Novel Fibrin Clot Components

identification, characterization and function

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“Live as if you were to die tomorrow.
Learn as if you were to live forever.”
— Gandhi

CONTENTS

Chapter 1

Introduction

COAGULATION

The main goal of blood coagulation is to diminish blood loss and to support vessel damage repair. The initial step in primary hemostasis is the adhesion of platelets to the exposed subendothelial extracellular matrix which contains several adhesive macromolecules such as collagen, von Willebrand factor (vWF), laminin, fibronectin and thrombospondin. These adhesive macromolecules are ligands for different platelet-surface receptors. Upon binding, platelets can be activated by different signal transduction pathways. Platelet adhesion and activation is followed by platelet aggregation, which involves fibrinogen binding to an integrin on the surface of the activated platelet [1,2]. Secondary hemostasis is initiated by exposure of tissue factor (TF) to blood by endothelial damage. TF forms a complex with coagulation factor VII which activates factor IX and factor X. Activated factor X (FXa) forms the prothrombinase complex together with activated factor V (FVa) on a phospholipid membrane surface, which is provided by, for example, activated platelets and microparticles, and which converts prothrombin into thrombin [3,4]. Thrombin can amplify its own generation by activating FXI, and FXIa activates FIX, that together with activated FVIII forms more FX. Thrombin is an important enzyme that cleaves fibrinogen into fibrin monomer. Fibrin monomers polymerize and the resulting network is stabilized by factor XIIIa-catalyzed cross-linking [5]. This tight fibrin network together with activated platelets occludes the site of vascular injury. After its formation, the clot will retract. The speed and degree of clot retraction are proportional to the number of platelets. Neither the significance of *in vivo* clot retraction for hemostasis as well as the mechanism of clot retraction are well understood. Retraction may improve the mechanical stability of clots [6]. To maintain a balance, the coagulation pathway is inhibited by several inhibitors. Tissue factor pathway inhibitor (TFPI) inhibits the TF-induced coagulation firstly by binding to FXa. Secondly, this TFPI-FXa complex binds to the TF-FVIIa complex resulting in the formation of an inactive quaternary complex. TFPI can also inhibit the initiation phase of coagulation in one step by binding to activated FX that is not yet released from its complex with TF-FVIIa [7]. Thrombin is directly inhibited by the serine protease inhibitor antithrombin as well as heparin cofactor II [8]. Thrombin can also bind to thrombomodulin and form a complex that activates protein C. Activated protein C (APC), with its cofactor protein S, has anticoagulant properties through proteolytic inactivation of factors Va and VIIIa [9] (figure 1).

FIBRINOLYSIS

The fibrin clot that is formed upon vascular damage must be removed when normal tissue structure and function are restored. A natural mechanism to dissolve a fibrin clot is

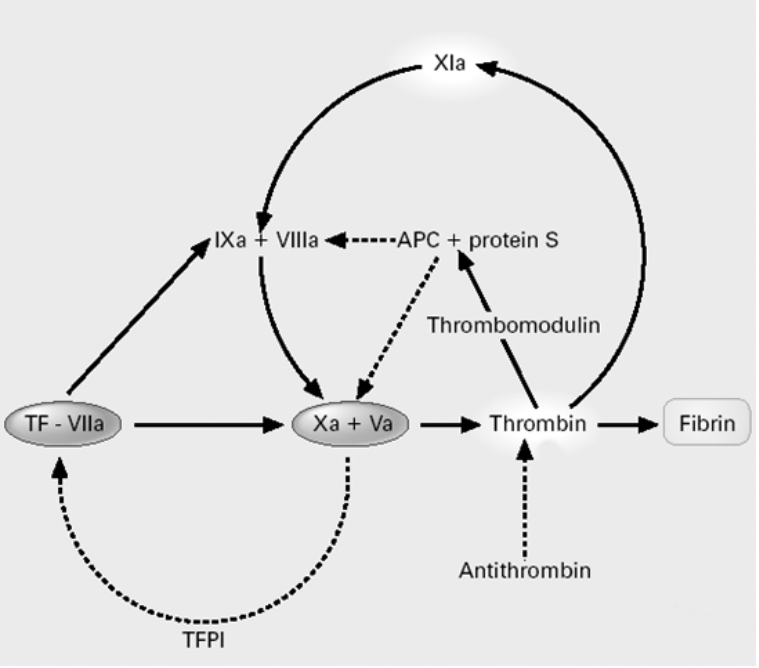

Figure 1: Schematic representation of the clotting cascade
The coagulation cascade is initiated by exposure of tissue factor (TF) to blood by endothelial damage. TF forms a complex with coagulation factor VII which activates factor IX and factor X. Activated factor X (FXa) converts prothrombin into thrombin. Thrombin can amplify its own generation by activating FXI and FXIa activates FIX that together with activated FVIII produces more FX. Thrombin cleaves fibrinogen into fibrin monomer. Tissue factor pathway inhibitor (TFPI) inhibits the coagulation by binding to the FXa in complex with TF and FVIIa. Thrombin is directly inhibited by the serine protease inhibitor antithrombin. Thrombin could also bind to thrombomodulin and form a complex that activates protein C. Activated protein C (APC), with its cofactor protein S, has anticoagulant properties through proteolytic inactivation of factors Va and VIIIa. Figure adapted from J.P. Vandenbroucke *et al.* [91]

the fibrinolytic system. Fibrin is digested into soluble fibrin degradation products by the enzyme plasmin in several cleavage steps [10]. The proenzyme plasminogen is converted into the active enzyme plasmin by plasminogen activators, generally tissue-type plasminogen activator (t-PA) or urokinase-type plasminogen activator (u-PA), by the cleavage of a single peptide bond [11]. The interactions of t-PA and plasminogen with fibrin accelerate plasminogen activation [12]. Partially degraded fibrin by plasmin exposes additional C-terminal lysine residues, which are binding sites for plasminogen and t-PA. This partial degradation of fibrin results in increased plasmin generation. Just like the coagulation pathway, the fibrinolytic system needs to be in balance and can therefore be inhibited by several inhibitors. Plasmin generation can be inhibited by plasminogen activator inhibitor-1 (PAI-1). Plasmin in the circulation is quickly inhibited by α_2-antiplasmin, facilitated by binding of α_2-antiplasmin to the lysine binding sites of plasmin [13]. Plasmin bound to fibrin is inhibited 100 times more slowly because the lysine binding sites are occupied by fibrin and are not accessible to α_2-antiplasmin [14]. The C-terminal lysine residues on

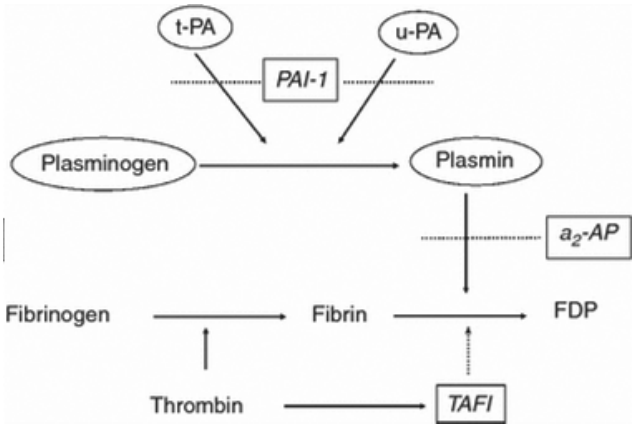

Figure 2: Schematic representation of the fibrinolytic system
Fibrin is digested into soluble fibrin degradation products (FDP) in several cleavage steps by the enzyme plasmin. Plasminogen is converted into the active enzyme plasmin by plasminogen activators, tissue-type plasminogen activator (t-PA) or urokinase-type plasminogen activator (u-PA). Plasmin is quickly inhibited by α_2-antiplasmin in circulation, facilitated by binding to the lysine binding sites [13]. Plasmin bound to fibrin is inhibited 100 times more slowly because the lysine binding sites are occupied by fibrin and are not accessible to α_2-antiplasmin. The C-terminal lysine residues on partially degraded fibrin can be cleaved off by activated thrombin-activatable fibrinolysis inhibitor (TAFIa) thereby inhibiting plasminogen binding and finally plasmin generation. TAFI can be activated by thrombin or more efficiently by thrombin in complex with thrombomodulin [13].

partially degraded fibrin can be cleaved off by activated thrombin-activatable fibrinolysis inhibitor (TAFIa) thereby inhibiting plasminogen binding and finally plasmin generation. TAFI can be activated by thrombin or more efficiently by thrombin in complex with thrombomodulin [15] (figure 2). The main enzyme that dissolves fibrin is plasmin but fibrin can also be degraded by other proteases, including elastase [16]. Elastase is excreted from leukocytes that can migrate into the blood clot and is mainly inhibited by α_1-antitrypsin (A1AT) [17].

