduction in antithrombin levels that were present in plasma from cirrhotic patients and the low plasma anti-Xa activity determined *in vitro*. In particular, patients with Child Pugh C

cirrhosis were characterized by antithrombin levels which were as low as those of subjects with the prothrombotic condition of genetic antithrombin deficit ($42\pm 14\%$ versus $52\pm 4\%$, respectively, $p=.06$). At low molecular weight heparin 0.35 UI/mL concentration *in vitro*, anti-Xa activity was significantly lower in Child Pugh B and Child Pugh C patients as compared to controls ($p<0.001$), as well as in patients with congenital AT defect as compared to controls ($p<0.001$). Despite low levels of antithrombin and anti-Xa activity, patients with cirrhosis showed a greater anticoagulant effect of low molecular weight heparin, with a mean endogenous thrombin potential reduction of $72.6\pm 11\%$ ($p=0.02$ versus controls). This increased susceptibility of cirrhosis patients with advanced stages of the disease may therefore actually warrant dose reduction of anticoagulation.

Riassunto

La cirrosi epatica avanzata è caratterizzata da alterazioni emostatiche importanti che possono portare a complicanze emorragiche o trombotiche. Nonostante siano presenti alterazioni pro-emorragiche come trombocitopenia e ridotti livelli dei fattori della coagulazione, sono presenti anche anomalie pro-trombotiche come la diminuzione di proteine anti-coagulanti, quali antitrombina, Proteina C ed S, ed incremento del Fattore VIII e Fattore von Willebrand .

Il sito più frequente di trombosi in pazienti cirrotici è la vena porta, risultato di vari fattori quali le alterazioni emostatiche sistemiche così come la stasi venosa locale. Tuttavia, l'endotelio, il terzo componente del triade trombotica, probabilmente gioca un ruolo importante nella genesi della trombosi in situ della vena porta. Nel presente studio, è stato analizzato l'endotelio della vena porta e comparato a quello della vena cava in pazienti cirrotici e non, per determinare il possibile ruolo delle alterazioni locali nello sviluppo della trombosi. Come principale proteina endoteliale anticoagulante, è stata studiata la trombomodulina tramite immunofluorescenza, rivelandone una ridotta presenza nell'endotelio della vena porta rispetto a quello della vena cava nei pazienti cirrotici.

D'altro canto, l'analisi immunocitochimica del Fattore VIII, con proprietà pro-coagulanti, ha rivelato che questa proteina endoteliale è presente in maniera continua e costante lungo il lumen della vena porta e della vena cava sia nei pazienti cirrotici che non. La diminuzione della trombomodulina può danneggiare le proprietà anticoagulanti dell'endotelio che, in presenza del Fattore VIII preservato, può portare allo sviluppo della trombosi.

La trombosi della vena porta rappresenta una complicanza rilevante nella storia naturale dei pazienti cirrotici, causando frequentemente un aumento della morbilità prima e della mortalità dopo il trapianto epatico.

L'ottenimento della ricanalizzazione tramite terapia anticoagulante è perciò importante, e nel presente studio è stata fatta un'analisi dei fattori che possono avere un impatto sull'efficacia della terapia con eparina a basso peso molecolare in pazienti cirrotici con questa complicanza. Si è dimostrato che l'anticoagulazione con eparina a basso peso molecolare è una strategia valida per la ricanalizzazione della vena porta, con un tasso di risposta del 65.2%, includendo ripermeazione completa in 24 dei 46 pazienti trattati, dopo una media di 4.5 mesi (± 3.1 mesi) di anticoagulazione. Nonostante lo status emostatico dei pazienti non correlava con la risposta all'anticoagulazione, l'intervallo tra lo sviluppo del trombo e l'inizio della terapia è stato l'unico fattore predittivo dell'efficacia terapeutica. Specificamente, l'età del trombo alla diagnosi (1.9 ± 1.2 mesi vs 6.3 ± 4.5 mesi, rispettivamente, $p < .001$) e l'intervallo tra lo sviluppo del trombo e l'inizio della terapia anticoagulante (3.2 ± 1.7 mesi vs 7.78 ± 4.5 mesi nel gruppo che ha ottenuto ricanalizzazione e nel gruppo che non ha ottenuto ricanalizzazione, rispettivamente, $p < .002$) sono stati i principali determinanti dell'efficacia terapeutica. Questo sottolinea l'importanza di una diagnosi precoce e di un opportuno inizio della terapia, per incrementare la probabilità di successo del trattamento anticoagulante.

Benché i livelli bassi di antitrombina, necessaria per l'azione dell'eparina, verificatesi in cirrosi possano teoricamente diminuire l'effetto anticoagulante, in questo studio si è dimostrata l'efficacia clinica dell'anticoagulazione con eparina a basso peso molecolare.

L'effetto anticoagulante dell'eparina a basso peso molecolare è stato esplorato *in vitro* utilizzando il test della trombino generazione, e concentrazioni di eparina dentro il range terapeutico sono state in grado

di ridurre la generazione della trombina, nonostante la spiccata riduzione nei livelli plasmatici di antitrombina e i bassi livelli di anti-Xa determinati *in vitro*. In particolare, i pazienti in classe C di Child Pugh si sono caratterizzati da livelli di antitrombina bassi quanto quelli presenti in pazienti con la condizione protrombotica di deficit genetico di questa proteina ($42\pm 14\%$ vs $52\pm 4\%$, rispettivamente, $p=.06$). Alla concentrazione *in vitro* di 0.35 UI/mL di eparina a basso peso molecolare, l'attività anti-Xa è stata significativamente più bassa in pazienti in classi di Child Pugh B e C rispetto ai controlli ($p<.001$), così come in pazienti con difetto genetico dell'antitrombina rispetto ai controlli ($p<.001$). Nonostante i ridotti livelli di attività anti-Xa, i pazienti cirrotici hanno dimostrato un maggiore effetto anticoagulante dell'eparina a basso peso molecolare, con una riduzione del potenziale endogeno di trombina di $72.6\pm 11\%$ ($p=0.02$ vs i controlli). Data l'incrementata suscettibilità dei pazienti cirrotici in stadi avanzati della malattia epatica, potrebbe essere necessaria la riduzione della dose di anticoagulazione.

Introduction

Multiple changes occur in the hemostatic system as a result of deranged liver function. Being the liver the primary site of synthesis of most coagulation factors, as well as of proteins which keep these mechanisms in check, severe derangement of its function has long been known to result in bleeding complications such as epistaxis, gastrointestinal bleeding from esophago-gastric varices, and gum bleeding, but also in thrombotic complications.

Routinely performed coagulation profile is abnormal in the majority of these patients, and clinical consequences of alterations in this complex interplay may lead to bleeding or to thrombosis. In patients with severe liver disease, hemostasis is affected due to diminished synthesis of factors II, V, VI, IX, X, XI, XIII, fibrinogen, protein C, protein S, oftentimes coexisting with Vitamin K deficiency due to malabsorption or malnutrition. Dysfibrinogenemia, enhanced fibrinolysis, impaired clearance of activated clotting factors, plasminogen activators, and fibrinogen degradation products all contribute to altered hemostasis in cirrhosis. Coagulation in patients with decompensated liver cirrhosis can also be affected by other factors like infections, endogenous heparinoids, renal failure, and endothelial dysfunction(1-3).

Procoagulant factors

While von Willebrand factor is synthesized by the endothelium (4) and Factor VIII is primarily synthesized by hepatic sinusoidal cells(5;6), the liver is the site of synthesis of fibrinogen and factors II, V, VII, IX, X, XI and XII (7). Thus the plasma concentration of factor VIII is not decreased with

liver disease, and may be even increased, as many chronic liver diseases are associated with chronic inflammation (8). This probably obeys to an increase in endothelial synthesis, a reduced clearance via low-density lipoprotein receptor-related protein (4), and increased levels of von Willebrand factor. However, the biological activity of the synthesized molecule is lower than the plasmatic concentration (9). Furthermore, Factor VIII is elevated in fulminant hepatic failure but decreased in disseminated intravascular coagulation (DIC) (10).

Vitamin K is an essential cofactor for the production of biologically active forms of the coagulation factors II, VII, IX and X, enhancing hepatic post-ribosomal conversion of certain glutamic acid residues in the protein precursors, to γ -carboxyglutamic acid (Gla). These active forms of the clotting factors chelate calcium at the Gla site, resulting in effective hemostatic function. When γ -carboxylation is impaired due to deficiency or antagonism of vitamin K, inert precursors are synthesized, (known as Proteins Induced by Vitamin K Absence [PIVKA]) and released into the blood stream (11). The clinical significance of these precursors is not clear. In the case of prothrombin, a specific and sensitive immunoassay has been developed which is able to detect small decreases in Gla content of this incomplete PIVKA prothrombin before any changes occur in conventional coagulation tests (12). In cholestasis, reduction of vitamin K absorption from the small intestine due to decreased bile salt production can be compensated with parenteral administration of vitamin K 10mg daily for 24-48 hours, but in parenchymal liver disease decreased levels of coagulation factors are dependent on a decreased synthesis, so that there is no improvement with vitamin K administration (13). Nevertheless 25% of patients with acute liver injury have a subclinical deficit of vitamin K which may benefit from parenteral administration, with corresponding improvement of the INR (14).

Anticoagulant Factors

Antithrombin III (ATIII) is a non-vitamin K-dependent glycoprotein synthesised by the liver but also by the endothelium (15). It has low concentration in patients with liver disease, probably due to reduced synthesis and/or increased consumption due to hyperfibrinolysis (16). ATIII replacement does not correct hyperfibrinolysis in patients with liver cirrhosis. Usually the ATIII deficit is mild and thrombosis as a complication is very rare, reported only sporadically (17).

Proteins C and S are vitamin K dependent glycoproteins synthesised mainly by hepatocytes (18). Therefore during acute or chronic liver disease, their concentrations can be decreased concomitantly with the other coagulation factors, but usually not below 20% of normal values (19). Genetic deficiency of protein C is rare in the general population, but found in 20% patients with Budd-Chiari syndrome (BCS). In patients with liver disease who also have genetic deficiency, plasma concentration is often lower than 20%. When there is severe liver disease, it can be difficult to exclude coexistent genetic deficiency as levels may be very low, due to very depressed synthesis (20). In this situation a concomitant finding of a normal level of factor II and protein C/factor VII ratio, can help to confirm a coexistent genetic deficit (21). In acquired deficiency of vitamin K, a defective protein C lacking γ -carboxyl (PIVKA) is produced (22). Protein C deficiency is not associated with extrahepatic portal vein thrombosis (23). Genetic deficiency of protein S is extremely rare, yet accounts up to 7% of patients with BCS or portal vein thrombosis (PVT), especially in series from Asia (24).

Overview of hemostasis alterations in cirrhosis

The fact that a rebalanced hemostatic status, which can be easily tipped towards bleeding or towards thrombotic complications not only is evident from laboratory studies, but also from the clinical point of view. It is ever clearer that patients with cirrhosis are not “auto-anticoagulated”, as previously thought, but actually have a greater risk than non-cirrhotic counterparts for developing thrombotic complications as well. Clinically, evidence is accumulating regarding a relative hypercoagulable state which is present in patients with cirrhosis. (Table 1).

Table 1. Evidence of thrombotic complications in patients with liver disease (25).

Disease state	Possible contributing etiologies
Portal vein thrombosis	Obstruction of flow Prothrombotic predisposition Infectious nidus from gastrointestinal tract Local inflammatory mediators
Deep vein thrombosis or pulmonary embolism	Imbalance in clotting cascade favoring coagulation Immobility of end-stage liver disease Infection and systemic inflammation
Progression of cirrhosis	Parenchymal extinction
Vascular prosthesis and extracorporeal circuit thrombosis	Mechanical obstruction Inflammatory mediators Abnormal platelet adhesion
Portopulmonary hypertension	Pulmonary endothelial dysfunction Microvascular pulmonary thrombosis Altered shear stress in the pulmonary vessels
Metabolic syndrome and non-alcoholic fatty liver disease	Venulitis and microthrombi with remodeling Atherosclerotic vascular changes Inflammation related to metabolic syndrome Factor level alteration with insulin resistance

From the point of view of laboratory parameters, patients with chronic or acute liver failure show profound abnormalities in their haemostatic and coagulation system(2;26).

Despite routine coagulation tests may be compatible with a bleeding tendency, both pro and anti-coagulation factors are affected, the latter of which are not well reflected in these tests (27). Reduced levels of FII, FIX, FXI, FXII are characteristic of the coagulation profile of a typical patient with cirrhosis, and levels are correlated to the severity of liver disease. However, anticoagulant factors are also decreased in cirrhosis, and therefore a new thrombotic-hemostatic balance is reached(2;28).

In fact, more global tests such as thrombin generation test (TG), which are able to detect the overall effect of deficient anticoagulant mechanisms in plasma of cirrhosis patients, have shown that endogenous thrombin potential in cirrhosis patients is not significantly different from that of healthy subjects(29). Specifically, when the thrombin generation test is performed in the presence of thrombomodulin, an endothelial receptor which catalyzes the thrombin-mediated conversion of Protein C into its active form activated Protein C, the amount of thrombin generated is similar between plasma from cirrhotic and from healthy subjects(30;31).

As mentioned above, aside from acquired coagulation-hemostatic defects, it has been demonstrated that genetic thrombophilias are more frequent in cirrhosis patients with PVT when compared to cirrhosis patients without PVT. In a study by Amitrano et al, the frequencies of Factor V Leiden and of Prothrombin A20210 polymorphism were reportedly 13% and 34.8% in cirrhotic patients with PVT, whereas frequencies were 7.5% and 2.5% in cirrhotic patients without PVT (32).

Regarding primary hemostasis, chronic liver disease is characterized by a variable degree of thrombocytopenia due to increased platelet destruction, increased splenic and/or hepatic sequestration, or to reduced levels of thrombopoietin and by altered platelet function due to defective thromboxane A2 synthesis, storage pool deficiency and abnormalities of the platelet glycoprotein Ib (33-39).

Different mechanisms compensate for reduced platelet number and function: von Willebrand factor is notably elevated in cirrhosis, probably as a result of its reduced clearance resulting from diminished levels of its cleaver ADAMTS13 and as a reflection of high levels of FVIII, to which it is bound when circulating in plasma(40). A summary of these alterations in the hemostatic-thrombotic systems is presented on table 2.

Table 2. Alterations in the hemostatic system in patients with liver disease that contribute to bleeding (left) or counteract bleeding (right)(27).

Changes that impair hemostasis	Changes that promote hemostasis
Thrombocytopenia	Elevated levels of von Willebrand factor (VWF)
Platelet function defects	Decreased levels of ADAMTS-13
Enhanced production of nitric oxide and prostacyclin	Elevated levels of factor VIII
Low levels of factors II, V, VII, IX, X, and XI	Decreased levels of protein C, protein S, antithrombin, α 2-macroglobulin, and heparin cofactor II
Vitamin K deficiency	
Dysfibrinogenemia	Low levels of plasminogen
Low levels of α 2-antiplasmin, factor XIII, and TAFI	
Elevated t-PA levels	

Thrombin generation

Thrombin is often seen as the end-product of the coagulation cascade and as such responsible for the final step, the conversion of the soluble protein fibrinogen into the insoluble fibrin clot. However, the coagulation cascade is actually composed of many feedback loops and inhibitory pathways that are all directly or indirectly influenced by the developing thrombin concentration, which renders thrombin pivotal throughout the entire coagulation-anticoagulation process.

The thrombin generation assay in plasma is a test which assesses coagulation globally, and is able to detect alterations both in coagulation and anticoagulation mechanisms (41) (Figure 1). In contrast to determination of thrombin generation in vivo, which detects byproducts of an ongoing thrombus formation such as prothrombin fragments 1-2 and d-dimer, the thrombin generation assay measures ex vivo the potential capacity of a certain sample to generate thrombin, triggered by tissue factor. The sample analyzed may be platelet-poor plasma (PPP), or platelet-rich plasma (PRP), and the interplay between all coagulation components is analyzed concomitantly, with the exception of the vessel wall. To this end, thrombomodulin and tissue factor are added to the assay, rendering the test a very close approximation to what occurs inside the vessel. Phospholipids are added as platelet substitutes when PPP is analyzed, providing a pro-coagulant surface. This method can monitor the initiation, propagation and decay phases of thrombin generation. In addition, this assay also measures the endogenous thrombin potential (ETP), which represents the activity of thrombin multiplied by the time for which it remains active in plasma(42).

Thrombin generated from the plasma sample after addition of a suitable trigger (tissue factor, recalcification of thawed plasma), is measured using

a fluorogenic substrate. The amount of generated thrombin is plotted against time to construct a thrombin generation curve (or thrombogram) (Figure 2). A computer program developed by Hemker and collaborators calculates the parameters which characterize the thrombogram i.e., the lag time, the peak height, the time to peak and the endogenous thrombin potential (ETP) (Figure 3) (43). The latter is the area under the thrombin generation curve and represents the total amount of thrombin formed over time, after exclusion of the contribution to amidolysis of the alpha2macroglobulin-thrombin complex that is unable to convert fibrinogen into fibrin, but retains amidolytic activity towards the thrombin-specific synthetic substrates. The fluorescent signal has the drawback of not being linear with product concentration(44). To compensate for this and for the effects of substrate consumption, the calibrated automated thrombogram (CAT) method has been developed, which continuously compares the signal from the experimental sample to that of a fixed known thrombin activity (45). This method allows visualizing the thrombin concentration in clotting PPP or PRP in 24 parallel experiments. In the present study, the Thrombinoscope™ software (Thrombinoscope BV) was used, together with the Thrombinograph™, a 96-well plate fluorimeter (Thermo Scientific). A thrombin calibrator was used for every sample, to correct for the inner filter effect, donor-to-donor variability in color of plasma, substrate depletion and instrumental differences.

The study of thrombin generation – performed either with clotting-based assays, or with chromogenic substrates – is an established tool in blood coagulation research –(43;46-49). It has been shown that the thrombin generation assay is able to detect thrombophilic phenotypes in subjects at risk of VTE (50-52). When this test is performed in the presence of thrombomodulin or activated protein C, alterations in the pathway of the natural anticoagulant protein C are unmasked.

In population-based studies Lutsey et al. demonstrated an increased risk for VTE (primarily idiopathic) for elevated peak values of TG (53). Furthermore, Tripodi and colleagues demonstrated a correlation between TG values and clinical risk assessment of VTE (low, medium, high risk of VTE), when thrombin generation assay was performed in the presence of thrombomodulin(54). In cirrhosis patients, studies performed using the thrombin generation assay have demonstrated an imbalance between pro- and anti-coagulant factors which tends towards hypercoagulability and is unveiled by the addition of thrombomodulin(55). Finally, the thrombin generation test has also been used to study the anticoagulant effect of multiple anticoagulant drugs, showing adequate sensibility to detecting their action (56).



Figure 1. Calibrated automated thrombogram system (CAT) by Thermo Electron. The instrument consists of a 96-well microplate fluorometer and Thrombinoscope™ package.