FIBRIN CLOT-BOUND PROTEINS

Several proteins are known to bind to fibrin and to change clot properties or clot function via their effect on fibrin formation and degradation [18]. For example, the binding of thrombin to fibrin results in a smaller quantity of active thrombin in the circulation. Reduced binding of thrombin to fibrin, which is seen in patients with fibrinogen Naples I, is associated with thrombosis [19]. As already stated, both tPA and plasminogen bind to fibrin, which results in a strongly increased plasminogen activation [12]. Lipoprotein(a) (Lp(a)) has structural similarities to plasminogen. Lp(a) can compete with plasminogen for fibrin binding and in so doing inhibit plasmin formation and eventually fibrinolysis

[20]. Binding of proteins to fibrin can have an effect on the structure of fibrin fibers. Binding of fibronectin to fibrin causes the fibrin network to have thicker fibers and larger pores [21]. Clots with thicker fibers and larger pores are broken down more rapidly than clots with thinner fibers and smaller pores [22]. There are several other proteins that are known to bind to fibrin including α_2-antiplasmin, plasminogen activator inhibitor-2 (PAI-2), hepatocyte-derived fibrinogen-related protein-1 (HFREP-1), albumin, fibroblast growth factor-2, vascular endothelial growth factor, interleukin-1β, activated factor X, TFPI, TAFI, vWF, thrombospondin, actin, factor V and factor XIII (FXIII) [23–30]. Some of these proteins are cross-linked to fibrin by FXIIIa, e.g. α_2-antiplasmin, fibronectin, PAI-2, TAFI, vWF, thrombospondin, actin and factor V. This thesis focuses on the novel fibrin clot components α_1-antitrypsin, carboxypeptidase N and high-density lipoprotein.

α_1-ANTITRYPSIN (α1AT)

A1AT is a member of the serine protease inhibitor (serpin) family and is predominantly produced in the liver. The serpin family also includes antithrombin, heparin cofactor II, PAI-1, PAI-2 and α_2-antiplasmin [31]. A1AT is folded into three β sheets and nine α helices, which surround the β sheet scaffold. Serpins interact with their target via the reactive center loop (RCL), which is exposed on the surface of the protein. A1AT, like most of the serpins, in its native, active conformation is not folded in its most stable state and is therefore termed metastable. Serpins use their metastable state to inhibit proteases. Specific residues in the RCL are recognized by a target protease, which binds and cleaves the RCL. Upon cleavage the RCL translocates the protease, which stays bound to the serpin via a covalent bond, to the opposite site of the serpin. During the translocation the RCL is incorporated into β sheet A. The result of this conformational change is that the protease becomes distorted and catalytically inactive, whereas the serpin becomes much more stable [32–34] (figure 3). The main function of A1AT is to inhibit neutrophil elastase. However, several proteases that play a role in coagulation and fibrinolysis may also be neutralized by A1AT. The main extracellular function of neutrophil elastase is the digestion of connective tissue. In general, neutrophil elastase is capable of digesting almost every type of matrix protein, including several types of collagen, fibronectin, proteoglycans, heparin, and cross-linked fibrin [35]. A1AT deficiency results in an imbalance between proteases and inhibitors within the lung and can finally result in emphysema or chronic obstructive pulmonary disease (COPD). Mutations in A1AT may also lead to polymerization of the protein. A1AT polymers may accumulate in the endoplasmatic reticulum of the hepatocytes which results in liver damage [36]. Another conformation of serpins is the latent form, which is formed when the uncleaved reactive loop is inserted into the β sheet A of the same molecule [37]. Polymerized and latent forms of A1AT are inactive.

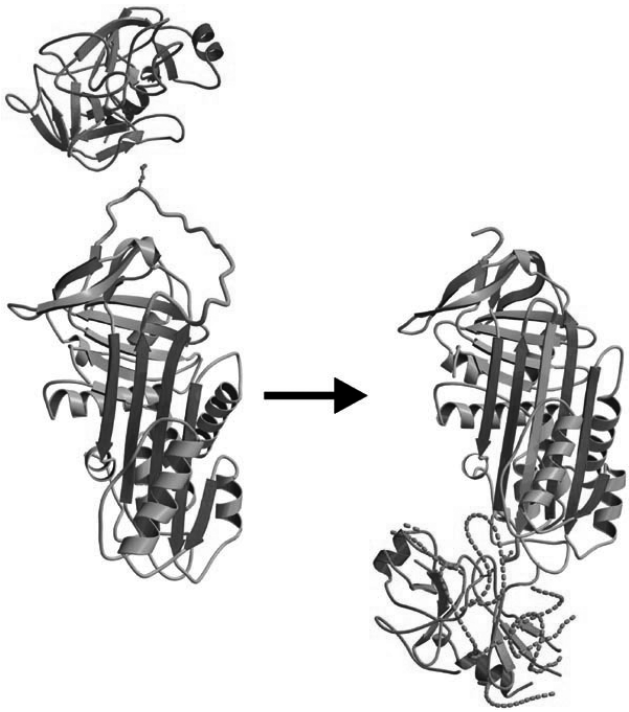

Figure 3: Inhibition of trypsin by A1AT
Specific residues in the reactive center loop (RCL) of A1AT are recognized by trypsin, which binds and cleaves the RCL. Upon cleavage the RCL translocates trypsin to the opposite site of A1AT. During the translocation the RCL is incorporated into β sheet A. The result of this conformational change is that trypsin becomes distorted and catalytically inactive, whereas A1AT becomes much more stable [34].

CARBOXYPEPTIDASE N (CPN)

CPN belongs to the family of zinc metallocarboxypeptidases and because it was discovered as an inhibitor of bradykinin it was originally called kininase I [38]. In addition to bradykinin, CPN can also inactivate the complement anaphylatoxins C3a, C4a and C5a by cleaving C-terminal lysine and arginine residues [39]. CPN is expressed in the liver and is constitutively active in the blood stream at a concentration of approximately 100 nM. The protein circulates in plasma as a tetramer with a mass of about 280 kDa, consisting of two heterodimers, each with a catalytic subunit and a regulatory subunit. The catalytic subunit is 55 kDa in its native form, but is *in vivo* proteolytically cleaved at the C-terminus to form a 48 kDa subunit, which are both present at a 1:1 ratio [40]. The regulatory subunit is not active, but stabilizes the protein and inhibits the clearance of the catalytic subunit from the circulation. This subunit is highly glycosylated, which accounts for about 27% of the protein's molecular mass. CPN is sensitive to proteolysis by e.g. plasmin, which can finally result in a more active protein [41]. The hydrolysis of the 83 kDa regulatory subunit

into a 73 kDa- and a 13 kDa fragment results in the dissociation of the tetramer in two heterodimers and the hydrolysis of the catalytic subunits results in increased CPN activity. CPN shares similar substrates with TAFIa including bradykinin and the anaphylatoxins C3a, C4a and C5a. The effect of CPN on fibrinolysis has not been extensively investigated in contrast to TAFI. However, Walker *et al.* [42] showed that CPN has antifibrinolytic properties, which are enhanced by limited proteolytic cleavage of CPN by plasmin. Proteolytic cleavage of CPN results in a long-term antifibrinolytic enzyme. There are no individuals known to have a complete deficiency of CPN. Two case reports of patients with low levels of CPN suggest that a CPN deficiency causes angioedema, probably due to elevated kinins [43,44].

HIGH-DENSITY LIPOPROTEIN (HDL)

HDL consists of a hydrophobic core with triglycerides and cholesteryl esters, surrounded by a hydrophilic phospholipid monolayer surface containing unesterified cholesterol and apolipoproteins. The most abundant apolipoproteins are apolipoprotein AI (apo AI) that comprises about 70% of all proteins and apo AII that accounts for about 10–15% of all the proteins in a HDL particle. The other 15–20% of the proteins include other apolipoproteins such as apo AIV, apo AV, apo CI, apo CII, apo CIII, apo E, apo J and apo M as well as antioxidants and enzymes [45]. Proteomic studies reveal that HDL is associated with proteins that are involved in processes such as lipid metabolism, hemostasis, protease inhibition, the immune system and the complement system [46,47]. HDL particles are heterogeneous in size and composition. Identification of the different subclasses is dependent on the technique that is used to isolate HDL. Using ultracentrifugation HDL particles can be separated on the basis of their density into a small dense HDL3 fraction and a larger, less dense HDL2 fraction. Using gradient gel electrophoresis 5 different subfractions can be identified on the basis of their particle diameter HDL2a, HDL2b, HDL3a, HDL3b and HDL3c. HDL can also be separated on the basis of their size and charge with native 2D gel electrophoresis into large cholesterol-rich HDL2a and HDL2b, small-sized HDL3a, HDL3b and HDL3c and very small-sized pre-β2-HDL [48].

Low plasma levels of HDL are a risk factor for arterial and venous thrombosis [49–51]. It is thought that the negative association is particularly due to the role of HDL in reverse cholesterol transport. HDL mediates the transport of cholesterol from peripheral tissues to the liver where the cholesterol is broken down. However, HDL has more properties including anti-oxidant properties, anti-inflammatory properties, anticoagulant properties and favorable effects on endothelial function [45]. HDL is thought to be anticoagulant by acting as a cofactor for the activated protein C pathway together with protein S [52]. However, this enhancement of the protein C pathway could also be due to a contamina-

tion of negatively charged phospholipid membranes in the HDL preparation [53]. Another mechanism by which HDL could be anticoagulant is that phosphatidyl serine loses its procoagulant properties when incorporated into HDL particles, because the surface area of HDL is too small to accommodate the complete prothrombinase complex [54]. HDL can also bind to platelets via the scavenger receptor B type I (SR-BI) [55] and inhibit platelet aggregation. However whether this is true for native HDL, specific HDL subfractions or oxidatively modified HDL is still controversial [56–59].

THROMBOSIS

Dysregulation of any of the components of the clotting cascade or the fibrinolytic system can potentially disrupt the hemostatic balance, leading to an increased tendency to either bleeding or thrombosis. Examples of arterial thrombosis include myocardial infarction, unstable angina pectoris, ischemic stroke and transient ischemic attack and examples of venous thrombosis are deep vein thrombosis, pulmonary embolism and splanchnic vein thrombosis. Multiple genetic and acquired risk factors contribute to the development of thrombosis. Some risk factors for arterial thrombosis may also play a role in venous thrombosis and the other way around [60,61]. Genetic factors for venous thrombosis include antithrombin deficiency, factor V Leiden mutation, prothrombin 20210A variant, protein C deficiency and protein S deficiency [62]. The latter four are also hemostatic risk factors for arterial thrombosis in addition to fibrinogen and thrombomodulin polymorphisms which lead to increased and decreased plasma levels respectively [60,63]. Classic acquired risk factors for arterial thrombosis are age, male sex, dyslipidemia, high blood pressure, cigarette smoking, diabetes and obesity [64]. The classic acquired risk factors for venous thrombosis include cancer, surgery, immobilization, fractures, pregnancy and oral contraceptives [62]. Acquired changes in levels of several plasma proteins such as plasminogen, β_2-glycoprotein, α_2-antiplasmin, C reactive protein, TAFI and albumin [65–69] are also associated with the risk of arterial thrombosis. Acquired changes in plasma protein levels that are associated with the risk of venous thrombosis include hyperhomocysteinaemia, TAFI and elevated levels of nearly all coagulation factors [70–72]. The pathogenesis of arterial and venous thrombosis can involve multiple gene-gene and gene-environmental interactions. Although there are several risk factors known, there are still a number of patients without a known thrombotic risk factor [73,74].

BUDD-CHIARI SYNDROME

The most common manifestations of venous thrombosis are deep venous thrombosis of the lower extremities and pulmonary embolism. Thrombosis at other locations including cerebral and abdominal veins is seen less frequently. When thrombosis involves the hepatic veins or the inferior vena cava, blocking the outflow of blood from the liver, it is referred to as Budd-Chiari syndrome (BCS) [75]. BCS is a rare vascular liver disorder with an incidence rate of about 1 in 1,000,000 that mostly affects young females [76]. Hematologic abnormalities, in particular myeloproliferative disorders, are the most common cause of BCS. Patients present with acute signs and symptoms of abdominal pain, ascites and hepatomegaly. Medical treatment for BCS patients includes anticoagulants and diuretics. More invasive treatment includes thrombolysis, percutaneous transluminal angioplasty, transjugular intrahepatic portosystemic shunting (TIPS) and orthotopic liver transplantation [77]. As is the case with thrombosis at other locations, various inherited and acquired factors have been identified that are associated with BCS [78–81]. Moreover, current evidence suggests that BCS is a multifactorial disease that often develops in the presence of more than one risk factor [82]. Although one or more underlying causes can be found in the majority of patients, there are still cases in which none of the known risk factors are present. Moreover, the site specificity of thrombosis is poorly understood [83].

CIRRHOSIS

Chronic liver disease is a pathological condition characterized by a gradual and progressive destruction of the liver tissue over time leading to advanced fibrosis and cirrhosis. The most common causes of chronic liver disease are alcohol abuse, viral infections, non-alcoholic fatty liver disease and exposure to hepatoxic drugs [84]. The liver plays a central role in hemostasis by synthesizing most coagulation factors, coagulation inhibitors and pro- and antifibrinolytic proteins. In addition, the liver is also responsible for the clearance of clotting factors and fibrinolysis factors [85]. Increased fibrinolysis is a common finding in patients with cirrhosis, possibly due to a disturbed balance in fibrinolysis factors. Cirrhosis patients have high plasma levels of t-PA and low levels of α_2-antiplasmin and TAFI which may cause acceleration of fibrinolysis [86–88]. It is still debatable whether disturbed plasma levels of hemostatic factors result in increased fibrinolysis. Lisman *et al.* [87] did not find evidence of increased fibrinolysis, whereas Colucci *et al.* [88] did provide evidence for increased fibrinolysis. Although both studies used a plasma clot lysis assay, there may be still some differences in the assay as well as the patient selection. Moreover, information about biological variation in the plasma clot lysis time is not sufficiently avail-

able to determine whether the samples have been tested in an optimal way to obtain solid data. Therefore, more research needs to be done on global fibrinolysis assays to investigate whether the observed changes in plasma levels of hemostatic factors result in increased fibrinolysis. Although bleeding is the most common clinical manifestation in cirrhosis patients a disturbed balance in the clotting cascade can also result in excessive thrombus formation. Whether bleeding or thrombosis occurs depends on which of these complex hemostatic mechanisms predominates [89,90].

OUTLINE OF THE THESIS

The aim of this thesis is to obtain more insight into the proteins that are present in a fibrin clot and their possible role in coagulation and/or fibrinolysis. Identification of novel players in hemostasis could help in determining new risk factors for thrombosis and additionally in understanding the pathogenesis of bleeding and thrombotic disorders.

To identify novel players in hemostasis we determine the most abundant non-covalent plasma clot-bound proteins using a proteomic approach. In chapter 2 we will describe the identification of 18 fibrin clot-bound proteins of which 9 were novel plasma clot components. Some of these proteins will be investigated in more detail. In chapter 3 we characterize the binding of A1AT to fibrin(ogen). In chapter 4 we describe in more detail the binding of CPN to fibrin(ogen) and in chapter 5 we investigate the possible role of HDL in hemostasis using thromboelastometry. We identify proteins that differ in their abundance in a plasma clot between BCS patients and controls in chapter 6. Cirrhosis patients have deviating levels of pro- and anti-fibrinolytic factors and it is unclear whether these deviating factors also translate into an enhanced fibrinolytic capacity. Using two different fibrinolysis assays, the plasma clot lysis assay and a whole blood clot lysis assay we show in chapter 7 whether cirrhosis patients have hyperfibrinolysis compared to controls. Finally we study the biological variation of the clot lysis time, measured with the plasma clot lysis assay in chapter 8. This information is not sufficiently available in the literature although the plasma clot lysis assay is widely used. In chapter 9 the data of this thesis will be summarized and discussed.

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Chapter 2

Identification of Fibrin Clot-bound Plasma Proteins

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ABSTRACT

Several proteins are known to bind to a fibrin network and to change clot properties or function. In this study we aimed to get an overview of fibrin clot-bound plasma proteins. A plasma clot was formed by adding thrombin, $CaCl_2$ and aprotinin to citrated platelet-poor plasma and unbound proteins were washed away with Tris-buffered saline. Non-covalently bound proteins were extracted, separated with 2D gel electrophoresis and visualized with Sypro Ruby. Excised protein spots were analyzed with mass spectrometry. The identity of the proteins was verified by checking the mass of the protein, and, if necessary, by Western blot analysis. Next to established fibrin-binding proteins we identified several novel fibrin clot-bound plasma proteins, including α_2-macroglobulin, carboxypeptidase N, α_1-antitrypsin, haptoglobin, serum amyloid P, and the apolipoproteins AI, E, J, and AIV. The latter six proteins are associated with high-density lipoprotein particles. In addition we showed that high-density lipoprotein associated proteins were also present in fibrinogen preparations purified from plasma. Most plasma proteins in a fibrin clot can be classified into three groups according to either blood coagulation, protease inhibition or high-density lipoprotein metabolism. The presence of high-density lipoprotein in clots might point to a role in hemostasis.

INTRODUCTION

Arterial and venous thrombosis are major causes of morbidity and mortality in the Western world. These thrombotic disorders are considered as separate diseases, with different pathology, pathophysiology, epidemiology and treatments. However, there is evidence that suggests that there is an association between venous and arterial thrombosis [1]. Multiple genetic and acquired risk factors contribute to the development of thrombosis. Some risk factors for arterial thrombosis may also play a role in venous thrombosis and the other way around [2,3]. Although there are several risk factors known, there are still a number of patients without a known thrombotic risk factor [4,5]. Moreover, the site specificity of thrombosis is poorly understood [6]. Identification of novel players in hemostasis can help in determining new risk factors and additionally in understanding the pathogenesis of thrombotic disorders.

Elevated fibrinogen is a risk factor for both arterial and venous thrombosis [4,7]. Several proteins are known to bind to fibrin and to change clot properties or clot function via effects on fibrin formation and degradation [8]. For example, the main enzyme in fibrinolysis, plasmin, is formed by activation of the zymogen plasminogen by tissue plasminogen activator (t-PA). The interactions of t-PA and plasminogen with fibrin accelerate plasminogen activation [9]. Lipoprotein(a) (Lp(a)) has structural similarities to plasminogen. Lp(a) can compete with plasminogen for fibrin binding and in so doing inhibit plasmin formation and eventually fibrinolysis [10]. Binding of proteins to fibrin can have an effect on the structure of fibrin fibers. Binding of fibronectin to fibrin causes the fibrin network to have thicker fibers and larger pores [11]. Clots with thicker fibers and larger pores are broken down more rapidly than clots with thinner fibers and smaller pores [12]. The binding of thrombin to fibrin results in a smaller quantity of active thrombin in the circulation. Reduced binding of thrombin to fibrin, which is seen in patients with fibrinogen Naples I, is associated with thrombosis [13]. There are several other proteins that are known to bind to fibrin including α_2-antiplasmin, plasminogen activator inhibitor-2 (PAI-2), hepatocyte-derived fibrinogen-related protein-1 (HFREP-1), albumin, fibroblast growth factor-2, vascular endothelial growth factor, interleukin-1β, activated factor X, tissue factor pathway inhibitor, thrombin-activatable fibrinolysis inhibitor (TAFI), von Willebrand factor, thrombospondin, actin, factor V and factor XIII (FXIII) [14–21]. Some of these proteins are cross-linked to fibrin by FXIIIa, e.g. α_2-antiplasmin, fibronectin, PAI-2, TAFI, von Willebrand factor, thrombospondin, actin and factor V.