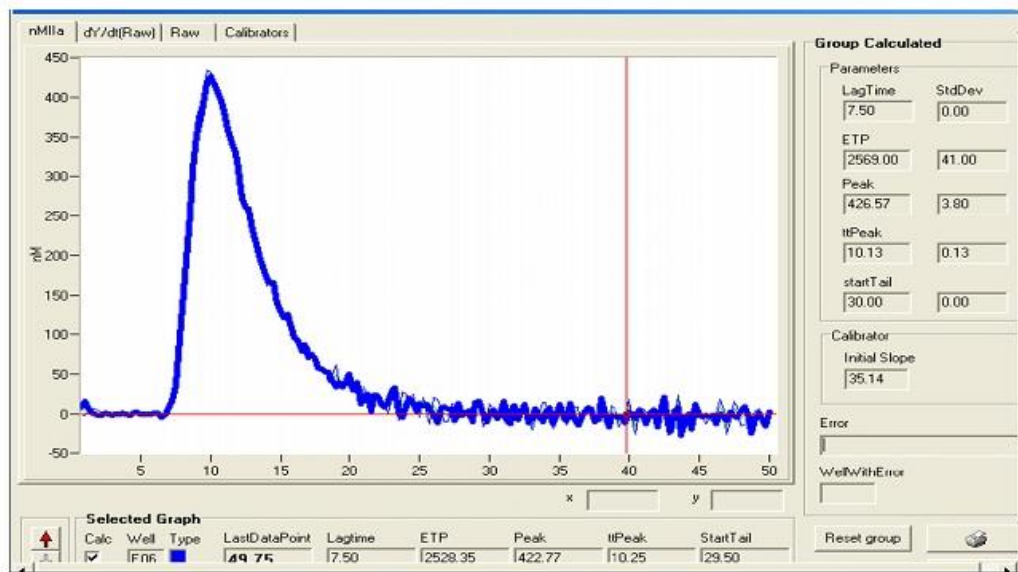


Figure 2. Typical thrombin generation curve (thrombogram).

The endogenous thrombin potential (ETP) corresponds to the area under the curve.

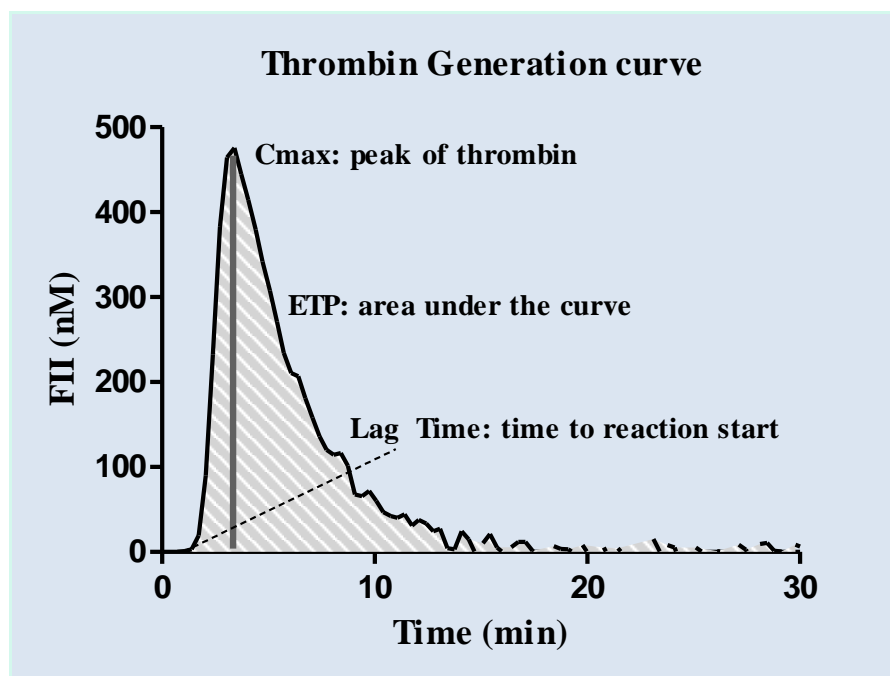


Figure 3. Thrombin generation curve and parameters.

Thrombin generated and subsequently degraded is plotted against time.

Clinical evidence of pro-thrombotic complications in cirrhosis

Venous thromboembolism

Patients with cirrhosis appear to have a higher incidence of unprovoked deep vein thrombosis and pulmonary embolism (DVT/PE) compared with the general population. Although there are no prospective studies that report on the incidence of venous thromboembolism (VTE) in patients with cirrhosis, a nationwide population-based study undertaken in Denmark evaluating more than 99000 patients with thromboembolism showed that cirrhosis and liver disease carry a greater risk of VTE (OR of 2.10, and of 3.58 if age < 55 years)(57). In a prospective cohort study with case-control analysis of 6550 patients with VTE in the United Kingdom, Huerta et al. found that in patients with chronic liver diseases, the odds ratio for PE was 1.75 (CI 0.91–3.36), and the odds ratio for DVT/PE combined was 1.65 (CI 0.97–2.82), demonstrating not only that these patients are not protected from thromboembolic events, but rather that cirrhosis is a predisposing condition(58).

Eight articles, of which two are case-control studies and six are retrospective studies, have been published up to date specifically aimed at investigating the incidence of DVT and PE in patients with cirrhosis (59-66). (Table 3)

Table 3. Prevalence of venous thromboembolism in patients with cirrhosis admitted for hospitalization (59-66).

Reference	Type of study	Study population (n)	Incidence of DVT/PE (%)	DVT number (%)	PE number (%)	DVT+PE number (%)
Northup <i>et al.</i> (2006)	Case control	21000	113 (0.5%)	74 (65.5%)	22 (19.5%)	17 (15%)
Garcia Fuster <i>et al.</i> (2008)	Retrospective	2074	17 (0.8%)	10 (59%)	6 (35%)	1 (6%)
Gulley <i>et al.</i> (2008)	Case control	963	18 (1.87%)	NA	NA	NA
Lesmana <i>et al.</i> (2010)	Retrospective	256	12 (4.7%)	12 (100)	0 (0%)	0 (0%)
Ali <i>et al.</i> (2010)	Retrospective	449798	8231 (1.8%)	4335 (0.9%)	3688 (0.8%)	208 (0,8%)
Dabbagh <i>et al.</i> (2010)	Retrospective	190	12 (6.3%)	NA	NA	NA
Wu <i>et al.</i> (2010)	Retrospective	649879	52881 (8.1)	NA	NA	NA
Aldawood <i>et al.</i> (2011)	Retrospective	226	6 (2.7%)	6 (100%)	0 (0%)	0 (0%)

DVT: Deep vein thrombosis; NA: data not available; PE: pulmonary embolism.
Adapted from(67).

Portal vein thrombosis

The portal vein is certainly the most common site for thrombus formation in patients with advanced liver disease. Moreover, non-neoplastic portal vein thrombosis (PVT) is more frequent in cirrhosis than in the general population, with a prevalence of 0.6 to 26% in patients without hepatocellular carcinoma, although the wide range in reported prevalence is owed to the different groups of cirrhotic patients studied and the different diagnostic method (68-74).

Risk factors for the development of portal vein thrombosis in cirrhosis

The occurrence of pathological thrombosis is determined by an alteration in the physiological equilibrium that regulates coagulation and anticoagulation dynamics, and shifts regarding any one of the components of Virchow's triad (venous stasis, endothelial injury and hypercoagulability) may result in thrombosis. Both local and systemic factors can result in the development of site-specific thrombosis, particularly PVT.

In patients with end-stage liver disease, venous stasis results from splanchnic vasodilatation and architectural derangement, which primarily involves the cirrhotic liver. In one prospective study regarding a cohort of 73 patients with cirrhosis, reduced portal flow velocity below 15 cm/s was the only independent variable that correlated with the risk of developing PVT at 1 year of follow-up(75). However, the prospective study by Francoz et al. on 251 patients with cirrhosis listed for liver transplantation failed to find a correlation between portal flow direction (hepatofugal or hepatopetal) and risk of development of PVT(70). Recent in vivo evidence shows that the ratio of the most important pro and anticoagulant factors, factor VIII and protein C, respectively, demonstrates a strong imbalance in favor of factor VIII in cirrhotics, indicating a hypercoagulable state(55).

In several series, elevated levels of factor VIII have been shown to be a risk factor associated with either primary or cirrhosis-associated PVT(76;77). This has been confirmed in a larger cohort that evaluated 58 non-cirrhotic patients with PVT, 27 cirrhosis patients with PVT and 200 with DVT, in whom a strong association between elevated levels of FVIII and the risk of PVT was demonstrated (odds ratio for thrombosis and FVIII levels above 129 UI/dl being 6.0 for cirrhosis)(78).

The possible role of inherited thrombophilic abnormalities has been advocated in several cross-sectional studies reporting a thrombophilic genotype in up to 9% of patients with cirrhosis and PVT. It has also been demonstrated that polymorphisms TT677 of methylene-tetrahydrofolate reductase, and G20210A in the prothrombin gene are significantly more frequent in this group than in controls(32;68;71). Although not demonstrated in all studies regarding the liver transplant population(79), in other studies, the risk of PVT has been shown to be independently associated with the severity of cirrhosis, being paradoxically more frequent in those patients with worsening indices of coagulation and those with worsening portal hypertension (i.e. ascites and encephalopathy)(73;80).

Furthermore, PVT is more likely to occur in late stages of cirrhosis, and the combination of sclerotherapy and history of abdominal surgery are associated with an increased risk of PVT, which is probably not the case of a causal relationship, but rather reflecting a more advanced disease stage (68). The presence of previous treatments for portal hypertension (sclerotherapy, TIPS, shunt surgery, previous splenectomy) and severity of liver disease classified as Child Pugh C are two factors which have been found to be significantly associated with an increased risk of PVT (73), and the former was also demonstrated in other studies analyzing risk factors for PVT in liver transplantation series(74;81).

Table 4 summarizes the most important reported risk factors for the development of PVT in patients who undergo liver transplantation (70;73;74;79;80;82-89).

Table 4. Risk factors for non-neoplastic portal vein thrombosis (PVT) in liver transplant (LT) candidates (70;73;74;79;80;82-89).

Study, year	Statistically associated risk factors	Investigated risk factors with no demonstrated association
Cherqui, 1993		Child Pugh classification, presence of ascites, previous variceal bleeding, number of endoscopic sclerotherapies
Ravaioli, 2011	Hepatic neoplasm (40.7% vs no neoplasm 30.7, p=0.05) Alcoholic cirrhosis (23.3% vs other etiology of cirrhosis 10.9%, p<.001)	Age, sex, presence of TIPS
Nonami, 1992	Postnecrotic cirrhosis (Alcoholic, viral, autoimmune, drug-induced, cryptogenic) Hepatic neoplasm (34.8% vs no neoplasm 11.4, p<.001) Portal hypertension, previous treatment for portal hypertension (TIPS)	Primary biliary cirrhosis, Primary sclerosing cholangitis, liver-based inborn errors of metabolism, biliary atresia.
Davidson, 1994	Autoimmune hepatitis (60% vs 8.6%, p<.03) Cryptogenic cirrhosis (33% vs 8.3%, p<.05) Hepatic neoplasm (27% vs 9%, p<.05)	
Gayowski, 1996		Etiology of liver disease, age, sex, Child Pugh classification, previous abdominal surgery, liver size
Yerdel, 2000	Male sex, previous treatment for portal hypertension (sclerotherapy, TIPS, shunt surgery, previous splenectomy), Child C classification, Alcoholic liver disease. The presence of any one of these factors was associated with a rate of PVT of 12.5% (vs 6.6% if none of these risk factors was present), p<.013	Age, previous upper abdominal surgery, hepatic neoplasm
Manzanet, 2001		Hepatic neoplasm, primary biliary cirrhosis
Francoz, 2005	Platelet count	MELD score Direction of portal vein flow Past medical history of variceal bleeding (47.4% vs 29.1%, p=.162)
Gimeno,2005		Primary and secondary biliary cirrhosis
Egawa, 2006	MELD in PVT patients was lower (mean 18.2) vs in non-PVT patients (mean 21.9), p=.0106	
Lladó, 2007		Age, sex, severity of liver disease (Child class), previous TIPS
Pan, 2009	Male sex (11.05% male vs 4.23% female, p<.01) Previous splenectomy (27.4% vs 8.7% without previous splenectomy, p<.01)	
Englesbe, 2010	MELD at transplant 21.8±8.7 in PVT patients vs 20.9±9 in non-PVT patients, p=.0044 Diabetes: 26.1% of PVT patients vs 21.4% in non-PVT patients, p=.0008 Age at transplant: PVT patients were 54±9 years old vs non-PVT patients 52.8±9.5, p=.0002 Previous abdominal surgery: 51.7% of PVT patients vs 42.8% in non-PVT, p<.0001	

Endothelial injury and development of portal vein thrombosis

Endothelial alterations of the portal vein in cirrhosis patients which can compromise natural antithrombotic mechanisms, together with the hemostatic plasmatic alterations as well as venous stasis, may play a key role in development of site-specific thrombosis of the portal vein.

Cirrhosis is being increasingly recognized as a condition characterized by vascular damage, which can result in significant impairment of the physiologic mechanisms that ensure vessel permeability and the myriad of its homeostatic functions (90). A generalized inflammatory state is perpetuated by altered substance hepatic metabolism, shear stress due to disturbed hemodynamics and volume distribution, and the presence of circulating bacterial products caused by bacterial translocation from the gut.

Evidence of altered vascular permeability has been demonstrated in animal models of cirrhosis and more recently in cirrhosis patients (91). Moreover, proteins and glycoproteins associated with endothelial injury and inflammation, such as heparin-like substances, are notably elevated in cirrhosis. There is evidence that cirrhosis -and its possible concomitant events such as bacterial translocation, portal hypertension, and ascites - determines a particular set of conditions that challenge the endothelium's physiologic anti-thrombotic and anticoagulant capacity.

Furthermore, there is evidence to support the concept of greater inflammation and endothelial injury within the portal vein with respect to other venous beds in cirrhosis patients(91). D-dimer, an established marker of inflammation, which is also associated with abnormal activation of the coagulation cascade, has been demonstrated to reach significantly higher levels in blood samples drawn from the portal vein when compared to levels in blood samples from the jugular vein of cirrhosis patients (92). A

similar pattern has also been demonstrated for levels of prothrombin F1+2, demonstrating significantly higher levels in the portal vein vs the jugular vein, which represents systemic venous blood (92). Moreover, elevated levels of glycosaminoglycans, constituents of the glycocalyx, are elevated in patients undergoing liver transplant for advanced cirrhosis, and probably reflect shedding from an injured endothelium (3).

Regarding the endothelial anticoagulant mechanisms, a fundamental role is played by thrombomodulin. This cell surface-expressed transmembrane glycoprotein, predominantly synthesized by vascular endothelial cells, is a critical cofactor for thrombin-mediated activation of protein C, an event further amplified by the endothelial protein C receptor, as part of the natural anticoagulant mechanisms. This protein, which is present in both large vessels and capillary endothelium, and has a crucial role in anticoagulation, has also been implicated in inflammation (93). In fact, this protein has also been studied with regard to endothelial injury, linking both inflammation and coagulation, conditions that present concomitantly in cirrhosis. This endothelial cell surface glycoprotein forms a 1:1 complex with thrombin, and this binding activates protein C approximately 1000x faster than thrombin alone. Activated protein C, in turn, together with Protein S, inactivates Factor Va and VIIIa. TM also modulates inflammation independently of activated protein C, by activating anti-inflammatory pathways, negatively regulating complement, and by facilitating the activation of tissue activatable fibrinolysis inhibitor (TAFI), which modulates both fibrinolysis and complement-mediated inflammation (94-97).

Clinical consequences of portal vein thrombosis in cirrhosis and rationale for treatment

Clinical manifestations of PVT vary from asymptomatic disease to a life threatening complication. In a study on 79 cirrhotic patients with newly diagnosed PVT, 39% presented with gastrointestinal bleeding (from varices or portal hypertensive gastropathy) and 18% had abdominal pain, amongst which 70% had intestinal infarction due to the extension of the thrombus into the mesenteric vein (71).

PVT may worsen portal hypertension and may lead to variceal bleeding, development or worsening of preexisting ascites, and increases mortality and morbidity after liver transplantation. As emerged from a recently published systematic review, the presence of non-neoplastic PVT at LT entails a greater 30-day mortality after surgery when compared to patients without PVT (10.5% vs 7.7%, respectively ($p=.01$))(98). One-year mortality is also increased in patients who undergo LT in the presence of PVT in contrast with those with patent portal vein at transplant (18.8% vs 15.3%, respectively ($p<.001$)). Furthermore, the presence of complete or Grade IV PVT according to Yerdel adversely affects outcome after liver transplantation, as shown in table 5.

Table 5. Early (30-day) mortality and 1-yr mortality in patients according to the grade of portal vein thrombosis (PVT) in liver transplant (LT) recipients with this complication (73;83;84;86;88;89;99-104).

Author, year of publication	LT recipients <i>n</i>	30-day mortality Number of patients (%)		1 year mortality Number of patients (%)	
		<u>Partial PVT</u>	<u>Complete PVT</u>	<u>Partial PVT</u>	<u>Complete PVT</u>
Ravaioli (2011)	91	1/50 (2)	5/41(12.2)	-	-
Suárez (2010)	48	-	-	5/28 (17.8)	9/20 (45)
Egawa (2006)	39	5/29 (17.2)	6/10 (60)	-	-
Azoulay (2002)	8	-	0/8 (0)	-	-
Manzanet (2001)	62	2/48 (4.2)	2/14 (14.3)	12/48 (25)	5/14 (35.7)
Davidson (1994)	14	0/7 (0)	1/7 (14.3)	-	-

	LT recipients <i>n</i>	30-day mortality Number of patients (%)				1 year mortality Number of patients (%)	
		<u>G I</u>	<u>G II</u>	<u>G III</u>	<u>G IV</u>	<u>G I - G II</u>	<u>G III - G IV</u>
Yerdel (2000)	63	3/24 (12.5)	8/23 (34.8)	2/6 (33)	5/10 (50)		
Pan (2009)	253	0/218 (0)		1/29 (3.4)	2/6 (33.3)		
Ceulemans (2005)	5				0/5 (0)		
Urbani (2002)	6				1/6 (16.7)		
Olausson (2001)	6				1/6 (16.7)		
Doenecke (2010)	193					2/13 (15.4)	5/11 (45.5)

G = grade; LT= liver transplant; PVT = Portal vein thrombosis

Although no longer considered an absolute contraindication for liver transplantation (LT), occlusive PVT still precludes LT in many centers and increases the complexity of LT(81;105;106). In PVT patients undergoing LT, operative time, rate of reoperation or postoperative complications, transfusion requirement, and length of hospital stay are increased(73), while survival rates, related to the extent of thrombosis, are decreased(70). Whereas terminal to terminal portal vein anastomosis, with or without low dissection, thrombectomy, and/or thrombendvenectomy can be appropriate for isolated PVT, more complex vascular reconstruction techniques are required when the thrombus extends into the superior mesenteric vein. Porta-cava hemitransposition is the most common option

during transplantation with PVT extending into the superior mesenteric vein(107), but is characterized by survival of 60% and 38% at 1 and 3 years, respectively, and entails a 30% bleeding complications related to residual portal hypertension after transplantation(108).

These recent studies underscore the need for a clear protocol for the treatment of PVT in patients with cirrhosis, regardless of whether LT might be a future prospect or not.

Hence, the aims of PVT management in patients with cirrhosis listed for LT are the achievement of complete or partial recanalization and the prevention of thrombosis progression. These goals can be achieved either through the placement of, a transjugular intrahepatic portosystemic shunt (TIPS), with or without local thrombolysis or thrombectomy(109;110) or employing anticoagulation(111).

The rationale for the use of anticoagulation to treat PVT in patients with underlying liver disease derives from larger experiences published in patients with PVT and no liver disease, and the rare event of spontaneous recanalization (112;113). In patients with non-cirrhotic PVT, recanalization rate is achieved in 40% of patients who receive early anticoagulation(112;114;115). Since recanalization has been shown to occur only within 6 months from the start of anticoagulation, the duration of anticoagulation is currently recommended for at least 3 and preferably for up to 6 months(112;114). Evidence for the use of anticoagulation to treat PVT in patients with cirrhosis is more scarce.

The development of thrombotic complications in cirrhosis patients warrants a therapeutic approach that is similar to the non-cirrhosis setting. However, the anticoagulant of choice is still not known and a suboptimal utilization of prophylactic fractionated heparin in cirrhotic patients has been

recently described(65). Several low-molecular-weight heparins (LMWHs) are currently available as anticoagulant drugs for the prophylaxis and treatment of thrombosis in non-cirrhotic patients. LWMHs act by stimulating antithrombin (AT)-mediated inhibition of factor Xa; those with saccharide chains above a critical length also exert an inhibitory effect on thrombin. Due to their pharmacokinetic properties, LMWHs have a much more predictable anticoagulant effect than unfractionated heparin. (Figure 4) This allows for monitoring of clotting times to be avoided and makes it possible to use weight-adjusted or fixed doses of LWMHs depending on the clinical setting. Since LMWHs exert their anticoagulant effect by means of AT, the reduction of plasmatic levels of this protein seen in patients with advanced liver disease could theoretically hamper its anticoagulant effect.

Recently, Bechmann et al have demonstrated that after administration of LWMH, cirrhotic patients reach lower levels of anti-Xa activity than controls, which correlate to the severity of liver disease(116). Although this finding could also suggest the need to increase the dose of LWMH in cirrhotics, Lisman and colleagues showed *in vitro* that the anti-Xa assay underestimates LWMH plasma levels in patients with cirrhosis(117;118).

Low molecular weight heparin (LMWH) has been shown to be effective for the treatment of PVT in 45-75% of patients; however it is still not clear which thrombus and patient characteristics, including hemostatic coagulation balance, are predictive of the response to anticoagulation therapy.

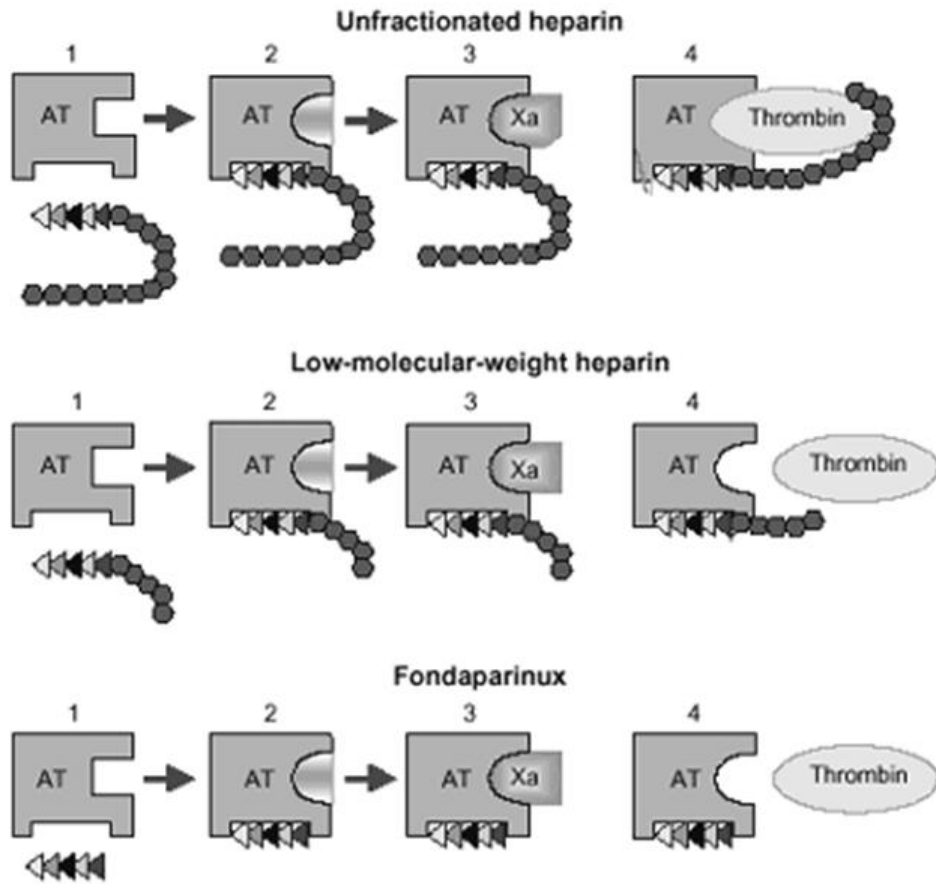


Figure 4. Action site of unfractionated heparin, low molecular weight heparin and fondaparinux.

Source: Am J Health-Syst Pharm © 2002 American Society of Health-System Pharmacists

Aims

Project I: Endothelial dysfunction in cirrhosis: characterization of structural and functional aspects of the portal vein which lead to in situ thrombosis.

1. To analyze the integrity of the endothelial lining in cirrhotic patients using specific markers for endothelial cells, such as FVIII, and to compare it to that of other systemic venous territories, as exemplified by the vena cava, and to compare these findings to those of the portal vein and vena cava of non-cirrhotic subjects.
2. To study the anticoagulant properties of the endothelium as represented by the distribution of thrombomodulin, the main endothelial anticoagulant protein, in the portal vein and vena cava of cirrhotic subjects and compare it to that of non-cirrhotic subjects.

Project II: Predictors of response to anticoagulant therapy in cirrhosis patients with portal vein thrombosis.

3. To assess hemostatic status in terms of pro- and anti-coagulant factors, as well as clinical characteristics of the thrombus and patients, as predictors of therapeutic efficacy of anticoagulation with low molecular weight heparin to treat portal vein thrombosis in patients with cirrhosis.

Project III: Anticoagulant response to low molecular weight heparin in plasma from patients with advanced cirrhosis.

4. To evaluate the effect of low molecular weight heparin on endogenous thrombin potential in plasma from patients with cirrhosis.
5. To correlate the anticoagulant efficacy of low molecular weight through thrombin generation assay with levels of anti-Factor Xa (activated Factor X) in plasma from patients with cirrhosis.
6. To correlate the anticoagulant effect of low molecular weight heparin *in vitro* with the severity of liver disease.
7. To correlate the efficacy of low molecular weight heparin *in vitro* with the determined plasmatic levels of coagulation factors and antithrombin in plasma from patients with cirrhosis.

I. Endothelial dysfunction in cirrhosis: characterization of structural and functional aspects of the portal vein which lead to in situ thrombosis

Materials and Methods

Venous samples

Venous samples from vena cava and portal vein were from obtained from adult subjects at transplant surgery in the case of cirrhosis patients, and during organ retrieval in non-cirrhosis donors (controls). All procedures were performed according to the Helsinki declaration. Criteria for exclusion of patients were: hepatocellular carcinoma (HCC), fulminant or acute hepatic failure, extrahepatic neoplasms, known genetic or acquired thrombophilia or overt thrombotic complications (DVT, PVT, or VTE) subsequently confirmed by determination of prothrombotic mutations (Factor V Leiden, prothrombin polymorphism G20210A) and pediatric LT recipients (<18 years of age). Criteria for exclusion of controls were similar to criteria used to exclude potential donors (neoplasm, abdominal trauma). As part of the protocol for organ retrieval, visual assessment by the surgeon and histological intraoperative analysis exclude the presence of significant liver disease.

Sample obtainment

Upon notice from the on-call transplant coordinator, the equipment necessary for sample transportation and preservation was prepared.

Vena cava and portal vein samples were obtained by sterile surgical excision of a 1 cm-wide circumferential specimen immediately after the liver explant in the case of cirrhotic patients, before immersion in the preserving UW (University of Wisconsin) cold solution, and after infusion of cold preserving UW solution in non-cirrhotic subjects, as part of the protocol in explant surgery. Specimens were received on sterile gauze.

Sample retrieval and conservation

Samples thus obtained were immediately prepared for conservation using a sterile technique. According to the position of the lumen, all samples were neatly and sharply sectioned transversally (perpendicularly to the luminal surface) into approximately 0.4 cm-diameter fragments for future analyses, obtaining fragments of the full-depth vessel wall. One fragment of each venous sample (vena cava and vena porta) was immediately conserved in buffered formalin, for immunohistochemical analysis. One fragment of each venous sample (vena cava and vena porta) was immediately embedded in optimal cutting temperature compound (OCT) frozen by isopentane, which was previously cooled using liquid nitrogen. The OCT-embedded samples were then placed into criovials and stored in liquid nitrogen until surgery was terminated and the team returned to the base hospital. Subsequently, frozen samples were immediately stored at -80°C and formalin-fixed samples were stored at room temperature until processing and analysis. All samples were processed within six months of procurement.

Immunohistochemical evaluation of FVIII

Formalin-fixed samples were processed for routine paraffin embedding. Serial 2 µm-thick slices were cut using a microtome, mounted onto clean slides and stored at room temperature. Thus obtained slices were then dewaxed in xylene (twice for 5 min each) and rehydrated through serial acohols (100%, 95%, 70%; two changes of 3 min each) to distilled water (twice for 5 min each), and air-dried for thirty minutes.

Immunohistochemical staining for factor VIII-related antigen (FVIII-RAG), as a marker for endothelial cells (119) was performed with rabbit anti-human Factor VIII conjugated with horseradish peroxidase antibody (Dako Cytomation, Denmark) diluted 1:100 in buffer containing 20 mM Tris-HCL pH 7.4 and 150 mM NaCl for two hours at room temperature. The antibodies were developed with 3, 3'-diaminobenzidine (DAB, Fluka, Milan, Italy), slides were then rinsed in phosphate buffered saline (PBS), and slides were mounted using coverslips with Vector ® mounting medium. Examination of the samples was performed with a light microscope Leica DMS 5000 (Leica, BM Medical, Padua, Italy). For this analysis all images were viewed and captured at 20 x and 40x magnification.

Immonofluorescence evaluation of TM

Cryostat sections were prepared from OCT-embedded frozen samples by cutting 4 µm-slices at -15° from frozen tissue bound to a chuck, and mounted on clean slides using static force. Mounted tissues were stored at -20°.

Before use, slides were air-dried for 30 minutes. For immunofluorescence analysis, tissue samples were fixed in 2% paraformaldehyde (PF) in PBS for 20 minutes at room temperature, treated with 50 mM NH₄Cl, and permeabilized with 0.5% Triton X-100 in PBS for 15 minutes. Tissues were then incubated for one hour with mouse anti-human TM (Diagnostica Stago, Asnières, France) at a concentration of 1:50, as the primary antibody. Following two rinsing procedures with PBS, tissues were subsequently incubated for one hour with goat anti-mouse IgG Fluorescein isothiocyanate (FITC)-conjugated antibody (Diagnostica Stago, Asnières, France) at a concentration of 1:400, as the secondary antibody.

Examination of samples was performed with a fluorescent microscope Leica IMDM 6000 (Leica, BM Medical, Padua, Italy). FITC fluorescence was visualized by excitation at 475-490 and emission at 530 nm. Cell nuclei staining was performed with Hoechst 1 µg/mL for 8 minutes at room temperature in the dark and fluorescence of nuclei was visualized by excitation at 330-385 nm with a 450 nm barrier filter. All samples were analyzed by differential interference contrast (DIC) objective. For this analysis, all images were viewed and captured at 20x and 40x magnification.

Results

I: Endothelial dysfunction in cirrhosis: characterization of structural and functional aspects of the portal vein which lead to in situ thrombosis

Four portal vein and four vena cava samples from adult patients with cirrhosis were obtained at liver transplantation, and four portal vein and four vena cava samples from non-cirrhosis adult controls were obtained at surgery for organ donation.

Characteristics of patients and controls

Mean age was 50.25 ± 15.1 in cirrhotic patients, vs 64.75 ± 14.6 in non-cirrhotic subjects. The etiology of liver disease was HCV in two patients, while one patient had autoimmune hepatitis/primary sclerosing cholangitis overlap syndrome, and the remaining patient had alcohol-related liver disease. MELD scores were 17, 38, 15, and 20 at liver transplant, and all four patients had evidence of portal hypertension, including refractory ascites in two, moderate ascites in the other two patients, and esophageal varices in three patients. Causes of death in organ donors were: cerebral anoxia in two, intracerebral hemorrhage in one, and non-abdominal trauma in the remaining patient. (Table 6).

Table 6. Characteristics of patients and controls. Samples of portal vein and vena cava were analyzed for each patient.

	Patients (n=4)	Controls (n=4)	P
Age (years)	50.25±15.1	64.75±14.6	0.47
Etiology of liver disease	HCV n=2, Alcohol-related n=1, Overlap syndrome n=1	-	
Cause of death	-	Cerebral anoxia n=2, Intracerebral hemorrhage n=1, Trauma n=1	
Platelet count (x10⁹/L)	104500±76483	168500±44762	0.2
INR	1.115±0.02	1.165±0.48	0.9
PT	67±5.3	78±4.9	0.45
PTT	28±2.8	27.25±5.67	0.85
Creatinine (mg/dL)	1.805±0.6	1.34±0.95	0.74
Hemoglobin (g/dL)	9.15±1.01	11.625±1.9	0.83
WBC (x10⁹/L)	4335±1723	12699.25±3704	0.87

WBC: white blood cells.

Immunohistochemistry

Sixteen samples (portal vein samples and vena cava samples from four cirrhosis patients and from four controls) were analyzed. FVIII was ubiquitously, consistently, and homogeneously present in all vessels studied, delineating the one-cell thick endothelial lining of the portal vein and vena cava samples in all patients, without any significant differences between patients and controls, nor between vena cava and the portal vein. (Figure 5).

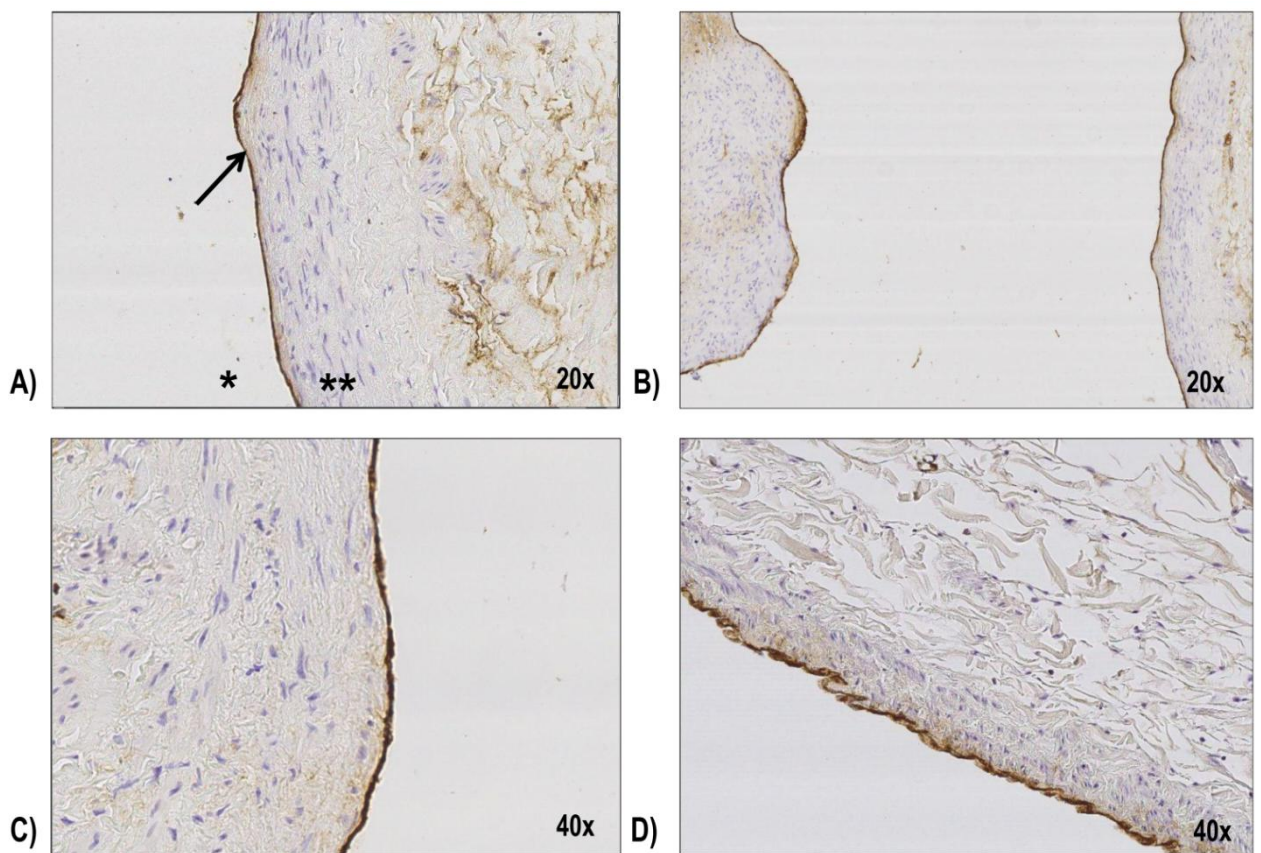


Figure 5. Immunohistochemical peroxidase staining for FVIII of portal vein and vena cava of a patient with cirrhosis and a non-cirrhotic control. A) Section of vena cava from control subject. B) Section of vena cava from cirrhotic patient. C) Section of portal vein from control subject. D) Section of portal vein from cirrhotic patient. *Vessel lumen. **Vessel wall, composed of collagen fibers and smooth muscle. Arrow: Endothelial lining. Objective 20x and 40x.

Immunofluorescence

TM was present in the endothelial lining of the portal vein in non-cirrhotic subjects. In contrast, the TM fluorescence signal was less intense in the analyzed portal vein sample of a cirrhotic patient. These results are preliminary, and represent the portal vein of three control cases and three cases of cirrhotic patients. (Figure 6).