In this study we aim to establish the protein composition of fibrin clots made from plasma. Changes in the protein composition can influence clot formation and breakdown and may therefore play a role in arterial and venous thrombosis. We identified 18 fibrin clot-bound plasma proteins by 2D gel electrophoresis followed by mass spectrometry.

Nine of them were novel plasma clot components of which six proteins are associated with high-density lipoprotein (HDL).

MATERIALS AND METHODS

Materials

Urea, thiourea, CHAPS, dithiothreitol (DTT) and iodoacetamide were obtained from Fluka (St. Louis, MO, USA). Aprotinin (Trasylol) was obtained from Bayer (Leverkusen, Germany). Tris (PlusOne), DeStreak, IPG buffer pH 3–10, immobiline strips, Ettan Spot Picker, IPGphore and Typhoon Trio apparatus were obtained from GE Healthcare (Uppsala, Sweden). The anchorchip plate, α-cyano-4-hydroxycinnamic acid matrix and the Ultraflex-II apparatus were from Bruker Daltonics (Bremen, Germany). Human thrombin and apolipoprotein AI purified from plasma were obtained from Sigma-Aldrich (St. Louis, MO, USA). Trypsin Gold was obtained from Promega Corporation (Madison, WI, USA). Bis-Tris (12%) Criterion XT precast gels, XT MOPS and XT MES buffer were from Bio-Rad (Hercules, CA, USA). Sypro Ruby was obtained from Invitrogen (Paisley, UK) and 0.45 µm nitrocellulose transfer membrane from Whatman (Dassel, Germany). The goat polyclonal IgG to human α_2-macroglobulin, the goat polyclonal IgG to human apolipoprotein AII and the goat polyclonal IgG to human apolipoprotein B were from Abcam (Cambridge, UK). The rabbit polyclonal IgG to human apolipoprotein AI was from Calbiochem (Darmstadt, Germany) and the goat polyclonal IgG to human apolipoprotein J was from Abgent (San Diego, CA, USA). The Odyssey apparatus and IRDye® 800CW secondary donkey-anti-goat and goat-anti-rabbit antibodies were obtained from Li-Cor Bioscience (Lincoln, NE, USA). Human fibrinogen (plasminogen, von Willebrand factor and fibronectin depleted) was obtained from Enzyme Research Laboratories (South Bend, IN, USA).

Plasma clot preparation

In vitro clots of 500 µl citrated platelet-poor plasma (pool from 10 healthy volunteers, Sanquin, location Leiden, the Netherlands) were prepared by adding calcium chloride (20 mM), thrombin (1 NIH U/ml) and aprotinin (100 KIU/ml) [15]. After 2 hours of incubation at room temperature, the clots were extensively washed by perfusing them with 10 ml Tris-buffered saline (50 mM Tris-HCl, 100 mM NaCl, pH 7.4) containing aprotinin (100 KIU/ml) at 4°C. Where indicated, the NaCl concentration was increased to 0.5 M. The clots were compacted by centrifugation, washed with deionized water and non-covalently clot-bound proteins were extracted with 150 µl rehydration buffer (7M urea, 2M thiourea,

4% (w/v) CHAPS, 0.5% (v/v) IPG 3–10 buffer) for 1 hour at room temperature. For optimal 2D gel electrophoresis 1% (v/v) DeStreak was added to the extract.

2D gel electrophoresis

Plasma clot extract was separated with 2D gel electrophoresis. The proteins in the 150 µl extract were separated in the first dimension with a 11 cm immobiline drystrip with a 3–10NL pH range by isoelectric focusing on the IPGphor with the following running protocol: 30V for 12 hours (rehydration), 1000V for 4 hours (gradient), 8000V for 5 hours (step-n-hold), with a 50 µA limit per gel. After isoelectric focusing the gel strip was equilibrated in buffer (6 M urea, 50 mM Tris-HCl pH 8.8, 20% (v/v) glycerol, 2% (w/v) SDS) with 1% (w/v) DTT for 15 minutes followed by a second equilibration step with equilibration buffer with 1% (w/v) iodoacetamide for 15 minutes. For the second dimension the gel strip was laid on a 12% Bis-Tris gel and run for 1h at 200V constant, using the XT MOPS buffer as running buffer. The proteins in the gel were visualized by Sypro Ruby staining according to manufacturer's instructions and scanned on a Typhoon Trio at an excitation wavelength of 532 nm and an emission wavelength of 610 nm.

Mass spectrometry analysis

The highly abundant proteins were analyzed with Matrix Assisted Laser Desorption/Ionization – Time of Flight (MALDI-ToF). Therefore proteins spots were excised with Spot Picker using a 2 mm picker head and destained in 30% (v/v) acetonitrile (ACN)/50 mM NH_4HCO_3. Destained gel pieces were vacuum-dried and rehydrated in 4 µl trypsin digest solution (75 µg/ml Trypsin Gold in 20 mM NH_4HCO_3, pH 8.0) for digestion overnight at room temperature. Peptide extraction was performed with 5 µl of 50% ACN/0.1% trifluoroacetic acid. The extracted sample was spotted on an anchorchip plate with saturated α-cyano-4-hydroxycinnamic acid matrix solution in 100% ACN (1:1). Digested peptide fragments were analyzed in a MALDI-ToF mass spectrometer using an Ultraflex-II apparatus. Flexanalysis 2.4 and BioTools 3.1 software were used for data processing. The mass spectra obtained were analyzed using peptide mass fingerprint spectra with the online Matrix Science Database with MASCOT software (www.matrixscience.com). The NCBInr database 20100624 (11299630 sequences; 3855426203 residues) was searched with the Mascot parameters set as follows: Taxonomy, *homo sapiens*; mass tolerance, 100 ppm; maximally one missed cleavage per peptide; fixed modification of carboxymethylation of cysteine residues; variable modification of partial oxidation of methionine residues. Mowse scores above NCBInr database threshold of 66 were considered significant ($p<0.05$).

For the less abundant proteins mass spectrometry analysis was done with nanoflow LC-MS/MS. Picked gel spots were subjected to in-gel reduction with DTT, alkylation with iodoacetamide and digestion with Trypsin Gold, essentially as described by Wilm *et al.*[22]. Nanoflow LC-MS/MS was performed on an 1100 series capillary LC system (Agilent Technologies, Santa Clara, CA, USA) coupled to an LTQ linear ion trap mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) operating in positive mode and equipped with a nanospray source. Peptide mixtures were trapped on a ReproSil C18 reversed phase column (Dr Maisch GmbH, Ammerbuch-Entringen, Germany; column dimensions 1.5 cm × 100 μm, packed in-house) at a flow rate of 8 μl/min. Peptide separation was performed on ReproSil C18 reversed phase column (Dr Maisch GmbH, Ammerbuch-Entringen, Germany; column dimensions 15 cm × 50 μm, packed in-house) using a linear gradient from 0 to 80% B (A = 0.1 % formic acid; B = 80% (v/v) acetonitrile, 0.1 % formic acid) in 70 min and at a constant flow rate of 200 nl/min using a splitter. The column eluent was directly sprayed into the ESI source of the mass spectrometer. Mass spectra were acquired in continuum mode; fragmentation of the peptides was performed in data-dependent mode. Peak lists were automatically created from raw data files using the Mascot Distiller software (version 2.1; MatrixScience). The Mascot search algorithm (version 2.2, MatrixScience) was used for searching against the NCBInr database (release NCBInr_20090808.fasta; taxonomy: *Homo sapiens*). The peptide tolerance was typically set to 2 Da and the fragment ion tolerance was set to 0.8 Da. A maximum number of 2 missed cleavages by trypsin were allowed and carbamidomethylated cysteine and oxidized methionine were set as fixed and variable modifications, respectively. The Mowse score cut-off value for a positive protein hit was set to 60. For identification as fibrin clot-binding protein the cut-off value for the emPAI score [23] was set at 0.15. Individual peptide MS/MS spectra with Mascot scores below 40 were checked manually and either interpreted as valid identifications or discarded.

Fibrinogen purification from plasma

Fibrinogen was purified from barium-adsorbed citrated plasma with immunoaffinity chromatography according to Takebe *et al.* [24] with some changes. In short, IF-1 antibody was conjugated to CNBr-activated Sepharose 4B according to manufacturer's manual. Barium-adsorbed citrated plasma was dialyzed against Tris-buffered saline (50 mM Tris-HCl, 100 mM NaCl, pH 7.4) and applied on the IF-1-conjugated Sepharose 4B column with 1 mM $CaCl_2$. The column was washed with 50 mM Tris-HCl, pH 7.4, containing 0.3 M NaCl and 1 mM $CaCl_2$ and eluted with 50 mM Tris-HCl, pH 7.4, containing 0.3 M NaCl and 5 mM EDTA. The optical densities at 280 nm of the fractions were measured.