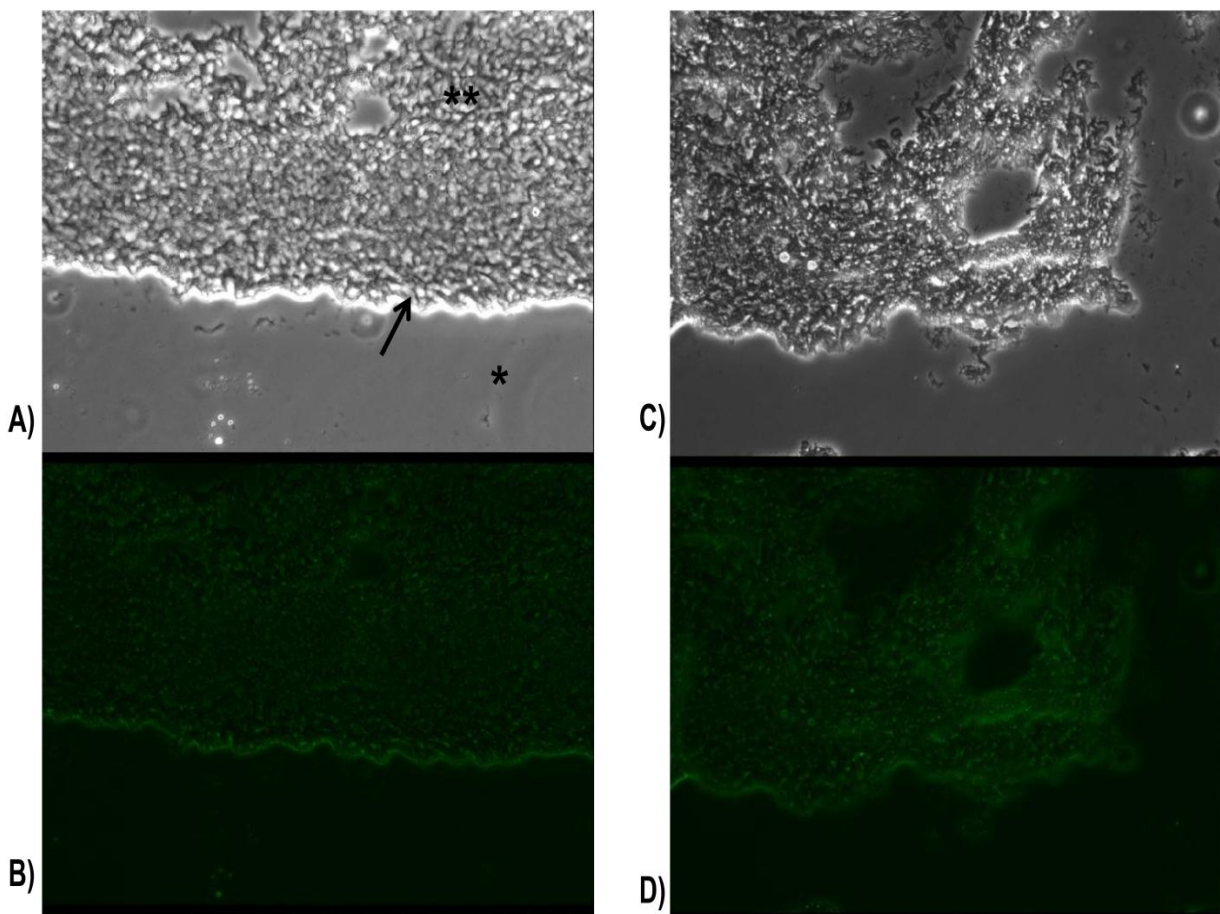


Figure 6. Immunofluorescence staining with FITC thrombomodulin (TM) on sections of portal vein from a control subject (left panel) and from a cirrhotic patient (right panel).

A;C) interference contrast. B;D) FITC staining of TM. *Vessel lumen.

**Vessel wall. Arrow: Endothelial lining which stains for TM).

Objective 40x magnification.

II. Predictors of response to anticoagulant therapy in cirrhosis patients with portal vein thrombosis.

Materials and Methods

Patients

A retrospective analysis of cirrhotic patients prospectively and consecutively evaluated and treated for thrombosis of the portal vein according to the local anticoagulation protocol at the Multivisceral Transplant Unit, Department of Surgical, Oncological, and Gastroenterological Sciences of the Padua University Hospital from January 2007 to October 2012 was performed. Exclusion criteria were: absence of underlying liver disease, present or recent (during the two weeks prior to evaluation) use of anti-platelet agents, and the presence of hepatocellular carcinoma. Patients with isolated thrombosis of the superior mesenteric vein or splenic vein, without involvement of the portal vein, were not included in the analysis. Upon initial evaluation, and as part of the local treatment protocol, every patient underwent clinical evaluation and blood sampling.

Definition of extent and age of thrombosis

At the first evaluation, all patients underwent computer tomography (CT) scanning or magnetic resonance imaging (MRI) to define the grade of

occlusion of the vessel(s) and determine the extension of thrombosis into the splenic vein and/or the superior mesenteric vein. Thrombosis was defined as partial or total, when thrombotic material occupied <90%, or ≥90% of the vessel lumen, respectively. Thrombus age was estimated based on past medical history, analysis of previous radiological studies, and radiological characteristics of the thrombus at diagnosis. Thrombus was arbitrarily defined as new if there was a recent episode of abdominal pain associated with radiological image of PVT compatible with fresh thrombus, with no evidence of collateral circulation at hepatic hilum on cross-sectional imaging. The thrombus was defined as recent (≤ 6 months) when imaging in the previous 6 months demonstrated no thrombosis and there was no established cavernous transformation of the portal vein. When signs of long standing thrombus were present (i.e. established cavernous transformation, defined as multiple small collaterals in and around the recanalizing or occluded main portal vein), the time interval was determined using previous radiological imaging demonstrating absence of thrombosis, and clinical history of previous diagnosis of PVT. Based on the thus obtained information, thrombus age was recorded as a discrete variable and also classified as ≤6 months or >6 months.

Thrombophilia screening

Upon initial evaluation (and before the start of anticoagulation), all patients underwent blood sampling for determination of: platelet count, PT, PTT, INR, Lupus Anticoagulant, anticardiolipin and anti-b2 glycoprotein I antibodies, levels of antithrombin, Protein C and S antigen and activity, activated protein C resistance, Factor VIII, Factor IX, Factor XI, Fibrinogen, and FVIII:PC Ratio. DNA analysis for Factor V Leiden (FVL) and Prothrombin G20210A mutations was also performed.

Prothrombin time (PT), INR and activated partial thromboplastin time (aPTT) were assessed with commercially available methods. Factor II, V, VII, VIII, IX, XI activities and fibrinogen levels were measured using commercially available reagents on BCT (Siemens, Germany) (120). AT activity was detected by a chromogenic method (Antithrombin III, Roche Diagnostic, Milan, Italy). Protein C anticoagulant and chromogenic activities were assessed using the Protein C and the Berichrom PC kits, respectively (Siemens, Germany) on BCT (Siemens, Germany), as previously reported (121). Protein S activity was measured using a coagulometric method (ProS IL, Milan, Italy) on ACL 9000 (IL, Milan, Italy) (122). Activated protein C resistance was measured using a “home-made” method on ACL 3000 (IL, Italy) as previously described (123). DNA analysis for Factor V Leiden (FVL) and Prothrombin G20210A mutations was performed as previously described (124). Lupus anticoagulant (LAC) was detected by PTT-LA (Diagnostica Stago, Asnieres, France) and DRVVT (Siemens, Marburg, Germany) on BCT (Siemens, Germany). The presence of LAC was established according to the guidelines of the “Scientific and Standardization Committee of the International Society on Thrombosis and Haemostasis: Subcommittee of the Lupus Anticoagulant/Phospholipid Dependent Antibodies” (125). Anticardiolipin and anti- β 2-glycoprotein-I antibodies both IgG and IgM were detected by commercially available ELISA kits (Orgentec, Mainz, Germany).

Anticoagulation protocol

According to the local protocol, before starting anticoagulation, all patients underwent full blood count, routine laboratory tests to evaluate coagulation and renal function, and endoscopic screening for esophageal varices. Patients with previous variceal bleeding and those with Grade II esophageal varices with red signs and grade III varices with or without red

signs completed variceal eradication by endoscopic band ligation at least 15 days prior to being started on anticoagulation. The study was conducted according to Declaration of Helsinki and patients enrolled in the treatment group gave their consent before starting the protocol.

The anticoagulation protocol consisted of administration of LMWH 1.5 mg/kg/day, and a 40% dose reduction was applied to patients with platelet count $<50 \times 10^9/L$. If serum creatinine was $>150 \text{ umol/L}$ or Creatinine Clearance $<50 \text{ mL/min}$ monitoring of anticoagulation was performed by assaying dosage of anti-Xa activity at 6 h after low weight molecular heparin (LWMH) administration. Anticoagulation was continued until recanalization or for a period of 12 months. In cases in which recanalization was not achieved after a year of therapy, anticoagulation was maintained at a prophylactic dose in order to avoid thrombus extension.

Follow-up and imaging

All patients were evaluated with abdominal Doppler ultrasound every two months during the first six months of therapy, and with CT scan/MRI and abdominal ultrasound every six months. Patency of the portal vein was assessed at 6 and 12 months from the start of anticoagulation therapy. Efficacy of anticoagulation therapy was defined as complete or $>50\%$ recanalization of the portal vein and its main branches.

Analyzed variables

The following patient variables were analyzed: age, sex, etiology of liver disease, severity of liver disease according to Child-Pugh class and MELD

score, INR, aPTT, PT, platelet count, patient status at timepoints 6 and 12 months (dead, alive, or transplanted), months of follow-up, plasmatic levels of FVIII, FIX, FXI, fibrinogen, antithrombin, protein S, protein C, calculus of FVIII:Protein C ratio, and the presence of FV Leiden mutation or Prothrombin G20210A polymorphism. The following variables associated to the thrombosis of the portal vein and the anticoagulation therapy were analyzed: grade of occlusion of the portal vein, extension to superior mesenteric vein and/or splenic vein and/or to intrahepatic branches, presence of portal cavernoma, estimated thrombus age at time of diagnosis, time interval between thrombus onset and start of anticoagulation therapy, achievement of recanalization or failure to recanalize, time interval between start of anticoagulation therapy and recanalization of the portal vein, progression or extension of thrombosis into splanchnic vessels, continuation of anticoagulation at a prophylaxis dose or lack of prophylactic anticoagulation after recanalization, the event of liver transplant, patency of portal vein at liver transplant, adverse events such as bleeding from esophageal varices or other sites, heparin-induced thrombocytopenia (HIT), and the need to discontinue anticoagulation therapy.

Endpoints

The endpoints were (i) complete or > 50% patency of previously thrombosed portal vein trunk or main branches; (ii) maintained patency of superior mesenteric vein and splenic veins; (iii) progression of thrombosis into the portal vein or extension into splenic or mesenteric veins; (iv) bleeding, intestinal infarction, liver transplantation or death.

Statistical analysis

Quantitative variables are expressed as the mean \pm SD and qualitative variables as absolute and relative frequencies. Comparisons between groups of quantitative and qualitative variables were made by the Wilcoxon and Chi square tests respectively. Multivariate analysis of variables associated with recanalization was performed by discriminant analysis. Time to recanalization rates were assessed using Cox models. Comparisons of recanalization rates with risk factors were made by the log rank test. All tests were two-sided, and $P < 0.05$ was considered significant. Data handling and analysis were performed with SPSS version 12.0 software (SPSS Inc., Chicago, IL, USA).

Results

II. Predictors of response to anticoagulant therapy in cirrhosis patients with portal vein thrombosis

Medical records for all patients treated with anticoagulation for PVT and who were evaluated at the Portal Hypertension Clinic of the Multivisceral Transplant Unit of the Padua University Hospital from January 2007 to October 2012 were reviewed. Forty-six patients were included in the study, and were followed for a mean of 25.2 months (range 3-68 months) from the time of initial evaluation for PVT at our center and until the closure of the study (October 2012).

Patient and thrombus characteristics

The characteristics of the study population and of the thrombosed vessels are shown in table 7. Mean age was 59 years (range 41-80), patients were predominantly of male sex (34/46), and mean MELD score was 12.8 (± 4.2). Estimated thrombus age at diagnosis was ≤ 6 months in 39/46 (84.8%) of cases. Regarding the grade of portal vein occlusion, PVT was partial in 36/46 (78%) of cases. Twenty-one patients (45.7%) had portal cavernoma, and PVT was extended into the superior mesenteric vein or splenic vein in 14/46 patients, and to the intrahepatic branches in 11 patients.

Thrombophilic mutations were found in 4 patients (heterozygosity for FV Leiden n=2, heterozygosity for Prothrombin G20210A n=1, and the presence of both mutations in heterozygosity in one single patient).

Recanalization under anticoagulation protocol

Fifteen patients underwent banding of esophageal varices before starting anticoagulation (mean number of sessions to eradication was 2 (range: 1–3)). Thirty of the 46 treated patients (65.2%) responded to anticoagulation after a mean of 4.5 months (± 3.1 months) of anticoagulation (Figure 7); 26 during the first 6 months of therapy and 4 in the following 6 months of therapy. Only 2/11 patients in whom the interval between thrombus onset and start of anticoagulation therapy was greater than 6 months presented recanalization. Complete recanalization was achieved in a total of 24 patients, while in 6 patients recanalization was partial. (Figure 8).

Table 7. Characteristics of cirrhotic patients with portal vein thrombosis (PVT) treated with anticoagulation and PVT characteristics.

Studied variable	P
Mean age ± SD	59 (41-80)
Sex (Male/Female)	34/12
Etiology of liver disease	Alcohol 15/46 HBV/HCV 22/46 Cholestatic/ Cryptogenic 9/46
Child-Pugh Class A/B/C	21/19/6
Thrombus age at diagnosis (mean ± SD)	3.46 ± 3.58 months
Thrombus age at diagnosis	
≤6 months	39
>6 months	7
Interval between thrombus onset and start of anticoagulation	4.76 ± 3.89 months
Interval between thrombus onset and start of anticoagulation	
≤6 months	35
>6 months	11
Extension to splanchnic vessels	
Superior Mesenteric Vein	11 (23.9%)
Splenic Vein	4 (8.7%)
Extension to intrahepatic branches (yes/no)	11 (23.9%)
Occlusion grade (total/partial)	10/36
Presence of cavernoma	21 (45.6%)
Full-dose therapy	24 (52.2%)
Reduced-dose therapy	22 (47.8%)
PT	67.7 ± 15.8
INR	1.3 ± 0.19
aPTT	36.02 ± 7.67
Factor VIII	164.6 ± 57.9
Factor IX	82.3 ± 28.1
Factor XI	66.5 ± 31.1
Fibrinogen	249.2 ± 146.4
Antithrombin	65.5 ± 22.8
Protein S	80 ± 21.1
Protein C	48.3 ± 19.5
FVIII:PC Ratio	3.65 ± 1.46
Platelet count (x10 ⁹ /L)	68.2 ± 30.8

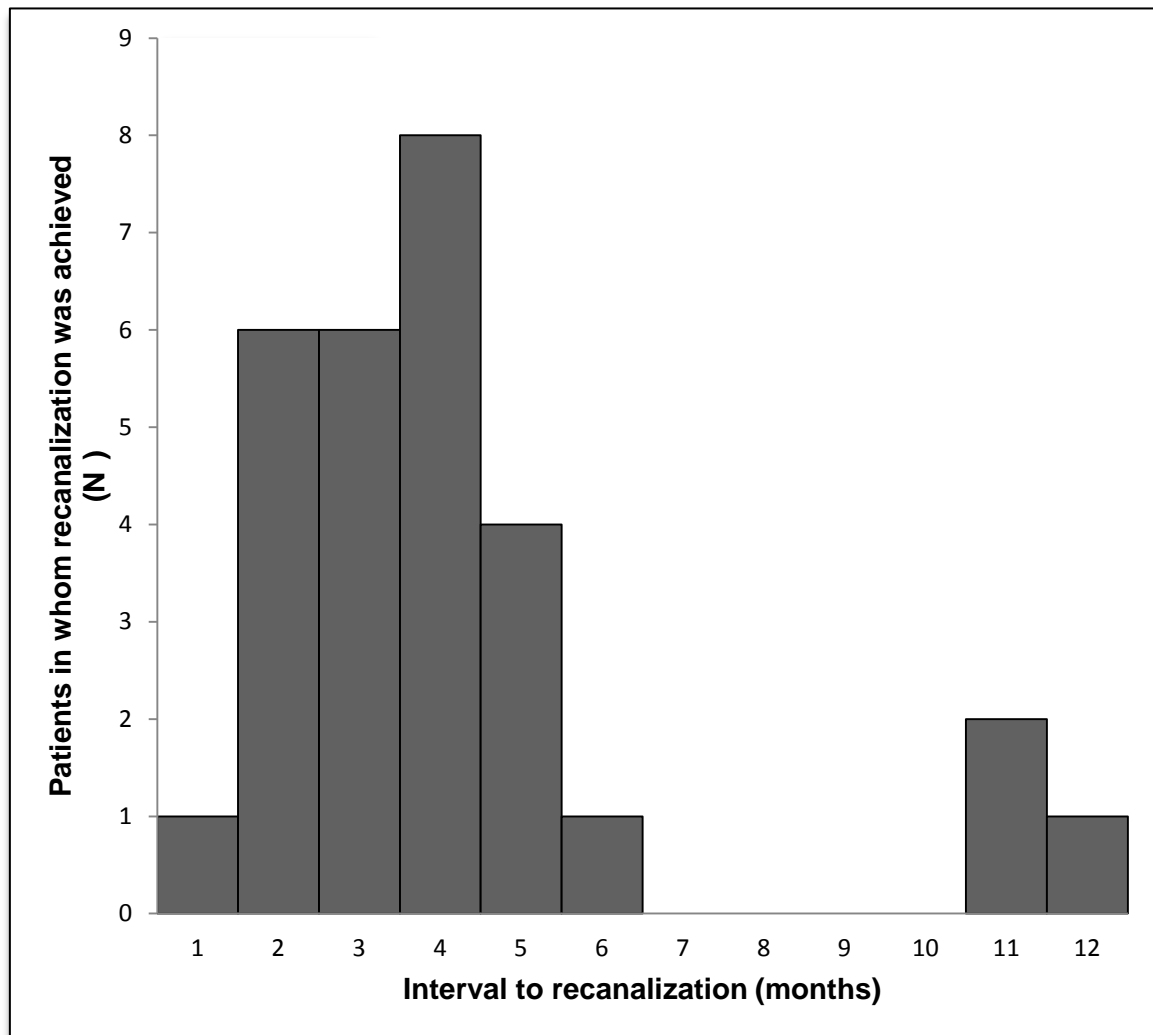


Figure 7. Frequency of recanalization of portal vein at different months after the start of anticoagulation treatment.

Mean = 4.5 months; standard deviation = 3.1; N = 30.

Adverse effects of anticoagulation therapy were observed in 8 patients: bleeding from esophageal varices n = 2, hematuria n = 1, epistaxis n = 1 (did not require suspension of anticoagulant therapy); cerebral hemorrhage n = 1 and HIT (heparin-induced thrombocytopenia) n = 3, in all of whom anticoagulation therapy with heparin was suspended.

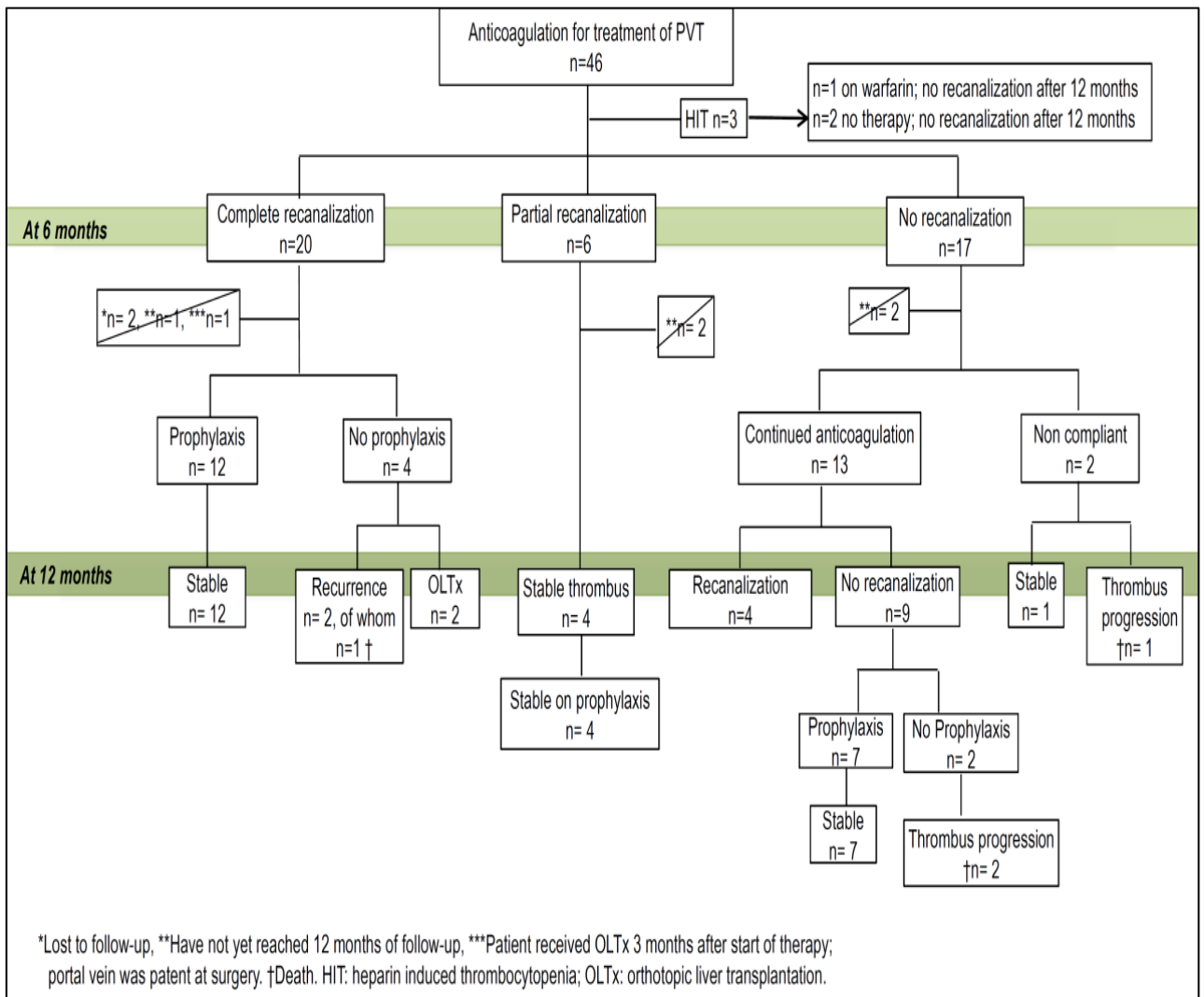


Figure 8. Therapeutic efficacy of anticoagulation for the treatment of portal vein thrombosis in cirrhosis patients after 6 and 12 months of anticoagulation therapy.

Factors associated with response to anticoagulation

Table 8 summarizes the patient and thrombus characteristics in the group of patients in whom recanalization was achieved compared to the group of patients in whom anticoagulation therapy did not result in recanalization of

the portal vein, and analyzed variables are compared between the two groups.

Thrombus age at diagnosis (1.9 ± 1.2 months vs 6.3 ± 4.5 months in the recanalization group and in the non-recanalization group, respectively, $p < .001$), the interval between thrombus onset and start of anticoagulation (3.2 ± 1.7 months vs 7.78 ± 4.5 months in the recanalization group and in the non-recanalization group, respectively, $p < .002$), and the presence of intrahepatic branch extension of the thrombosis ($p = .025$) were the only variables that correlated with response to anticoagulation therapy.

At logistic regression analysis, the interval between thrombus onset and start of anticoagulation therapy (≤ 6 months vs > 6 months in responders vs non-responders) correlated with the probability of recanalization ($p = .0051$). A longer interval between thrombus onset and start of anticoagulation therapy was associated with therapeutic failure (HR 0.734 (risk describes the probability of recanalization)).

At survival analysis using Kaplan Meier curves, the recanalization portal vein showed no significant impact on transplant-free survival. (Figure 9) However, mortality was higher in patients who did not present recanalization ($p = .03$); of five deaths in all the study population, four were related to PVT: one patient had thrombosis recurrence after recanalization had been achieved, but no prophylactic anticoagulation had ensued; one death occurred in a patient who never achieved recanalization due to non-compliance to therapy, and two other deaths occurred in patients in whom no recanalization had been achieved, while one death occurred in a patient who had adequately responded to therapy, but died on the waiting list for LT of hepatic insufficiency.

Table 8. Clinical characteristics of patients and portal vein thrombosis in patients in whom recanalization was achieved and in patients in whom recanalization was not achieved with anticoagulation.

Studied variable	Recanalized PVT n = 30	Not-Recanalized PVT n = 16	P
Mean age ± SD	59.5 ± 11.2	58.1 ± 9.8	NS
Sex (Male/Female)	22/8	12/4	NS
Etiology of liver disease	Alcohol 11/30 HBV/HCV 15/30 Cholestatic/ Cryptogenic 4/30	Alcohol 4/16 HBV/HCV 7/16 Cholestatic/ Cryptogenic 5/16	NS
Child-Pugh Class A/B/C	15/14/1	6/5/5	NS
Thrombus age at diagnosis (mean ± SD)	1.9 ± 1.2 months	6.3 ± 4.5 months	.001
Thrombus age at diagnosis ≤6 months	30	10	.001
>6 months	0	6	
Interval between thrombus onset and start of anticoagulation	3.2 ± 1.7 months	7.78 ± 4.5 months	.002
Interval between thrombus onset and start of anticoagulation ≤6 months	28	7	.001
>6 months	2	9	
Extension to splanchnic vessels			
Superior Mesenteric Vein (yes/no)	9/21	2/14	NS
Splenic Vein (yes/no)	4/26	0/16	
Extension to intrahepatic branches (yes/no)	4/26	7/9	.025
Occlusion grade (total/partial)	6/24	4/12	NS
Presence of cavernoma	12	9	NS
Full-dose therapy	13	11	NS
Reduced-dose therapy	17	5	NS
PT	69.2 ± 14.2	66.7±18.4	NS
INR	1.3 ± 0.1	1.4±0.2	NS
aPTT	35.6 ± 7.6	36.3±7.5	NS
Factor VIII	158.4 ± 39.0	177.2 ± 90.4	NS
Factor IX	83.5 ± 25.9	82.5 ± 35.2	NS
Factor XI	67.4 ± 26.0	68 ± 41.9	NS
Fibrinogen	233.1 ± 89.0	263.1 ± 227.9	NS
Antithrombin	63.9 ± 18.8	69.8 ± 30.2	NS
Protein S	79.7 ± 19.3	84.2 ± 23.7	NS
Protein C	51.7 ± 16.5	44.2 ± 24.3	NS
FVIII:PC Ratio	3.3 ± 1.2	4.2 ± 1.7	NS
Platelet count (x10 ⁹ /L)	84.5 ± 38.2	58.1 ± 23.4	NS

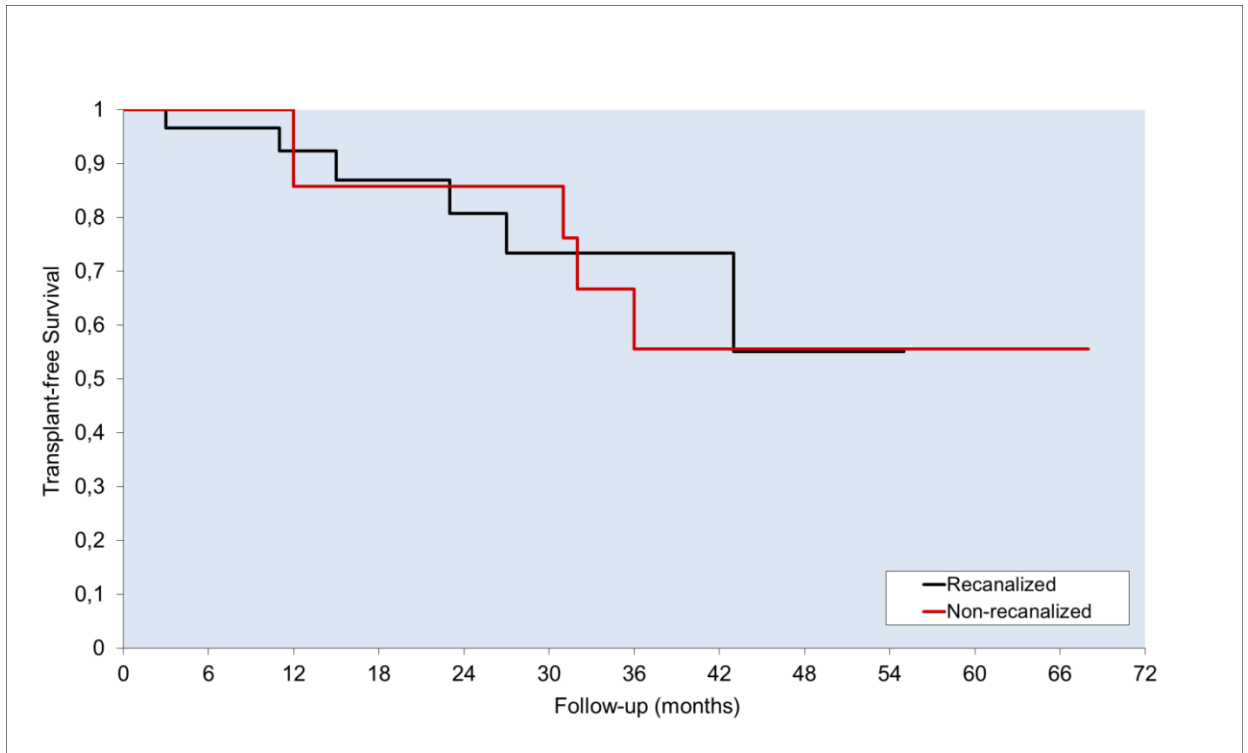


Figure 9. Transplant-free survival in cirrhosis patients stratified according to response to anticoagulation therapy for the treatment of portal vein thrombosis (PVT).

III. Anticoagulant response to low-molecular-weight heparin in plasma from patients with advanced cirrhosis.

Materials and Methods

Patients

Thirty patients admitted to the Multivisceral Transplant Unit, Department of Surgical, Oncological and Gastroenterological Sciences of the Padua University Hospital, who had been previously diagnosed with cirrhosis were included in the study, and were prospectively and consecutively enrolled for each class of Child Pugh until equal distribution of severity of liver disease was attained. Exclusion criteria were: use of antiplatelet agents, previous or ongoing anticoagulation treatment, presence of PVT and active hepatocellular carcinoma or extrahepatic neoplasm. Two cohorts of patients, ten with heterozygous inherited type 1 AT defect and ten healthy subjects were used as control groups. Patients with type 1 AT deficiency presented approximately 50% reduction of both AT antigen and activity and belonged to thrombophilic families that have been previously studied (126;127). None of the patients with inherited AT deficit presented other associated thrombophilic defects. Exclusion criteria for the control groups were: age younger than 18 years, pregnancy, presence of active cancer, ongoing anticoagulant or hormonal treatments, and history of previous thrombotic events. Informed consent according to the Helsinki Declaration was obtained from each patient before inclusion in the study.

Blood collection and plasma preparation

Fasting whole blood samples (15 mL) from each patient and control were drawn by clean venipuncture and collected into vacuum tubes containing 109 mmol/L trisodium citrate as an anticoagulant, at a blood to anticoagulant ratio of 9:1, using the BD-Vacutainer® system (Plymouth, UK). Platelet poor plasma was prepared by double centrifugation at 2000 g for 10 min. Plasma was aliquoted in plastic tubes, snap-frozen and stored at -80 °C until use.

Thrombophilia screening and evaluation of coagulation factors

Prothrombin time (PT), INR and activated partial thromboplastin time (aPTT) were assessed with commercially available methods. Factor II, V, VII, VIII, IX, XI activities and fibrinogen levels were measured using commercially available reagents on BCT (Siemens, Germany) (120). AT activity was detected by a chromogenic method (Antithrombin III, Roche Diagnostic, Milan, Italy). Protein C anticoagulant and chromogenic activities were assessed using the Protein C and the Berichrom PC kits, respectively (Siemens, Germany) on BCT (Siemens, Germany), as previously reported (121). Protein S activity was measured using a coagulometric method (ProS IL, Milan, Italy) on ACL 9000 (IL, Milan, Italy) (122). Plasminogen activity was evaluated by a chromogenic method (Berichrom Plasminogen, Siemens, Germany). Activated protein C resistance was measured using a “home-made” method on ACL 3000 (IL, Italy) as previously described(123). DNA analysis for Factor V Leiden (FVL) and Prothrombin G20210A mutations was performed as previously described (124). Lupus anticoagulant (LAC) was detected by PTT-LA (Diagnostica Stago, Asnieres, France) and DRVVT (Siemens, Marburg, Germany) on BCT (Siemens, Germany). The presence of LAC was

established according to the guidelines of the “Scientific and Standardization Committee of the International Society on Thrombosis and Haemostasis: Subcommittee of the Lupus Anticoagulant/Phospholipid Dependent Antibodies” (125) Anticardiolipin and anti- β_2 -glycoprotein-I antibodies both IgG and IgM were detected by commercially available ELISA kits (Orgentec, Mainz, Germany).

Addition of Enoxaparin to plasma samples

Enoxaparin (Clexane® (Sanofi-Aventis) at 0.5 UI/mL or 0.7 UI/mL final anti-Xa concentration was added to plasma samples of every cirrhotic patient, patients with genetic AT deficit, and controls.

Anti-Xa activity measurement

Anti Xa activity was measured at basal conditions and after addition of enoxaparin at a final concentration of 0.35UI/mL and 0.7 UI/mL of anti-Xa activity through an amidolytic method according to the manufacturer instructions, with the BCT (Siemens, Germany). A fixed concentration of bovine activated factor X (FXa) and a chromogenic substrate are added to the plasma sample. Hydrolyzation of the substrate mediated by FXa depends on the inhibition of FXa acted by the complex heparin-antithrombin present in the tested plasma sample. The hydrolysis of the substrate is inversely proportional to the LMWH concentration present in the plasma sample.

Thrombin Generation

Thrombin generation test (TGT) was performed on platelet-poor plasma (PPP) with the fluorimetric method described by Hemker, Calibrated Automated Thrombogram® (CAT)(44;45) and expressed as Endogenous thrombin potential (ETP). In this test, coagulation is activated on PPP, using the commercial trigger composed of recombinant tissue factor (TF) at a concentration of 5 pM and phospholipids at a concentration of 4µMol. Thrombin generation curves were calibrated with a thrombin Calibrator (Thrombinoscope BV, Maastricht, The Netherlands) at each cycle of the test.

A fluorogenic substrate (Z-Gly-Gly-Arg-AMC, BACHEM AG, Bubendorf, Switzerland) was dispensed in each well at a final concentration of 300 µM to allow a continuous registration of thrombin generation, fluorescence produced was read in real time by a fluorometer, Fluoroskan Ascent® (Thermo Labsystems, Helsinki, Finland). A software (Thrombinoscope BV, Maastricht, The Netherlands)® recorded fluorescence generated and created a curve of thrombin generation. The area under the curve is expressed as ETP, while the peak of the curve corresponds to the peak of thrombin generated (CMax.)

TG with determination of ETP was performed at basal conditions and after addition of enoxaparin at a final concentration of 0.35UI/mL and 0.7 UI/mL of anti-Xa activity. The anticoagulant effect of enoxaparin was expressed as the ratio between ETP after addition of anticoagulant (0.35 ETP ratio and 0.7 ETP ratio) and ETP obtained at basal conditions in each group of patients and controls.

TG tests were performed in the same way in another aliquot of plasma with the addition of soluble (TM) (Recombinant human thrombomodulin

(CD141), American diagnostica inc., Stamford, USA), at a final well concentration of 6nM.

Statistical Analysis

Continuous variables were expressed as mean \pm standard deviation (SD) or as medians and ranges. The nonparametric Mann-Whitney *U* and Kruskal-Wallis H test were used to test for differences between median values when appropriate. Spearman's correlation coefficient was used to assess correlation between continuous different variables and Pearson Chi-square or Fisher's exact test with Pearson Correlation Coefficient for dicothomus variables. *P* values of .05 or less were considered statistically significant. All analyses were performed with SPSS version 11.5 software (Chicago, IL).

Results

III. Anticoagulant response to low-molecular-weight heparin in plasma from patients with advanced cirrhosis.

Patient characteristics and coagulation profile

The main characteristics of the study population are reported in Table 9. Thirty patients with cirrhosis, (24 males and 6 females), were included, and they were equally distributed according to the severity of liver disease as expressed by the Child Pugh classes (10 Child Pugh A, 10 Child Pugh B, and 10 Child Pugh C patients). Ten patients with heterozygous type 1 AT defect and 10 healthy subjects were included as controls.

Table 9. Demographic and clinical characteristics of cirrhosis patients, subjects with genetic antithrombin defect, and healthy controls.