Western blot analysis

To detect α_2-macroglobulin, a clot extract was made and separated by 2D gel electrophoresis as described above. The proteins were transferred from the 12% Bis-Tris precast gel to a nitrocellulose membrane by semi-dry blotting at 0.33 mA constant for 1 hour. The membrane was incubated with block buffer (PBS, 1% BSA, pH 7.4). After the blocking step the membrane was first incubated with the α_2-macroglobulin antibody (20 µg/ml) diluted in block buffer containing 0.1% Tween 20 and then with the secondary antibody IRDye® 800CW donkey-anti-goat diluted 10.000 times in 5% milk and 0.1% Tween 20 in PBS, pH 7.4. All incubation steps were performed for 1 hour at room temperature. To visualize the protein, the membrane was scanned on an Odyssey scanner.

To detect fibrinogen-bound proteins, fibrinogen in the fractions of the IF-1 column experiment described above and fibrinogen from Enzyme Research Laboratories were analyzed with SDS-PAGE. Reduced samples of 30 µg fibrinogen were run on a 12% Bis-Tris precast gel with XT MES buffer for 1h at 200V constant and analyzed with Western blotting as described above. The different apolipoproteins were detected by using the specific apolipoprotein AI antibody (1000 times diluted), apolipoprotein J antibody (0.5 µg/ml) and apolipoprotein AII antibody (1 µg/ml).

The amounts of apolipoprotein AI and apolipoprotein B present in plasma clot extracts were estimated using Western blot analysis. For quantification of apolipoprotein AI a clot extract and different concentrations of purified apolipoprotein AI were run on a Tris-HCl gel (15%) and for apolipoprotein B quantification a clot extract and different concentrations of purified low-density lipoprotein (LDL) were run on a Tris-HCl gel (5%). LDL was purified according to Redgrave *et al*. [25]. Calibration curves were made of the different concentrations of apolipoprotein AI and apolipoprotein B which were used to estimate the amount of apolipoprotein in the plasma clot extract. Western blot analysis was done as described above using a specific apolipoprotein AI antibody (1000 times diluted) and a specific apolipoprotein B antibody (1 µg/ml).

RESULTS

To investigate the protein composition of a fibrin clot, *in vitro* plasma clots were made by adding $CaCl_2$, thrombin and aprotinin to platelet-poor citrated plasma. Unbound proteins were washed away and non-covalently bound proteins were extracted, separated with 2D gel electrophoresis and visualized with Sypro Ruby (Figure 1A). Spots that were identified using mass spectrometry were reproducibly detected in at least 7 out of 10 2D gels. The high-abundant protein spots (spots 7, 8 and 15) were analyzed with MALDI-ToF mass spectrometry and the other protein spots were analyzed with nanoflow

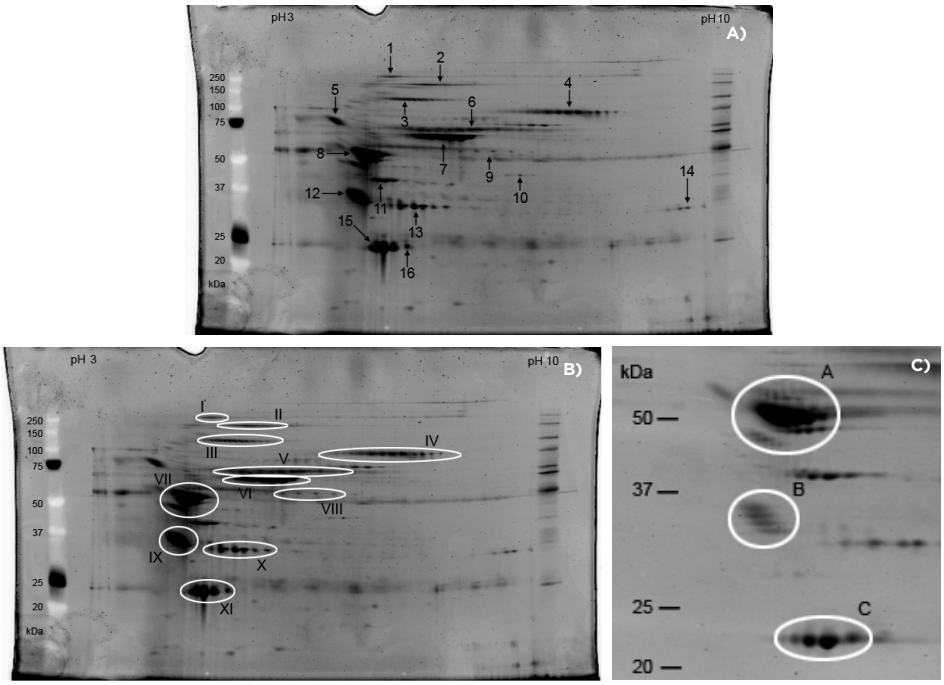

Figure 1. An overview of non-covalently fibrin clot-bound plasma proteins.
Plasma clots were made by adding $CaCl_2$, thrombin and aprotinin to platelet-poor citrated normal plasma, unbound proteins were washed away and bound proteins were extracted. These proteins were separated with 2D gel electrophoresis and visualized by Sypro Ruby. **A)** The numbers and arrows indicate the protein spots that were excised from gel and analyzed with mass spectrometry. **B)** The trains of spots that resemble the same protein are indicated by white ellipses. They include: fibronectin (I), α_2-macroglobulin (II, III and VIII), plasminogen (IV), FXIII A chain (V), albumin (VI), α_1-antitrypsin (VII), apolipoprotein J (IX), apolipoprotein E, HFREP-1 (X) and apolipoprotein AI (XI). **C)** A zoomed image of the 2D gel with a lower fluorescent signal. The isoforms of α_1-antitrypsin (A), apolipoprotein J (B) and apolipoprotein AI (C) are indicated by white ellipses.

LC-MS/MS. This resulted in the identification of 18 different proteins that were present in a plasma clot. Detailed information from Mascot analysis is shown in table 1. Several of the proteins identified were not previously described as plasma clot components including α_2-macroglobulin, carboxypeptidase N (CPN), α_1-antitrypsin, haptoglobin, serum amyloid P and the apolipoproteins AI, AIV, E and J. The latter six proteins are associated with the HDL particle. In addition we identified proteins that have previously been described as fibrin clot-bound proteins, including fibronectin, plasminogen, factor XIII, HFREP-1, actin and thrombin.

The majority of the identified spots belong to a train of spots, as is seen for example for protein spot 4 in figure 1A. These trains most likely represent different isoforms of the same protein. On the basis of multiple mass spectrometry analyses of different spots from different gels we identified several trains of spots (figure 1B). To visualize low-abundant fibrin clot-bound proteins on the 2D gel, the laser intensity in figure 1A was set high.

Table 1: Mascot analysis of fibrin clot-bound proteins

Spot	Accession #	Description	Mowse score	Seq. cov. (%)	Mw	Tot. pept.	Uniq. pept.	pI
1	gi|16933542	Fibronectin	2025	20	262656	33	30	5.49
2	gi|46812315	α_2-macroglobulin	2206	29	167505	43	33	6.06
3	gi|46812315	α_2-macroglobulin	1996	24	167505	46	29	6.06
4	gi|190026	Plasminogen	2240	49	93233	39	34	7.04
5	gi|51173528	carboxypeptidase N, polypeptide 2	864	29	61433	30	13	5.72
6	gi|119395709	coagulation factor XIII, A1 polypeptide	864	21	83267	39	15	5.75
	gi|33451	immunoglobulin heavy constant mu	555	21	51506	27	9	5.92
7	gi|4502027	albumin (*)	280	54	71317	28	25	5.92
8	gi|15080499	α_1-antitrypsin (*)	162	45	46864	15	13	5.36
9	gi|46812315	α_2-macroglobulin	621	7	167505	10	8	6.06
	gi|113584	immunoglobulin heavy constant alpha 1	282	13	38486	5	4	6.08
10	gi|4503011	carboxypeptidase N, polypeptide 1	210	7	52538	4	3	6.86
11	gi|4501887	actin, gamma 1	693	33	42108	20	10	5.31
	gi|178759	apolipoprotein AIV	424	14	45307	6	6	5.23
	gi|306882	haptoglobin	209	8	45860	3	3	6.24
12	gi|177827	α_1-antitrypsin	373	12	46787	7	7	5.42
	gi|338305	apolipoprotein J	344	16	36997	7	5	5.74
13	gi|178849	apolipoprotein E	1124	53	36302	32	17	5.65
	gi|22023090	HFREP-1	448	26	36640	8	7	5.58
14	gi|38018090	thrombin	599	23	34072	11	8	8.52
15	gi|4557321	apolipoprotein AI (*)	153	49	30759	18	15	5.56
16	gi|178775	apolipoprotein AI	723	44	28944	15	11	5.45
	gi|149673887	immunoglobulin light chain	442	40	23665	7	5	6.97
	gi|337758	serum amyloid P	306	18	25495	4	4	6.10

Protein spots shown in Fig. 1A were analyzed by mass spectrometry. Proteins with an asterisk were analyzed with MALDI-ToF and the other protein spots were analyzed with nanoflow LC-MS/MS. Accession number of the NCBInr database, protein description, Mowse score, sequence coverage (%), calculated molecular weight (Mw), total identified peptides, unique identified peptides and the calculated pI are given.
HFREP-1; hepatocyte-derived fibrinogen related protein-1.

The disadvantage of the resulting high fluorescent signal was that the high-abundant proteins displayed a saturated signal. Therefore we show in figure 1C a zoomed image of the high-abundant proteins with a lower fluorescent signal. Although α_1-antitrypsin (spot A in figure 1C) mainly varied in its isoelectric point, several minor species with higher and lower molecular mass were also detected.