Characteristics	Cirrhotic patients			AT defect <i>n</i> = 10	Healthy controls <i>n</i> = 10	<i>P</i> -value
	Child Pugh A <i>n</i> = 10	Child Pugh B <i>n</i> = 10	Child Pugh C <i>n</i> = 10			
Age (yrs)	55 ± 5	58,2 ± 3	52,4 ± 6	47.2 ± 7	44.8 ± 5	.12
Sex (male)	7	9	8	6	4	.14
Etiology of liver disease						
Alcoholic (n)	4	3	5	-	-	.65
HCV (n)	2	2	4	-	-	.5
HBV (n)	1	0	0	-	-	.35
Alcoholic+HCV (n)	3	5	1	-	-	.14
Serum bilirubin mg/dL	1.4 ± 0.5	1.9 ± 1.4	3.6 ± 2.1	-	-	.03
INR	1.3 ± 0.3	1.7 ± .04	1.9 ± 0.6	1.2 ± 0.2	1 ± 0.6	.01
Serum albumin (g/L)	3.3 ± 0.6	3.0 ± 0.4	2.8 ± 0.7	3.9 ± 0.6	3.8 ± 0.14	<.001
Serum creatinine (mg/dL)	0.6 ± 0.2	1.05 ± 0.6	0.8 ± 0.1	0.8 ± 0.1	0.7 ± 0.1	.07
Hemoglobin (g/dL)	10.5 ± 2.3	13.1 ± 2.4	11.3 ± 1.7	13.7 ± 1.5	13.3 ± 1.6	.05
Leucocytes (10 ⁹ /L)	3.8 ± 2.6	4.5 ± 3.2	4.2 ± 2.4	6.4 ± 1.5	7.3 ± 1.9	<.001
Platelets (10 ⁹ /L)	130 ± 56	114 ± 400	120 ± 62	260 ± 10	270 ± 13	<.001

AT: antithrombin; HBV: Hepatitis B Virus HCV: Hepatitis C Virus.

Plasma activity of coagulation factors and inhibitors, platelet count, aPTT, INR and PT are shown in Table 10. Patients with AT deficiency had lower levels of AT, and patients with liver cirrhosis showed a statistically significant prolongation of aPTT and INR, a decrease in PT and all measured coagulation proteins, except for factor VIII, as compared to controls. The reduction in levels of plasmatic factors was consistent with the severity of liver disease. In particular, patients with Child Pugh C cirrhosis were characterized by lower AT activity levels than patients with AT inherited defect (42±14% versus 52±4%, respectively, p=.06) (Figure 10)

No patient or control had antiphospholipid antibodies and none was carrier of factor V Leiden and/or prothrombin G201210A mutation.

Table 10. Coagulation parameters in controls, cirrhotic patients, and patients with antithrombin defect type 1.

	Platelet count (X10 ⁹ /L)	PT (%)	INR	PTT (sec)	FVIII (%)	FIX (%)	FXI (%)	Fibrinogen (mg/dl)	AT [†] (%)	Protein S [†] (%)	Protein C [†] (%)	Plasminogen (%)	FII (%)	FV (%)	FVII (%)	FX (%)
Controls	268 ± 134	98 ± 9	1 ± 0.6	26.2 ± 2.8	115.6 ± 34	121.8 ± 20	119.8 ± 27	393 ± 128.7	103.9 ± 9	122.7 ± 14	123.2 ± 24	92 ± 8	84 ± 8	88.4 ± 7	92.2 ± 3	84.7 ± 9
Child A	108 ± 56	88 ± 5	1.3 ± 0.3	1.3 ± 0.3	142 ± 38	95 ± 25	117 ± 108	258 ± 116	76 ± 25	83 ± 19	69 ± 34	78 ± 21	77 ± 27	65 ± 19	70 ± 23	76 ± 19
Child B	123 ± 40	50.5 ± 4	1.7 ± 0.4	1.7 ± 0.5	158 ± 50	71 ± 27	55 ± 30	164 ± 64	55 ± 22	78 ± 22	44 ± 28	55 ± 22	53 ± 28	39 ± 19	51 ± 35	68 ± 25
Child C	113 ± 62	45.5 ± 6	1.9 ± 0.6	1.9 ± 0.6	196 ± 34	68 ± 32	41 ± 19	191 ± 107	42 ± 14	83 ± 12	27 ± 16	52 ± 21	41 ± 28	40 ± 22	30 ± 11	64 ± 17
AT Defect	260 ± 103	96 ± 4	1.2 ± 0.2	27.4 ± 3	107 ± 11	118.4 ± 13	121.1 ± 12	480 ± 181	52 ± 4	115.7 ± 12	121.3 ± 21	88 ± 4	86.2 ± 2	101.1 ± 2	89.5 ± 8	86.5 ± 7
P	.03	<.001	<.001	<.001	<.001	<.001	<.001	<.001	<.001	<.001	<.001	<.001	<.001	<.001	<.001	ns

[†]Coagulometric activity.

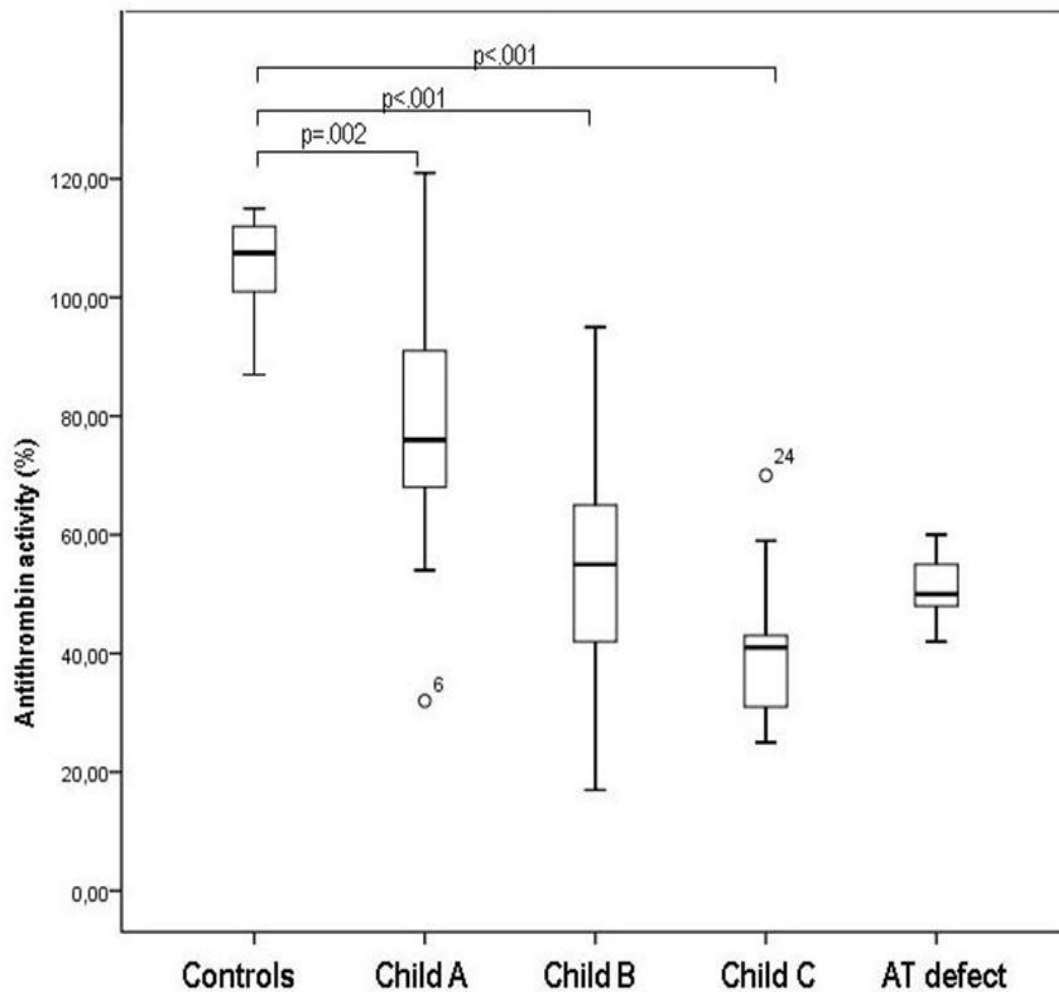


Figure 10. Plasmatic coagulometric antithrombin activity in healthy controls, cirrhotic patients distributed according to Child Pugh class, and in subjects with genetic antithrombin defect type 1.

Determination of anti-Xa activity in plasma

After the addition of LMWH to plasma samples at an estimated final concentration of LMWH 0.35 UI/mL, measured values of anti-Xa activity were 0.36 ± 0.04 UI/mL in healthy subjects, 0.34 ± 0.05 UI/mL, 0.27 ± 0.6 UI/mL, 0.21 ± 0.09 UI/mL in Child Pugh A, Child Pugh B, and Child Pugh C

class cirrhotic patients, respectively, and 0.34 ± 0.01 UI/mL in patients with congenital AT defect. At LMWH 0.35 UI/mL concentration, anti-Xa activity was significantly lower in Child Pugh B and Child Pugh C patients as compared to controls ($p < 0.001$), as well as in patients with congenital AT defect as compared to controls ($p < 0.001$). Likewise, at LMWH 0.7 UI/mL, anti-Xa activity was significantly lower in Child Pugh B and Child Pugh C cirrhotics (0.59 ± 0.03 and 0.51 ± 0.08 UI/mL) and in patients with AT defect (0.64 ± 0.04 UI/mL) than in controls (0.72 ± 0.009 UI/mL) ($p < 0.001$), and in Child Pugh C cirrhotics than in patients with AT defect ($p < 0.001$).

Thrombin generation

When thrombin generation was performed without addition of enoxaparin, patients with cirrhosis showed decreased endogenous thrombin potential, expressed as ETP (nMol*min), as compared to controls and to patients with congenital AT defect, independently of the severity of liver disease (Figure 11).

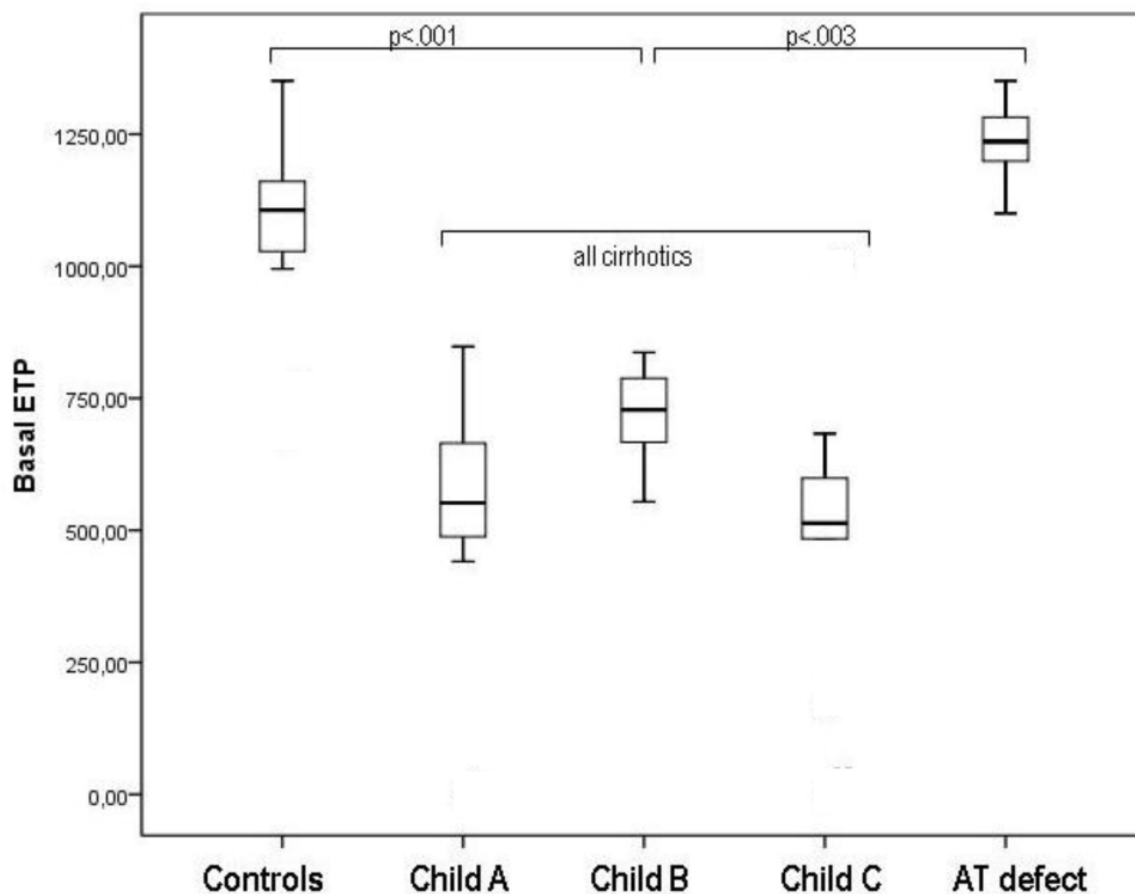


Figure 11. Native endogenous thrombin potential (ETP) in healthy controls, cirrhotic patients according to Child class, and subjects with genetic antithrombin defect type 1.

After the addition of enoxaparin at a final concentration of 0.35 IU/mL, there was a significantly greater decrease in thrombin generation in controls than in patients with congenital AT defect, with a mean ETP reduction of $51.6 \pm 14\%$ and $30.8 \pm 10\%$, respectively ($p = .003$). Despite low levels of AT and anti-Xa activity, patients with cirrhosis showed a greater anticoagulant effect of LMWH, with a mean ETP reduction of $72.6 \pm 11\%$ ($p = 0.02$ versus controls).

The decrease in thrombin generation with LMWH in cirrhotic patients mirrored the severity of liver disease. Although Child Pugh class C patients had AT levels similar to patients with heterozygous AT defect, they exhibited the greatest reduction in ETP, with a mean reduction of $82.6 \pm 10\%$. On the contrary, patients with AT defect were characterized by an apparent low response to LMWH with a mean reduction of ETP by $30.8 \pm 10\%$ with respect to basal conditions.

In cirrhotic patients, the anticoagulant response to LMWH at an estimated concentration equal to 0.35 IU/mL of anti-Xa activity expressed as ETP ratio correlated with all coagulation parameters tested, except for INR.(Table 11).

Table 11. Correlations between coagulation parameters and ETP ratio 0.35 in cirrhotic patients.

PT	$r = 0.45$	$P = 0.04$
INR	$r = -0.44$	$P = 0.19$
PTT	$r = -0.5$	$P = .007$
Fibrinogen	$r = 0.37$	$P = .05$
Antithrombin	$r = 0.64$	$P < .001$
Protein C	$r = 0.4$	$P = .03$
Plasminogen	$r = 0.5$	$P = .008$
Factor II	$r = 0.55$	$P = .003$
Factor VII	$r = 0.43$	$P = .019$
Factor X	$r = 0.42$	$P = .03$

ETP: endogenous thrombin potential

Interestingly, the plasma AT activity level was the parameter that most strongly correlated with the response to LMWH expressed as ETP ratio ($r=.64$, $p<0.001$) (Figure 12).

No correlation was found between ETP ratio 0.35 and severity of liver disease expressed as Child Pugh score, or serum bilirubin, albumin or creatinine levels.

When thrombin generation was performed after addition of enoxaparin at a final concentration of 0.7 UI/ml, ETP was not measurable in 9 (4 in Child Pugh B and 5 in Child Pugh C) out of 20 patients with more advanced cirrhosis because of complete abolition of thrombin generation, as compared to 1 out of 10 controls. In the remaining samples, in which generated thrombin reached a measurable threshold, the anticoagulation was almost complete in all groups, with ETP ratio 0.7 of 0.26 ± 0.08 , 0.19 ± 0.06 , 0.10 ± 0.08 in Child Pugh A, B, and C cirrhotic patients respectively), compared to 0.15 ± 0.15 in controls. Patients with heterozygous AT defect showed “resistance” to LMWH, with an ETP ratio of 0.56 ± 0.47 .

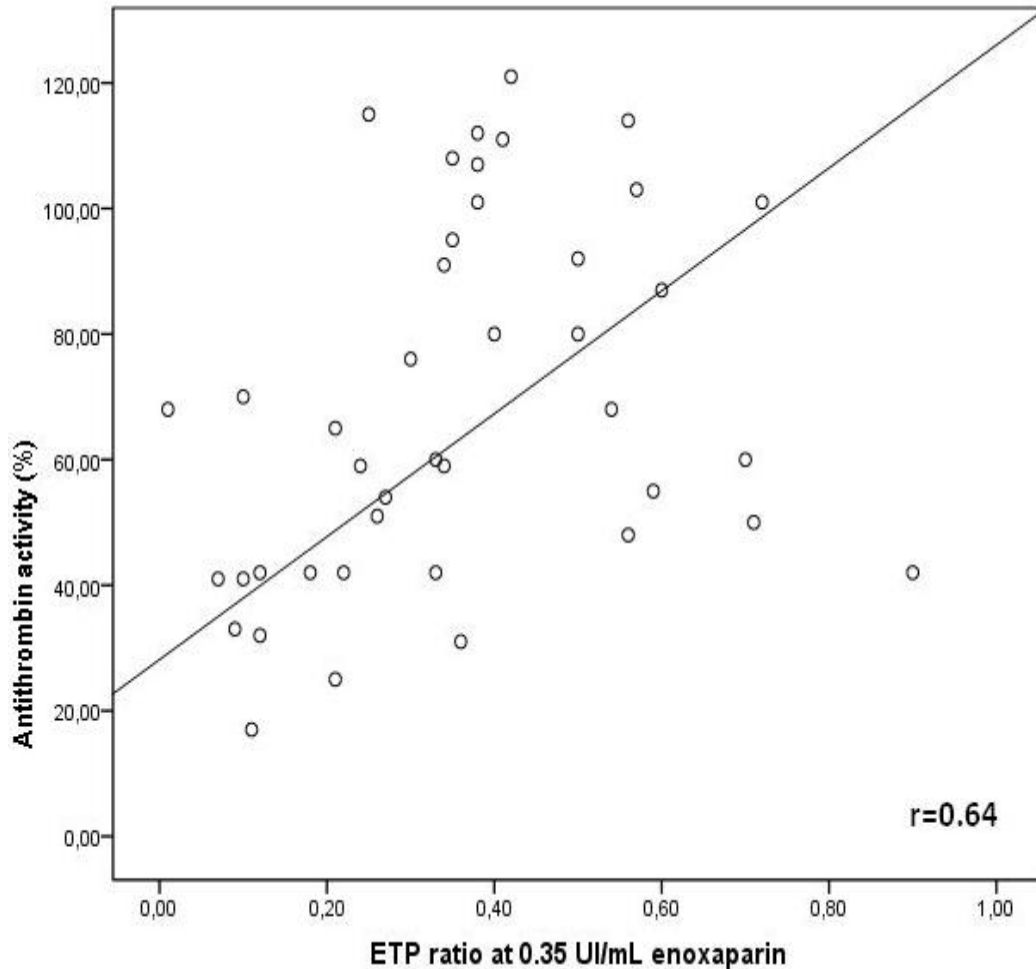


Figure 12. Correlation between endogenous thrombin potential (ETP) ratio at 0.35 UI/mL and plasmatic antithrombin levels.

In native plasma, amongst patients with cirrhosis, only those in Child Pugh class C showed a significant increase in the ETP ratio with and without (TM), compared with controls and patients with AT genetic defect (0.97 ± 0.03 vs 0.85 ± 0.05 , and 0.87 ± 0.06 , respectively, $P < .05$). (Figure 13). After the addition of enoxaparin at a final concentration of 0.35 IU/mL, the ETP ratio with and without TM mirrored the results obtained in the native samples (Figure 14). Moreover, when thrombin generation was performed after the addition of enoxaparin at a concentration of 0.7 IU/ml in samples with the addition of TM, ETP was not measurable in 7 cirrhotic patients

(three Child Pugh B and four Child Pugh C), and again in one of the controls, similarly to the results obtained in samples without the addition of TM.

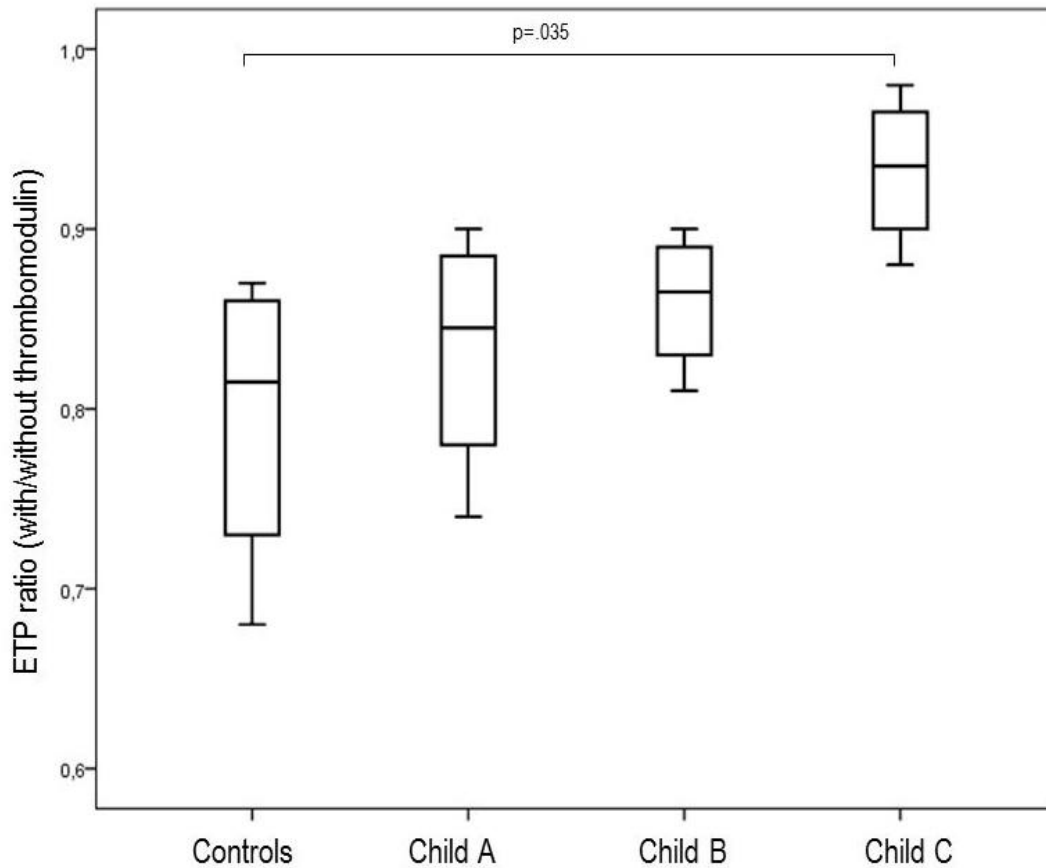


Figure 13. Distribution of values of ETP ratio between thrombin generation assessed with and without TM for healthy subjects and patients with cirrhosis stratified according to classes of Child Pugh.

ETP=Endogenous Thrombin Potential, TM=Thrombomodulin

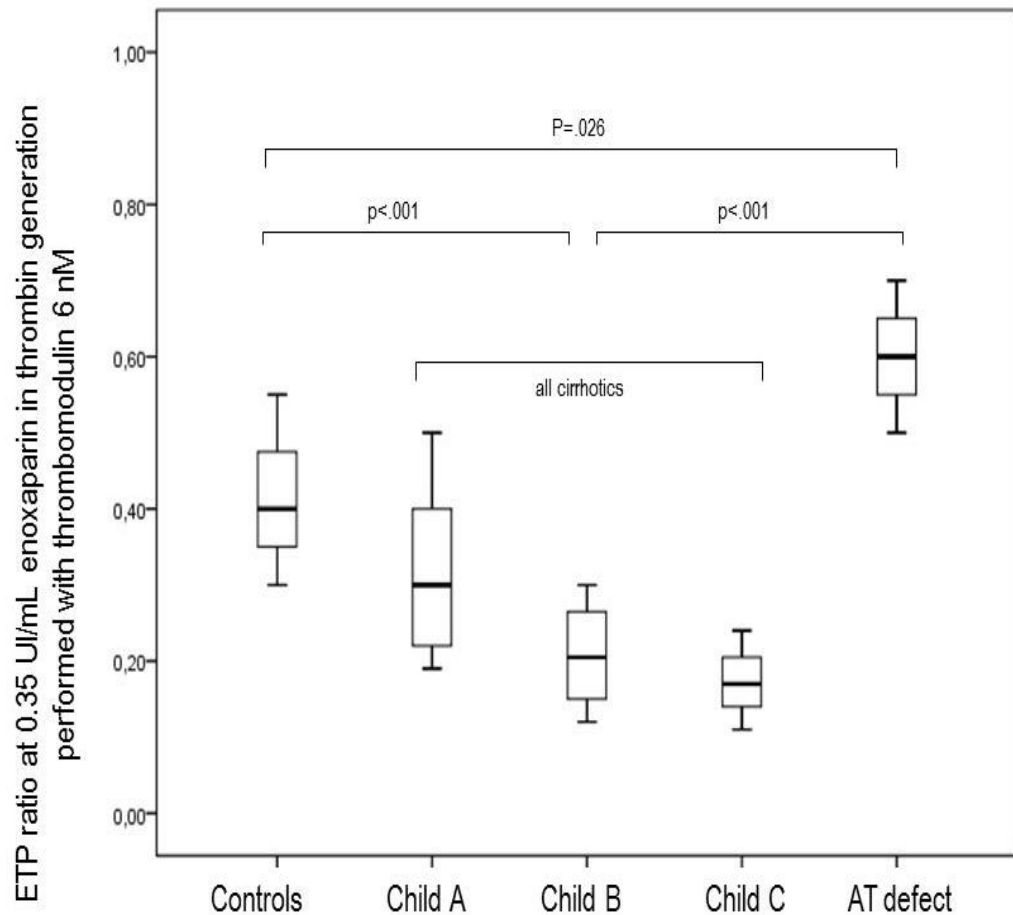


Figure 14. The ETP ratio of plasma with 0.35 UI mL enoxaparin to native plasma in samples with 6 nM TM in healthy controls, cirrhotic patients according to Child Pugh classes and subjects with a genetic antithrombin (AT) defect type 1.

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Discussion

According to Virchow's triad, the three key role players in the formation of thrombosis are: blood stasis, alterations of the coagulation cascade, and endothelial dysfunction. In cirrhosis patients, the most common site of thrombosis is the portal vein, probably reflecting the consequence of all three mechanisms. Venous stasis present in portal hypertension due to the architectural derangement of the cirrhotic liver, likely combines with an endothelium that has lost, partially or completely, its anticoagulant properties. The characteristic altered coagulation status of advanced liver disease, characterized by diminished levels of pro- but also anti-coagulant circulating factors, probably also contributes to the establishment of portal vein thrombosis.

The arm-to-tongue circulation time of the blood (~30 seconds) is short compared with a whole blood clotting time, so thrombin formed in flowing blood in vivo is rapidly diluted and inactivated before clotting can occur. Thrombin only builds up in unstirred boundary layers at cell surfaces and in the stagnant plasma caught in a clot or an aggregate, and transport by diffusion will therefore tend to govern reaction rates (42). Within the portal vein of cirrhosis patients, flow is severely hampered, with inversion of flow direction being a relatively common finding (128). In the study by Zocco and collaborators, where portal flow velocity was determined using Doppler ultrasound in cirrhotic patients who were subsequently followed for the development of PVT, patients who developed PVT had a mean basal value of portal flow velocity of 11.8 ± 2.6 cm/s, and a velocity below 15 cm/s was the only independent predictor of occurrence of PVT(75). Notably, however, low flow in the portal vein (19.6 ± 5.7 cm/s) was also found in all cirrhotics, also in those who had not developed PVT one year after initial Doppler evaluation. Venous stasis can favor the initiation of the

coagulation cascade, which, in the presence of an endothelium that has lost its anticoagulant properties, may result in thrombus formation.

Actual development of thrombosis has been demonstrated to injure the venous wall, causing a reaction that has been described as similar to wound healing, associated with increased procollagen, total collagen, and metalloproteinases (129).

Not only existing thrombosis, however, but also hypertension, has been experimentally shown to induce measurable changes. Hayashi et al. used a rabbit model in which left femoral venous pressure was elevated by constriction of the external iliac vein (130). Compared with the contralateral normal vein, the femoral vein, which experienced the higher intravascular pressure (16 vs 7 mmHg), displayed increased wall thickness and elevated vascular tone.

Regarding the integrity of the endothelium's anticoagulant functions, TM plays a fundamental role and was therefore studied. TM is an integral membrane glycoprotein that has a major role in the regulation of intravascular coagulation(131). This glycoprotein can change the function of thrombin, the main procoagulant enzyme, to an anticoagulant through activation of the protein C pathway(132). In turn, activated protein C in the presence of protein S inactivates factor VIIIa and factor Va, and thereby inhibits further formation of thrombin.

High levels of circulating TM have been demonstrated after induction of liver damage in animal models and other diseases with systemic inflammation(93;133). Circulating TM has been found to be either normal or elevated in patients with liver disease, and to be correlated with endothelial damage, without showing, however, a direct relationship with

the degree of portal hypertension. A correlation with severity of liver disease has been described, however (134).

In the study by Remkova and collaborator (135), the Authors explored plasmatic levels of homocysteine in relation to soluble TM and von Willebrand Factor (vWF) as markers of endothelial dysfunction in 71 patients with varying severity of chronic liver disease, and compared them to those of 51 healthy subjects. Homocysteine, TM, and vWF were all elevated in patients with chronic liver disease, but only the last two markers showed a statistically significant correlation with increasing severity of liver disease. Circulating levels of TM, determined using ELISA, were increased in steatosis ($P=0.029$), Child A cirrhosis ($P=0.0010$), and Child B and Child C cirrhosis ($P<0.0001$, respectively), whereas circulating levels of vWF, determined using the same methodology, were elevated in Child A cirrhosis ($P=0.0003$) as well as in Child B and C cirrhosis ($P<0.0001$, respectively).

In the present study, immunohistochemical analysis of portal vein samples in cirrhotic patients demonstrated low levels of endothelial TM. Although apparently contradictory with respect to the above mentioned study, elevated circulating TM levels can be a consequence of endothelial damage, where previously bound TM is shed into the bloodstream. Although TM is mostly located on endothelial cells of arteries, veins, and capillaries, there is a small amount of circulating, soluble TM, and albeit its function is unclear, there is evidence that its presence signifies endothelial-cell damage(136;137). A positive association between soluble TM and different manifestations of endothelial damage, such as coronary heart disease and peripheral occlusive arterial disease, has been shown(138). Furthermore, although dealing with arterial damage rather than venous endothelial injury, in a study by Blann and collaborators(139), it was reported that among 54 survivors of myocardial infarction, soluble

TM was directly related to the risk of a recurrent cardiovascular event during 49 months of follow-up.

In cultured human umbilical vein endothelial cells, the group of Kapiotis et al (140) demonstrated that TM was downregulated by the pyrogens IL-1, TNF, and lipopolysaccharides to less than 50% of TM activity of untreated cells. Interestingly, TM downregulation was completely neutralized when these mediators were co-incubated with IL-4, of known anti-inflammatory properties. In this same report, it was shown that lipopolysaccharide infusion reduced TM messenger RNA to less than 40% that of untreated cells, parallel to the decrease in TM surface activity, and this effect was partly antagonized by IL-4, which in part protects endothelial cell surface against pyrogen-induced procoagulant changes. As evidenced by this study, surface TM may be regulated at the transcriptional level. In the rabbit model, Semeraro and collaborators demonstrated decreased levels of TM messenger RNA after lipopolysaccharide infusion in rabbits, coupled to normal levels of the actual protein (141). In another study, Terada et al demonstrated that endothelial injury induced by lipopolysaccharide infusion reduced TM in the endothelia of lung, liver, and peritubular capillaries in rats(142).

The present is the first study that has been performed which analyzes the endothelium lining the portal vein of cirrhotic patients, demonstrating that TM is reduced at this level, with conservation of FVIII. Diminished levels of TM may lead to insufficient activation of Protein C, and therefore hampering of Factor V and Factor VIII blockade, the latter of which is present within the endothelium of the portal vein, as hereby demonstrated.

Regarding interactions between anticoagulant components, endothelial protein C receptor (EPCR), which is ubiquitously expressed on the endothelium of all arteries and veins, especially those of large and medium caliber like the portal vein and hepatic vein, promotes, together

with TM, protein C activation by increasing at least by 5-fold the catalytic efficiency of the thrombin-thrombomodulin complex. Although it has been demonstrated that soluble levels of EPCR are increased in chronic liver disease (143), this may be not sufficient to compensate for the low levels of TM that have been found in the present study. It is possible, moreover, that endothelial damage diminishes not only TM, but also EPCR, further compromising the anticoagulant mechanisms of the endothelium. This issue has not yet been explored, and future studies in this direction could help clarify the interactions of these proteins with the other anticoagulant components within the endothelium of the portal vein and other venous territories in cirrhosis patients.

Factor VIII (FVIII), on the other hand, is a pro-coagulant factor which is ubiquitously expressed in the endothelium. It is an essential cofactor for FIXa-mediated catalysis of factor X to Xa. In its soluble form, FVIII is notably increased in cirrhosis, and the most widely accepted mechanism is the increase of von Willebrand factor, to which it binds when in circulation. An established marker of endothelial dysfunction, the elevation in FVIII has been shown to parallel the increase in severity of liver disease (144-146).

As demonstrated in the present study, portal vein endothelium in cirrhosis patients, as well as systemic venous territories as represented by the vena cava, do not show differences with regard to the presence of FVIII, which appears consistently conserved. The coupling between diminished TM and conserved FVIII may represent hampering of a protective anticoagulant mechanism by the first, and the maintenance of a pro-coagulant mechanism by the latter, which possibly favor the development of PVT.

It is possible that the one-cell thick endothelial lining of the portal vein and of veins in the systemic circulation is structurally conserved (as demonstrated by positivity of FVIII), but that it lacks functional dynamics such as that established with the overlying glycocalyx. Likewise, other functional alterations may coexist, such as demonstrated by diminished presence of TM.

The analysis of more samples will be useful in confirming these results, which represent a preliminary approach, and represent an invaluable step in perfecting preservation techniques, opening a vast array of possibilities regarding analysis of portal vein endothelium as a specific site for thrombosis.

Portal vein thrombosis is a common complication in cirrhosis, with a reported prevalence of up to 25% in patients without HCC and 33% in those with HCC (74). Despite it being frequently encountered in end stage liver disease and therefore in liver transplant candidates, definitive guidelines for the treatment of PVT before transplantation are not available (147).

Actual experience in the treatment of thrombotic complications in cirrhosis patients is limited. In a retrospective study analyzing 17 cirrhosis patients with VTE, it is reported that 11 patients were treated only with LMWH and six patients received LMWH during the first week, followed by Vitamin K Antagonists (VKA). Eighty-three percent of patients (14 of 17) presented bleeding complications, six of which were severe and required blood transfusions, corresponding to one patient treated with LMWH and five patients treated with VKA. For this reason, anticoagulation was withdrawn in most patients, and only three remained on anticoagulant therapy for 6 months(60).

Notwithstanding existing evidence in favor of avoiding thrombosis progression, current guidelines do not comprise treatment strategies for management of patients in the waiting list for liver transplantation. To date, 135 cirrhotic patients with PVT in 4 published cohorts have been treated with anticoagulation, resulting in a portal vein recanalization rate ranging from 46% to 85% (70;148-150).

In the prospective study by Francoz et al (70), 19 cirrhosis patients evaluated for liver transplantation, 18 with partial and one with complete PVT, were treated with LMWH followed by VKA, and were compared against a historical control group of 10 patients who received no anticoagulation. All but two anticoagulated patients in Child class B or C, and fourteen had had previous variceal bleeding. The rate of complete recanalization was 42.1% at the end of follow up, but few details were provided about the time interval between the diagnosis of thrombosis and anticoagulation, and the time interval between the start of anticoagulation and recanalization. It is noteworthy that patients with complete PVT at the time of liver transplantation had significantly lower survival after transplant. This further confirms the fact that at least in patients with cirrhosis and PVT listed for liver transplantation, anticoagulation should be used.

In the second study by Amitrano et al. therapeutic dose (200UI/KG/die) of LWMH was used in 29 patients with cirrhosis and PVT. Recanalization was attained in 33.3% of cases within 6 months of treatment, but reached a 75% rate in those patients who continued anticoagulation treatment beyond 6 months, with time to recanalization which ranged from 1 to 17 months(148). This percentage is exceptionally high in comparison with the efficacy of anticoagulation in a non-cirrhotic population with PVT (27%-45.4%) and cannot be explained either by the time interval to anticoagulation, nor by the presence/absence of underlying thrombophilic conditions, which were not investigated by the authors.

In the retrospective study by Delgado et al, analyzing data from 55 cirrhosis patients with PVT who received anticoagulant therapy for acute or subacute thrombosis (n=31), or due to progression of previously known PVT (n=24), partial or complete recanalization was achieved in 33 patients, and early initiation of anticoagulation was the only factor which was significantly associated with anticoagulant response. After stopping anticoagulation, 5 of the 13 patients who had had recanalization on anticoagulant therapy presented rethrombosis of the portal vein a median of 1.3 months after cessation of anticoagulation. The Authors of this study recommend the lifelong maintenance of anticoagulant therapy after thrombus disappearance (151).

Senzolo and collaborators recently published a prospective study proposing an algorithm for the treatment of PVT in cirrhosis including the use of anticoagulation and transjugular intrahepatic portosystemic, and report on several patients which are comprehended in the present study population, but also on the group of patients from Royal Free Hospital that constituted the untreated arm (152). In that study, 35 patients were included in the protocol and 33 were anticoagulated with the standard therapeutic dose of nadroparin (95 anti-Xa U/Kg body weight td), with a 40% dose reduction with platelet count below $50000 \times 10^9/L$. A complete recanalization rate of 36% was reported, and partial response to anticoagulation therapy was seen in 27% of patients. Mean interval between starting of anticoagulation and recanalization was 5.5 ± 2.6 months (range 1-10).