The identification by mass spectrometry was verified by comparing the theoretical molecular mass of the proteins, obtained from the NCBInr database, with the apparent mass on the 2D gel. For most protein spots the theoretical molecular mass was similar to the mass estimated from the gel pattern. However, of three protein spots (2, 3 and 9 in figure 1A) identified as α_2-macroglobulin only one spot (spot 2) corresponded with the theoretical mass of the protein of 167 kDa. With Western blot analysis, using α_2-macroglobulin

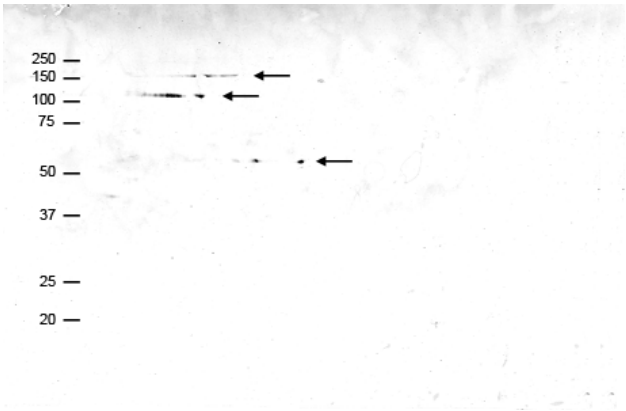

Figure. 2. Western blot analysis with specific α_2-macroglobulin antibodies
Fibrin clot-bound plasma proteins were separated with 2D gel electrophoresis and analyzed with Western blot analysis using specific α_2-macroglobulin antibodies. The arrows indicate the three different α_2-macroglobulin trains that were also identified as α_2-macroglobulin with mass spectrometry (protein spots 2, 3 and 9 in figure 1A and table 1). The molecular mass of the protein marker is indicated in kDa.

specific antibodies, the identity of the three protein spots and their isoforms in the same trains were confirmed to be α_2-macroglobulin (figure 2).

It was a remarkable observation that two-thirds of the novel plasma clot components appeared to be HDL-associated proteins. Next to the presence of these proteins in a fibrin clot we detected the presence of HDL-apolipoproteins in fibrinogen that we purified from plasma (figure 3) and in commercially available fibrinogen purified form plasma (data not shown). This suggests that HDL was bound specifically to fibrinogen and thereby to the plasma clot, which was also supported by the finding that HDL-apolipoproteins present in a plasma clot could not be washed out by increasing the NaCl in the washing buffer from

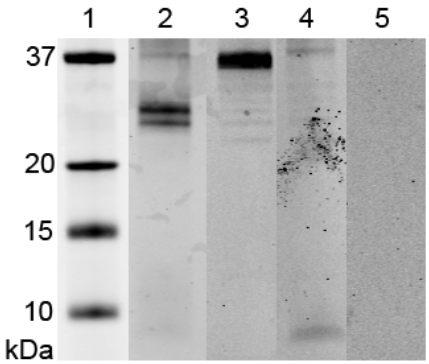

Figure. 3. Western blot analysis for apolipoproteins in purified fibrinogen
Fibrinogen was isolated from plasma with immunoaffinity chromatography and run on SDS-PAGE. Different apolipoproteins were detected with Western blot analysis using specific antibodies. Lane 1: protein marker, lane 2: apolipoprotein AI (Mw=28,900), lane 3: apolipoprotein J (Mw=37,000), lane 4: apolipoprotein AII (Mw=8,700). Only the relevant section of the gel is shown.

0.1 M to 0.5 M (data not shown). The amount of apolipoprotein AI present in a washed plasma clot, estimated from Western blot analysis of clot extracts was about 3 µg/ml. This corresponded to approximately 8 µg HDL per ml plasma. The amount of apolipoprotein AI was much higher than apolipoprotein B (about 0.05 µg/ml), which corresponded to approximately 0.3 µg LDL per ml plasma clot.

DISCUSSION

In this study, using 2D gel electrophoresis and mass spectrometry, we identified 18 different fibrin clot-bound proteins, which are not cross-linked to fibrin by FXIIIa. Several of these protein have not been described before as plasma clot components.

Eleven out of the 18 fibrin clot-bound proteins can be classified into three groups related to their function: blood coagulation, protease inhibition and HDL metabolism. Plasminogen, factor XIII and thrombin are involved in blood coagulation while α_2-macroglobulin and α_1-antitrypsin are protease inhibitors [26,27]. Plasma proteins that are associated with HDL and play a role in its metabolism are haptoglobin, serum amyloid P and the apolipoproteins AI, AIV, J and E [28–31]. The presence of actin as a plasma clot component could be due to small amounts of platelets present in the platelet-poor plasma. However, actin can also be released into the bloodstream by dying cells or tissue damage [32].

The intensity of the stained spots suggests that the 18 identified plasma proteins represent nearly the entire protein material non-covalently bound to a fibrin clot. However with this approach we do not visualize the very low-abundant proteins. For example t-PA, a known fibrin-binding protein, was not observed. A second limitation of 2D gel electrophoresis is that high molecular weight proteins are underrepresented [33]. However, we did observe the high molecular weight proteins fibronectin and α_2-macroglobulin with 2D gel electrophoresis. In addition, with 1D gel electrophoresis (SDS-PAGE) and protein staining we did not observe any additional high molecular weight protein in a plasma clot extract (data not shown).

To verify the identification of the protein spots by mass spectrometry the theoretical molecular mass of the identified protein was compared with the apparent molecular mass on the 2D gel. For most proteins the theoretical molecular mass was comparable with the observed molecular mass, given that the theoretical mass does not take into account several post-translational modifications like glycosylation. Three different protein spots on the 2D gel of figure 1A were identified as α_2-macroglobulin and the molecular mass of only one protein spot was comparable with the theoretical mass of the protein. However, all protein spots identified as α_2-macroglobulin were confirmed with Western blot analysis. It has been shown that when α_2-macroglobulin is heated at

high temperatures or at lower temperatures under denaturing conditions, two polypeptide chains of 125 kDa and 62 kDa can be produced [34]. Although we did not heat the clot extract in the preparation for 2D gel electrophoresis, the molecular weights of the two α_2-macroglobulin fragments we observed match the molecular weights of the fragments produced upon heating.

Very recently, after we finished the research described here, a paper was published that described complement C3 as a novel plasma clot component [35]. Most proteins identified were related to coagulation and inflammation, while we identified mainly proteins that were related to coagulation, protease inhibition and HDL metabolism. There are some clear differences between the two proteomic approaches. The most important difference is that Howes *et al*. [35] described the total protein composition of the whole clot, thereby also identifying proteins that are crosslinked via FXIIIa, while we focused on non-covalently plasma clot-bound proteins. Identifying proteins by examining the whole clot is technically more challenging because of the high abundance of fibrin compared to the other plasma clot components.

Two-thirds of the newly identified fibrin clot-bound proteins are associated with HDL suggesting that HDL particles have affinity for fibrin, which is specific for HDL and not for LDL because only low amounts of apolipoprotein B were present in a fibrin clot. In addition, this apolipoprotein B most likely comes from bound lipoprotein(a), which can bind with its apolipoprotein(a) to fibrin [10]. The presence of HDL-proteins in purified fibrinogen suggested affinity of HDL to fibrinogen as well. These findings are in line with the detection of fibrinogen in purified HDL preparations [29,36,37]. What the role is of the binding of HDL to a fibrin clot is not known. However, recent studies have shown that HDL levels are negatively associated with both arterial and venous thrombosis [38–40], for which the exact mechanism is not known. HDL is a reverse cholesterol transporter, which is considered to be the most important property of HDL in preventing atherosclerosis. Several other properties can contribute to the atheroprotective effect of HDL including antioxidant, anti-inflammatory, antiproliferative, antithrombotic and vasodilatory properties [41]. HDL consists of a heterogeneous population of particles containing different types and amounts of (apolipo)proteins and lipids. The existence of different subpopulations in HDL is consistent with the fact that HDL has multiple biological activities [42]. It is possible that the HDL particle present on a fibrin clot as identified in this study represents a distinct subfraction of HDL.

A direct role of HDL in coagulation or fibrinolysis is not yet clear. It was suggested that HDL enhances the activated protein C pathway [43], but this may be due to the contamination of negatively charged phospholipid membranes [44]. Another possible mechanism that can play a role in the anticoagulant effect of HDL is that anionic phospholipids lose their procoagulant properties when incorporated into HDL [45].

In conclusion, we have identified several novel plasma clot components of which two-thirds were associated with HDL particles. This suggests that the presence of HDL on a fibrin clot may be of importance in clot formation or fibrinolysis and may play a role in hemostasis and thrombosis.

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Chapter 3

Identification and characterization of α_1-antitrypsin in fibrin clots

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Submitted

ABSTRACT

Background & Objectives: Preliminary studies indicated that α_1-antitrypsin (A1AT) is the most abundant protein that is non-covalently bound to fibrin clots prepared from plasma. The aim of this study was to identify and characterize fibrin(ogen)-bound A1AT.

Methods & Results: Plasma clots were prepared and extensively washed with saline. Clot-bound A1AT could only be extracted using denaturing agents such as urea, thiourea or SDS, pointing to a strong association. Purified fibrinogen, containing A1AT as a contaminant, was gel filtered which showed that the A1AT was fully bound to fibrinogen. A specific ELISA detected the presence of A1AT-fibrinogen complexes both in purified fibrinogen and in pooled normal plasma. Finally, fibrin-Sepharose chromatography indicated that A1AT purified from plasma contained a small fraction of fibrin-binding A1AT. To study the inhibitory activity of fibrin(ogen)-bound A1AT, both fibrinogen containing A1AT and washed plasma clots were incubated with increasing amounts of elastase. SDS-PAGE and Western blotting showed the generation of A1AT-elastase complex as well as cleaved A1AT. The inhibitory activity of fibrin(ogen)-bound A1AT was also demonstrated by measuring elastase-induced lysis of fibrin clots.

Conclusion: Fibrin clots contain strongly bound A1AT, which is functionally active as a serine protease inhibitor (serpin). This A1AT might play a role in the local regulation of proteases involved in coagulation or fibrinolysis and represent a novel link between the inflammatory and hemostatic systems.

INTRODUCTION

Activation of the blood coagulation system eventually results in the conversion of fibrinogen into fibrin. Fibrinogen is a heterogeneous glycoprotein of about 340 kDa that occurs in plasma at concentrations of 2–4 mg/ml [1]. The molecule is composed of two identical subunits, each consisting of one Aα-chain, one Bβ-chain and one γ-chain. Thrombin cleaves the fibrinopeptides A and B from the amino-terminal ends of the Aα-chain and Bβ-chain, respectively. The resulting fibrin monomers polymerize to insoluble fibrin polymers that form a fibrin network, which is stabilized by cross-linking by the action of coagulation factor XIII. The fibrin network is essential for the physical stability of blood clots and thrombi. Fibrin is also actively involved in its formation and degradation (fibrinolysis). This is mediated by the specific binding of a number of coagulation factors and in particular of proteins of the fibrinolytic system [2]. Binding of proteins to fibrin could also affect the final fibrin structure and play a role in tissue repair [3]. Some of the fibrin-binding proteins are covalently cross-linked to fibrin by the action of factor XIII.

α_1-Antitrypsin (A1AT, also known as α_1-proteinase inhibitor) is the best studied inhibitor of the serpin superfamily of serine protease inhibitors and is referred to as SERPINA1 [4]. The prime target of A1AT is elastase which is released by leukocytes at sites of inflammation. Recent studies suggest that A1AT may exhibit other anti-inflammatory properties as well. Genetic deficiencies of A1AT are associated with lung emphysema [5] and various chronic diseases [6]. A1AT does not only inhibit elastase, but also a wide variety of other serine proteases, including those of the hemostatic system. A clear role of A1AT in either bleeding or thrombosis has not yet been established so far. Recent studies on A1AT and other serpins focus on protein structure and function [7]. Mutations in serpins may result in the formation of pathological polymers [8]. A classic example is the Z mutant of A1AT which forms polymers in the endoplasmic reticulum of hepatocytes and may cause cirrhosis.

In our studies on the plasma proteins that bind to the fibrin matrix of a plasma clot, we preliminarily observed a high abundance of A1AT [9–11]. In the present study we show that A1AT is the most abundant protein non-covalently bound to plasma clots and characterize the A1AT-fibrin(ogen) complex biochemically.

MATERIALS AND METHODS

Materials

Purified A1AT (Zemaira), a therapeutic protein (purity >90%) derived from human plasma was obtained from CSL Behring (King of Prussia, PA, USA). Human leukocyte elastase (purity >95%) was obtained from Elastin Products Company (Owensville, MO, USA), and

aprotinin (Trasylol) from Bayer (Leverkusen, Germany). Human thrombin and bovine serum albumin (BSA) were products of Sigma-Aldrich (St Louis, MO, USA). Purified fibrinogen (plasminogen, von Willebrand factor and fibronectin free) was obtained from Enzyme Research Laboratories (South Bend, IN, USA). In some experiments, in-house prepared fibrinogen and fibrinogen from Chromogenix (Instrumentation Laboratory, Milano, Italy) were used, as indicated. Destreak, IPG buffer, immobiline strips and IPGphor (for 2D gel electrophoresis) were from GE Healthcare (Little Chalfont, Buckinghamshire, UK). Trypsin Gold was obtained from Promega Corporation (Madison, WI, USA).

Partially A1AT-depleted fibrinogen was prepared by running 20 mg fibrinogen (1 mg/ml) through a 1 ml Sepharose column to which 5 mg goat anti-A1AT IgG (GA1AT-IG of Affinity Biologicals, Ancaster, Ontario, Canada) was coupled. The residual amount of A1AT was about 15%.

Pooled normal plasma was prepared from citrated platelet-poor plasma from 10 apparently healthy donors. Pooled normal plasma without free A1AT was prepared by 25% ammonium sulfate precipitation and by dissolving the precipitate in 5 mM sodium citrate.

Plasma clot preparation

In vitro clots of 500 µl citrated pooled normal plasma were prepared in a pipette tip by adding calcium chloride (20 mM), thrombin (1 NIH U/ml) and aprotinin (100 KIU/ml) [9]. After 2 hours of incubation at room temperature, the clots were extensively washed by perfusing them with 10 ml Tris-buffered saline (50 mM Tris-HCl, 100 mM NaCl, pH 7.4) containing aprotinin (100 KIU/ml) at 4°C. In some experiments, the NaCl concentration was increased to 0.5 M, as indicated. The clots were transferred to Eppendorf tubes and compacted by centrifugation, washed with deionized water and non-covalently clot-bound proteins from 4 clots were extracted for 1 hour at room temperature with 150 µl rehydration buffer (7M urea, 2M thiourea, 4% (w/v) CHAPS, 0.5% (v/v) IPG 3–10 buffer) for 2D gel electrophoresis or with SDS sample buffer without reducing agent for SDS-PAGE. Alternatively, washed and compacted clots were completely dissolved with SDS sample buffer with reducing agent and analyzed by SDS-PAGE to study covalently clot-bound proteins by Factor XIII.

2D gel electrophoresis and mass spectrometry

Plasma clot extracts were separated with 2D gel electrophoresis after addition of 1% (v/v) DeStreak. The proteins in the 150 µl extracts were separated in the first dimension on an 11 cm immobiline drystrip with a 3–10 or 4–7 pH range by isoelectric focusing on the IPGphor with the following running protocol: 30V for 12 hours (rehydration), 1000V for 4 hours (gradient), 8000V for 5 hours (step-n-hold), with a 50 µA limit per gel. After

isoelectric focusing the gel strip was equilibrated in buffer (6 M urea, 50 mM Tris-HCl pH 8.8, 20% (v/v) glycerol, 2% (w/v) SDS) containing 1% (w/v) DTT for 15 minutes followed by a second equilibration step with equilibration buffer containing 1% (w/v) iodoacetamide for 15 minutes. For the second dimension the gel strip was laid on a 12% Bis-Tris Criterion gel and run for 1h at 200V constant, using the XT MOPS buffer as running buffer (Biorad Laboratories, Hercules, CA, USA). The proteins in the gel were visualized by staining with Sypro Ruby (Invitrogen, Paisley, UK). Spots were excised, in-gel digested with trypsin and analyzed by MALDI-TOF mass spectrometry using an Ultraflex-II apparatus (Bruker Corporation, Billerica, MA, USA). Flexanalysis 2.4 and BioTools 3.1 software were used for data processing. The mass spectra obtained were analyzed using peptide mass fingerprint spectra with the online Matrix Science Database with MASCOT software (www.matrixscience.com). The NCBInr database 20100624 (11299630 sequences; 3855426203 residues) was searched with the Mascot parameters set as follows: Taxonomy, *homo sapiens*; mass tolerance, 100 ppm; maximally one missed cleavage per peptide; fixed modification of carboxymethylation of cysteine residues; variable modification of partial oxidation of methionine residues. Mowse scores above the NCBInr database threshold of 66 were considered significant ($p<0.05$).

SDS-PAGE

SDS-PAGE was performed according to Laemmli [12]. To detect A1AT-elastase complex a phosphate-based SDS-PAGE sample buffer and Bis-Tris gels were used as described below. Calibration was performed by running Precision Plus Protein Standards (Dual Color, Biorad Laboratories).

Western blot analysis

Proteins separated by 2D gel electrophoresis or SDS-PAGE were transferred to a nitrocellulose membrane by semi-dry blotting at 0.33 mA constant for 1 hour. Two different procedures for immunostaining were followed. In the first procedure the membrane was blocked with PBS, pH 7.4 containing 1% (w/v) BSA and 0.1% Tween 20, then incubated with 0.5 µg/ml sheep anti-A1AT IgG conjugated to HRP (Abcam, Cambridge, UK) in block buffer and finally stained with BM blue POD substrate (Roche, Basel, Switzerland). In the second procedure the membrane was blocked with PBS, pH 7.4 containing 1% (w/v) BSA, then incubated with goat anti-A1AT IgG (GA1AT-AP of Affinity Biologicals, 2 µg/ml) in block buffer and finally incubated with the secondary antibody IRDye® 800CW donkey-anti-goat IgG (LI-COR Biosciences, Lincoln, NE, USA) 10.000-fold diluted in 5% milk and 0.1% Tween 20 in PBS, pH 7.4. All incubation steps were performed for 1 hour at room temperature.

ELISAs

A1AT concentrations were determined using a matched-pair antibody set for ELISA of human A1AT and a protocol from Affinity Biologicals. Purified A1AT was used for calibration. Fibrin degradation products were determined using a matched-pair antibody set for ELISA of human fibrinogen and a protocol from Affinity Biologicals. Purified fibrinogen was used for calibration. An ELISA for A1AT-fibrinogen complexes was developed by combining the capture antibody from the A1AT ELISA and the detecting antibody from the fibrinogen ELISA.