The present study, which aside from broadening the study population, explores the coagulation profile as a possible predictor of response to anticoagulation, largely confirms these results, with a response rate of 65%. Complete recanalization rates are higher (52%), at the expense of a

smaller percent representation of partial recanalization rates (13%). This can be explained by the fact that the present study includes recently evaluated and treated patients, who presented for evaluation with a recent thrombus onset, due to the ever-increasing awareness of the feasibility and importance of anticoagulation treatment in these patients, which is reflected on increased referral to tertiary centers. The present study also confirms that the time interval between diagnosis of PVT and anticoagulation is the strongest predictor of response to anticoagulant treatment.

These data are similar to those obtained in the non-cirrhotic population with PVT reported by Plessier et al (112), in which the one year recanalization rate was 38%, with no patient obtaining recanalization when anticoagulation was started beyond 12 months from the diagnosis of PVT. Interestingly, in this series evaluating non-cirrhotic patients with PVT, the presence of ascites and thrombotic splenic vein involvement were independently associated with failure to obtain recanalization.

This probably confirms that chronicity of the thrombus (clinically associated with the appearance of portal hypertension in non-cirrhotic patients) is associated with the lack of efficacy of anticoagulation. In contrast with the study by Amitrano and collaborators, anticoagulation was continued for another 6 months in the case of those patients who had not achieved recanalization after the first 6 months(148).

Importantly, an aspect that could contribute to the lack of response to anticoagulation in some patients can be owed to the fact that a worse stage of liver disease can preclude a prolonged anticoagulation treatment. However, lack of studies do not allow for clarification of this bias; present evidence does not indicate that patients followed have had to abandon anticoagulant therapy prematurely due to increasing severity of liver

disease, or have perished while on anticoagulant therapy, thus reducing the available time for thrombus resolution. Arguably, liver disease is less severe in patients who are able to tolerate anticoagulation for several months before undergoing liver transplant, and this could represent an inherent bias. However, in the few studies published, patients with severe liver disease have been included (Child C cirrhosis), and this has not hampered the efficacy of anticoagulation, and comparability of groups anticoagulated vs non-anticoagulated have been corroborated, with the exception of variceal bleeding being less frequent in groups undergoing anticoagulation(70;148).

In the present study, thrombosis progression was observed in one of four patients who did not complete 12 months of anticoagulation, within 2 months of anticoagulation cessation. Of 9 non-responders who completed 12 months of anticoagulation, two had thrombosis progression after 3 and 4 months of anticoagulation cessation, respectively, and in the absence of prophylaxis. Two other patients presented thrombosis recurrence after 1 and 4 months of anticoagulation cessation after obtainment of complete recanalization, in the absence of prophylaxis. In contrast, no patient on prophylaxis presented thrombus recurrence, 16 after complete or partial response to therapy, and 7 after a course of 12 months of anticoagulation without obtainment of recanalization.

These data are similar to those reported by Amitrano et al (148), where thrombus progression was reported in 2/5 of non-responders, and in 1/10 of non-responders in the study by Francoz et al(70). In patients who were not anticoagulated, thrombus extension was observed in 6/10 patients.

In the present study, albeit 86.7% of patients in whom recanalization was achieved this event occurred within the first 6 months of therapy, in 4/30 patients this event was attained in the subsequent 6 months of

anticoagulation, which supports the notion that it is worthwhile to continue anticoagulation therapy beyond the initial 6 months, for a similar period of time.

The present study underlines the two important steps which must be met in the management of this complication in cirrhosis: early diagnosis and early treatment. Early start of anticoagulation therapy after thrombus formation was the most important predictor of efficacy of anticoagulation for the treatment of PVT in cirrhosis patients, and from this derives the importance of not delaying diagnosis. An old, organized thrombus is less likely to disappear on anticoagulation therapy with respect to a fresh thrombus.

This is in line with what reported by Delgado et al (153), in that the only factor that was statistically associated with the therapeutic efficacy of anticoagulation was the delay in start of treatment (15 ± 27 days in responders vs 55 ± 86 days in non-responders, $p=.042$). This group also confirmed the lack of association between efficacy of anticoagulation therapy and studied factors such as age, gender, history of previous variceal bleeding, history of encephalopathy, previous episodes of portal hypertension decompensation, Child-Pugh score, platelet count ($\times 10^9/L$), renal function, presence of an underlying thrombophilia, presence of symptoms at diagnosis, extension of PVT into the splachnic vasculature, and presence of ascites.

Similarly, the presence of thrombophilia, as well as the coagulation status, as described by the determination of coagulation factors does not seem to influence response to anticoagulation. In the present study, in fact, response to anticoagulation therapy neither thrombus progression nor recurrence were associated with coagulation factors nor thrombophilic defects. However, it is noteworthy that these tests offer only an

approximation of the actual hemostasis-coagulation dynamics taking place inside the portal vein. On the other hand, Amitrano and collaborators did not find any differences in clinical features or extension of portal thrombosis between early or late responders and those that were non-responders to anticoagulation (148).

In the present series, aside from a long-standing thrombus, the extension of thrombosis into intrahepatic branches was another negative predictors of response. The latter was associated with increasing severity of liver disease, although the correlation did not reach statistical significance, but this is probably due at least in part to the small number of patients with this particular condition.

Interestingly, response to anticoagulation was independent of the stage of cirrhosis and of the use of a full dose or a reduced dose of anticoagulation, which is dictated by platelet number and therefore correlates with severity of liver disease. These data support the notion that even patients with advanced cirrhosis may benefit from anticoagulation, and that if a full dose is not possible, recanalization can still be attempted with dose reduction.

In the present study, neither occlusion grade nor extension to other splanchnic vessels were associated with the efficacy of anticoagulation. This probably reflects the fact that acute thrombosis can debut as an extensive thrombus, and thrombus extension is not necessarily a temporal becoming of a chronic thrombosis, and that acutely formed, fresh thrombi are more susceptible to the action of anticoagulants. In the study by Francoz and collaborators (70), factors that were associated with response to anticoagulation were not specifically sought for; however, the only case of complete PVT was found in the group of non-responders. However, only one of 19 treated patients had complete occlusion, and therefore no conclusions can be drawn from this data.

Regarding the importance of opportune treatment, this relies partly on diagnosing PVT within the first 6 months of its onset. Doppler ultrasound is the first-line method used to diagnose PVT, whereas CT scan and MRI have largely substituted angiography as a confirmation method in the last decades. In 409 patients with surgically confirmed PVT from thirteen studies (73;82;85;86;99;106;154-160), preoperative imaging detected the presence of PVT in 53% of the cases. The reported rate of preoperative detection of PVT ranged from 21% (99) to 87%(156;158), and this could depend primarily on two factors: the grade of thrombosis and each center's radiological follow-up protocol, which determines the time interval between imaging and transplant. Whereas sensitivity for grade III-IV PVT ranges from 92% to 100% (83;88;161;162), sensitivity in partial PVT and Grades I-II PVT is much lower (14.3% to 50%)(83;99;161).

The timely diagnosis of PVT is crucial in determining a successful outcome of anticoagulant therapy, and therefore, periodic evaluation with abdominal Doppler ultrasound is mandatory. There is no evidence, however, to recommend very short imaging intervals, as it has been shown that ultrasound and CT scan every 2 months does not improve the rate of detection of partial PVT (160;161). The widely used 6-month interval seems to represent the optimal compromise.

Bleeding, especially life-threatening hemorrhage such as variceal bleeding, is the most feared complication of anticoagulant therapy, although it has been demonstrated that hemodynamic factors, such as variceal pressure, are the greatest determinants of the likelihood of these events, and not the coagulation status(163).

The choice of using LWMH seems safe in patients with advanced liver disease. In the study by Francoz et al, only one of the 19 patients treated

with anticoagulation presented upper gastrointestinal bleeding, but in this case the bleeding episode was caused by a post ligation ulcer, and anticoagulation did not have to be discontinued in any patient (70). In the study by Amitrano and collaborators, of 28 patients treated with anticoagulation, none presented side effects such to consider interruption of therapy; mild anemia due to portal hypertensive gastropathy was seen in two patients, improving after iron supplementation (148). Delgado et al report bleeding related to anticoagulation (variceal bleeding in all) in 5 of 55 treated patients, and platelet count below $50 \times 10^9/L$ was the only factor significantly associated with a higher risk of a hemorrhagic complication (153). At a prophylactic dose in the setting of primary prevention of PVT, LMWH was proven safe in a recently published prospective study in which enoxaparin 4000 UI/day was administered to 34 Child B and C cirrhosis patients for 12 months. No hemorrhagic events were reported, and suspension of therapy was necessary in only one patient because of thrombocytopenia (164).

In contrast, Garcia Fuster and collaborators reported bleeding complications in 85% of patients (35% of them requiring transfusions) in a series of 17 cirrhosis patients treated with vitamin K antagonists for DVT (60). Moreover, an early cohort study evaluating 29,000 INR measurements during a period of 6 months, demonstrated that underlying liver disease or alcohol abuse were independently correlated with risk of excessive anticoagulation ($INR \geq 6$) (165). Landefeld and collaborators retrospectively evaluated predictors of major bleeding in hospitalized patients receiving initial long term warfarin therapy. Among 411 patients evaluated for different risk factors, bleeding episodes significantly correlated with the presence of worsening liver function (166).

In the present study, one serious adverse event (a non-fatal cerebral haemorrhage) occurred which was possibly related to anticoagulation.

Risk factors correlated with the underlying liver disease were absent (PLT count was $110 \times 10^9/L$ while INR and creatinine were within the normal range).

Heparin induced thrombocytopenia occurred in three patients, and a similar high frequency has been reported with the use of LMWH in splanchnic vein thrombosis (167). LWMH was suspended in all three patients: one was switched to warfarin, and two patients did not receive any anticoagulation treatment. In all three patients the thrombosis remained stable (there was no thrombus progression and no recanalization was observed).

Attainment and maintenance of portal vein patency did not have an impact on transplant-free survival, but this is in part due to the fact that PVT is more frequent in patients with more advanced cirrhosis, who have a greater probability of being transplanted. If only the event death as such is considered, rethrombosis and thrombus progression entailed a mortality of 80%, and only one of the five deaths in the present series occurred in a patient who had adequately responded to therapy. However, this study was not powered to show survival differences, and prolonged follow-up studies are needed to assess survival.

Regarding secondary prophylaxis after achieving recanalization, 12 patients continued prophylactic anticoagulation, and remained free of rethrombosis at 6 months' follow-up. In contrast, of four patients who had achieved complete recanalization but did not continue anticoagulation prophylaxis, two had recurrence of the thrombosis, and in one case this led to death. Anticoagulation prophylaxis was also continued in 6 other patients after achievement of partial recanalization, and of the four patients evaluated six months later, none had presented thrombus recurrence. Of 9 patients in whom recanalization was not achieved even after 12 months of therapy, seven continued anticoagulation prophylaxis,

and remain stable after 4, and 7 months of follow-up, respectively. This is in contrast with the two patients in whom recanalization was not achieved after 12 months of therapy, and in whom prophylactic anticoagulation was not continued; these two patients presented progression of the thrombus into splanchnic vasculature and one into the vena cava, and led to death in both cases.

Although the recommendation to continue anticoagulation in patients with PVT who do not obtain recanalization cannot be made based on current evidence, the efficacy of anticoagulation in preventing progression of thrombosis to a total splanchnic vein thrombosis should be taken into consideration, especially in patients who are potential or future candidates for liver transplantation.

Patients with cirrhosis are prone to develop thrombotic complications including PVT and DVT. Despite these complications may warrant anticoagulant therapy, current treatment guidelines do not provide specific indications for anticoagulation in patients with liver disease and thrombosis.

Furthermore, low levels of antithrombin, which is necessary for the action of low molecular weight heparin, could theoretically hamper the anticoagulant effect of these drugs(116), and data on their use in patients with cirrhosis are still limited. Clinically, however, low molecular weight heparin is effective in the treatment of thrombotic complications in these patients, notwithstanding low levels of antithrombin and low levels of anti-Xa activity. Moreover, in a small *in vitro* study by Lisman and collaborators(117), it was demonstrated that the anti-Xa assay substantially underestimates the LMWH mass present in plasma from patients with cirrhosis, and actually a fixed amount of LMWH is as effective in cirrhotic plasma as it is in plasma from normal pool plasma.

Thrombin generation has been recently used to investigate haemostatic balance in patients with liver disease and can be useful to evaluate the efficacy of LMWH in this setting of patients.

The present study represents an evaluation of the *in vitro* effect of LMWH on thrombin generation in cirrhotic patients, in controls and in a group of AT-deficient patients.

Child Pugh C patients demonstrated the greatest response to LMWH, as compared to controls. On the contrary, patients with heterozygous AT deficiency showed an *in vitro* resistance to the effect of LMWH, despite similar plasma levels of AT as compared to Child Pugh C cirrhotics. This increased effect of LMWH was also evident when a therapeutic dose of LMWH was used (0.7 U anti-Xa/mL), which completely inhibited thrombin generation in approximately about one-third of cirrhotic patients and resulted in a thrombin generation similar to controls in the remaining cirrhotic samples.

When the experiments were repeated in samples with the addition of TM in order to unveil the acquired protein C deficiency in patients with cirrhosis, the response to the addition of LMWH did not change. Moreover, the proportion of plasma samples from patients with more advanced cirrhosis (Child C) whose thrombin generation was completely abated after the addition of LMWH at 0.7 IU/ml was not different between samples with and without TM. This confirms the notion that, in cirrhotic patients, the newly rebalanced hemostatic equilibrium due to the concomitant decrease in pro-coagulant and anticoagulant factors can be easily perturbed.

Contrary to what is expected, in cirrhotics the effect of LMWH inversely correlated with AT levels. Thus, the lower the level of AT, the higher the anticoagulant response to LMWH. This may be due to the concomitant reduction of all procoagulant factors, in particular of factor Xa which is the target of LMWH inhibition. On the contrary, subjects with inherited AT deficiency had normal plasma levels of all procoagulant factors. As a consequence, the reduced effect of LMWH caused by low AT levels, was not counterbalanced by the reduction of other procoagulant factors and possibly results in the *in vitro* LMWH resistance.

In agreement with Bechman et al, the present study shows that plasma from patients with chronic liver disease present with lower anti-Xa activity when a similar dose of LMWH is added, as compared to control plasma. However, we also found that in the presence of a lower anti-Xa activity, plasma from patients with end stage liver disease exhibited a more pronounced response to anticoagulation as compared to control plasma. Therefore, patients in Child Pugh C class, despite very low plasma anti-Xa activity, demonstrated the highest anticoagulation effect, as opposed to what can be seen in patients with isolated AT defect.

This apparently conflicting data can be explained by considering the characteristic of the method used to measure anti-Xa activity in the plasma sample.

As the test is performed by adding fixed amounts (in excess) of FXa to the sample, the result of the reaction depends only on LMWH-AT complex generation and concentration. Adding a fixed amount of LMWH in all samples, the differences in the levels of anti-Xa activity depend merely on AT plasma levels. Patients with AT deficiency showed similar anti-Xa levels to those of Child A cirrhotic patients, and not significantly different from that of controls; this could be due to the difference in the levels of

coagulation factors in plasma samples. Theoretically, the test mechanism implies saturation of Xa, and holds true for plasma of patients with normal AT level. The test's results could be influenced by decreased levels of endogenous FXa, as well as by the reduced levels of other coagulation factors.

Therefore, anti-Xa determination does not seem to be a suitable test for monitoring anticoagulation in cirrhotic patients, since low anti-Xa levels are not indicative of a reduction in response to LMWH. On the contrary, the present findings show that thrombin generation assay can be useful in the evaluation of anticoagulant effect of heparin in cirrhotic patients with impaired clotting system.

However, these findings need to be further confirmed *in vivo*. As the matter of fact, an additional influence on pharmacokinetics and distribution of LWMH in cirrhotic patients with ascites may derive from altered water content and body mass index, thus leading to unpredictable LWMH concentrations.

It is also important to consider that different LWMHs might not have the same effect in patients with cirrhosis, due to the different ratio of inhibition of factor Xa and thrombin. This ratio can vary from 2.2 to 4.1 among commercially available LWMH. For example, enoxaparin, which is characterized by an anti- Xa/anti- factor IIa ratio of 3.8, might exert a different effect than dalteparin, which has a ratio of 2.7.

Conclusions

Aside from the reduction of flow characteristic of portal hypertension and cirrhosis, there seems to be an apparent imbalance between pro- and anti-coagulant factors that could, together with other local factors, contribute to the development of PVT.

The structure of the endothelial lining of the portal vein in cirrhosis patients seems to be conserved, and its aspect regarding the endothelial marker FVIII is similar to that of other venous territories, exemplified by the vena cava, as well as to that of portal vein and vena cava endothelial lining of non-cirrhotic subjects. That is, the endothelium of the portal vein in cirrhosis patients seems to be characterized by a normal representation of FVIII, which is one of the most important drivers of TF-independent coagulation.

On the contrary, the anticoagulation mechanism mediated by TM seems to be underrepresented in the portal vein endothelium of cirrhotic patients when compared to that of non-cirrhotic subjects. This imbalance, together with the mechanical alterations of portal vein flow, may be responsible for the elevated incidence of PVT in cirrhosis patients. Further studies are needed regarding other protective mechanisms such as glycosaminoglycans and endothelial Protein C receptor.

When PVT develops in cirrhosis patients, anticoagulant therapy with LMWH is effective in 65.2% of cases, with achievement of complete portal vein patency in 52.2% of patients. Furthermore, recanalization is achieved during the first 6 months of anticoagulation therapy in most patients, but as recanalization is reached in yet another, albeit small group of patients who continue anticoagulant therapy beyond 6 months, it is worthwhile to continue anticoagulation for 12 months.

In particular, the hemostatic systemic status does not seem to have an impact on the response to anticoagulant therapy, but rather, the interval between PVT onset and start of anticoagulation therapy. The continuation of anticoagulation at a prophylactic dose for 6 months after recanalization seems to be effective in preventing recurrence of PVT after suspension of anticoagulation at a therapeutic dose.

Cirrhosis patients therefore demonstrate an adequate clinical response to anticoagulant therapy, notwithstanding their low antithrombin levels, actually showing a greater susceptibility to increasing concentrations of LMWH in advanced cirrhosis, apparent from sharply abated thrombin generation curves and decreasing endogenous thrombin potential.

Henceforth, patients with cirrhosis may have an enhanced anticoagulant response to LWMH, which increases with the severity of liver disease. Anti-X activity is not a good marker of response to LWMH in patients with liver disease and should not be used in clinical practice to monitor the anticoagulant effect.

Finally, dose adjustment of LWMHs might be warranted according to the Child Pugh class in order to avoid excessive anticoagulation and possible bleeding complications. Clinical studies on the *in vivo* effect of LMWH in cirrhotic patients with thrombosis are needed to further validate this hypothesis.

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