Gel filtrations

Purified fibrinogen (15 mg) or purified A1AT (4.5 μg) were gel filtered on Superdex 200 prep grade (GE Healthcare) at 4°C using an Econo-column (1.0 x 120 cm) of Biorad Laboratories and 0.05 M Tris-HCl buffer pH 7.5 containing 0.15 M NaCl and 0.01 % (v/v) Tween 20 as the eluent. In some experiments, the composition of the eluent was changed, as indicated. Fractions of about 1.5 ml were collected. The void volume and total volume were determined with Blue dextran (1 mg/ml) and acetone (10 mg/ml), respectively. Additional calibrators were plasminogen (Mr 92,000), BSA (Mr 66,000), ovalbumin (Mr 45,000) and ribonuclease A (Mr 13,700).

Fibrin(ogen)-Sepharose chromatography

Purified fibrinogen (50 mg) was coupled to 10 ml CNBr-activated Sepharose 4B, according to the instructions of the manufacturer (GE Healthcare). Five ml of fibrinogen-Sepharose was converted into fibrin-Sepharose by incubating a 25% (v/v) gel suspension with 10 NIH U/ml thrombin for 4 hrs at room temperature [13]. After extensive washing, the gel was treated with D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone to block residual amounts of thrombin. Chromatography was performed with 1 ml columns (1.2 x 1.0 cm) of fibrinogen-Sepharose or fibrin-Sepharose in 50 mM Tris-HCl, pH 7.4 containing 100 mM NaCl, 4 mM $CaCl_2$, 1% (w/v) BSA and 0.1% Tween 20 (column buffer) at room temperature and fractions of 1 ml were collected. Purified A1AT (200 μg in 5 ml of column buffer) was applied onto the column, which was then washed with 15 ml column buffer. Elution of bound A1AT was achieved with 2M KSCN in column buffer. Both the breakthrough fractions and the elution fractions (after dialysis) were separately rechromatographed on the same column.

Detection of A1AT-elastase complex by SDS-PAGE and Western blotting

Purified A1AT (17 ng) or purified fibrinogen containing A1AT (34 µg) were incubated with 0, 5, 10 or 20 ng elastase in 20 µl PBS containing 0.001%(v/v) Tween 20 for 1 hr at room temperature. Ten µl 3x SDS-PAGE sample buffer without reducing agent (188 mM sodium phosphate, pH 7.0, containing 9% (w/v) SDS and 35% (v/v) glycerol) was added to each incubation and the mixtures were incubated at 95°C for 5 min and analyzed by SDS-PAGE (20 µl per lane), using 10% Bis-Tris gels with the matching XT MOPS Buffer as running buffer (Biorad Laboratories). A sample buffer containing phosphate instead of Tris was used for optimal stability of serpin-proteinase complexes during SDS-PAGE [14] and Bis-Tris gels were used because pilot experiments had shown that cleaved A1AT was better separated from intact A1AT on these gels than on Tris-glycine gels (Laemmli system). Western blotting was performed with goat anti-A1AT IgG using the above-described Odyssey system.

A similar experiment with fibrin-bound A1AT was performed using 4 plasma clots of 200 µl prepared as described above. The clots were washed with 0.5 ml 0.15 M NaCl containing aprotinin (100 KIU/ml) followed by 10 ml of PBS containing aprotinin (100 KIU/ml) and then permeated with 200 µl PBS containing 0.001%(v/v) Tween 20 and 0, 36, 72 and 144 ng elastase, respectively and incubated for 30 min at room temperature. Finally the clots were permeated with 500 µl PBS containing 0.001%(v/v) Tween 20 and the first 200 µl aliquots were collected, mixed with 100 µl 3x SDS-PAGE sample buffer and analyzed as unbound A1AT. The clots were transferred to Eppendorf tubes, compacted by centrifugation and bound proteins were extracted for 1 hour at room temperature with a mixture of 200 µl PBS containing 0.001%(v/v) Tween 20 and 100 µl 3x SDS-PAGE sample buffer to obtain clot-bound A1AT. Unbound and clot-bound A1AT were incubated at 95°C for 5 min and analyzed by SDS-PAGE (39 µl per lane) and Western blotting as described for purified A1AT and purified fibrinogen containing A1AT.

Inhibition of fibrin clot lysis

The inhibitory activity of fibrin-bound A1AT was studied by comparing the elastase-induced degradation of fibrin clots prepared from purified fibrinogen containing fibrinogen-bound A1AT with the degradation of fibrin clots prepared from partially A1AT-depleted fibrinogen. Clots of 100 µl were prepared by mixing the fibrinogen preparations (0.4 mg/ml, final concentration) with varying amounts of elastase (0 - 0.8 µg/ml, final concentration) and thrombin (1 NIH U/ml, final concentration) in 50 mM Tris-HCl, pH 7.4 containing 100 mM NaCl, 2 mM $CaCl_2$, 3% (w/v) BSA and 0.1% (v/v) Tween 20. The clots

were incubated for 6 hrs at 37°C and centrifuged. The amount of solubilized fibrin was determined in the supernatant by using an ELISA for fibrinogen.

RESULTS

Identification of A1AT in fibrin clots

In order to investigate which proteins are associated with fibrin clots, pooled normal plasma was clotted and the resulting clots were washed with Tris-buffered saline. Bound proteins were extracted with rehydration buffer containing urea, thiourea, CHAPS and IPG-buffer for analysis with 2D gel electrophoresis. The main protein spot on the 2D gel at about 52 kDa (Fig. 1) was identified by mass spectrometry as α_1-antitrypsin (A1AT). This identification was confirmed by Western blot analysis using anti-A1AT IgG (Fig. 1 upper inset). The spot consisted of several isoforms of A1AT, as was demonstrated by performing the isoelectric focusing in the first dimension at pH 4–7 instead of pH 3–10 (Fig. 1 lower inset). The six main forms were identified as A1AT by mass spectrometry with Mowse scores of 167–308. The estimated isoelectric points of these isoforms were 4.8–5.1.

In a subsequent experiment plasma clots were washed with Tris-HCl buffer containing either 0.5 M NaCl or 0.1 M NaCl. SDS-PAGE of the two clot extracts revealed protein bands of A1AT of equal intensity (not shown), indicating that clot-bound A1AT did not

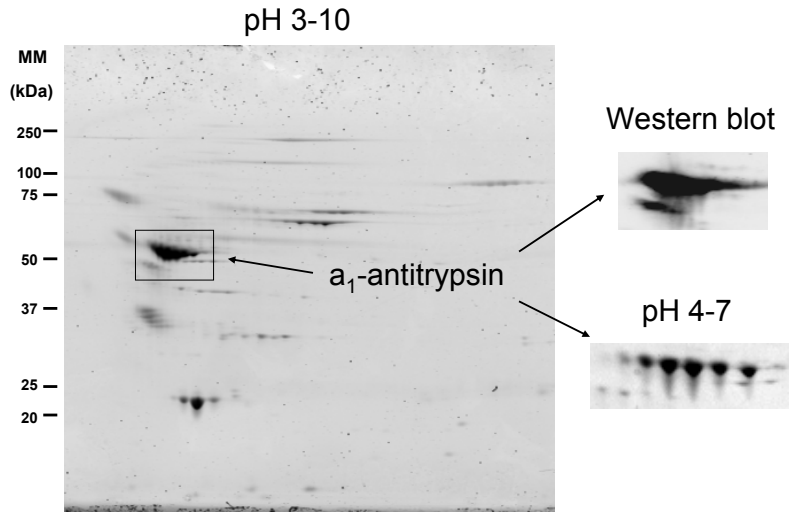

Figure 1. 2D Gel electrophoresis (pH gradient of 3-10) of proteins extracted from a washed fibrin clot prepared from pooled normal plasma. The main protein spot was identified as α_1-antitrypsin (A1AT) by mass spectrometry. This was confirmed by Western blotting using anti-A1AT IgG in the Odyssey system (upper right panel). The main spot was separated into different isoforms of A1AT by 2D gel electrophoresis using a pH gradient of 4-7 (lower right panel).

substantially dissociate at high ionic strength. Washed plasma clots were also extracted with SDS sample buffer containing a reducing agent, which completely dissolved the clots. SDS-PAGE followed by Western blotting using anti-A1AT IgG-HRP showed just one main band at about 52 kDa and no band(s) at 100–120 kDa, indicating that no covalent complex of A1AT with one of the three polypeptide chains of fibrin existed (not shown). This implied that A1AT was only bound to the clot in a reversible manner and was not covalently cross-linked by Factor XIII.

The amount of A1AT present in a washed plasma clot, as estimated by ELISA after extraction with rehydration buffer, was about 0.6 μg A1AT per ml of plasma clot which corresponds with 0.25 μg A1AT per mg of fibrin(ogen).

A1AT in purified fibrinogen preparations

While studying the composition of purified fibrinogen preparations, we observed that these preparations were contaminated with A1AT. This was confirmed in Fig. 2 showing an SDS-PAGE followed by Western blotting of three different fibrinogen preparations along with various amounts of purified A1AT. Comparison of the intensities of the bands indicated that the fibrinogen preparations contained about 0.25–0.50 μg A1AT per mg of fibrinogen. This was comparable with the amount of A1AT bound to a washed plasma clot.

To further characterize the A1AT present in purified fibrinogen preparations, purified fibrinogen was gel filtered on a Superdex 200 column using a Tris buffer containing 0.15

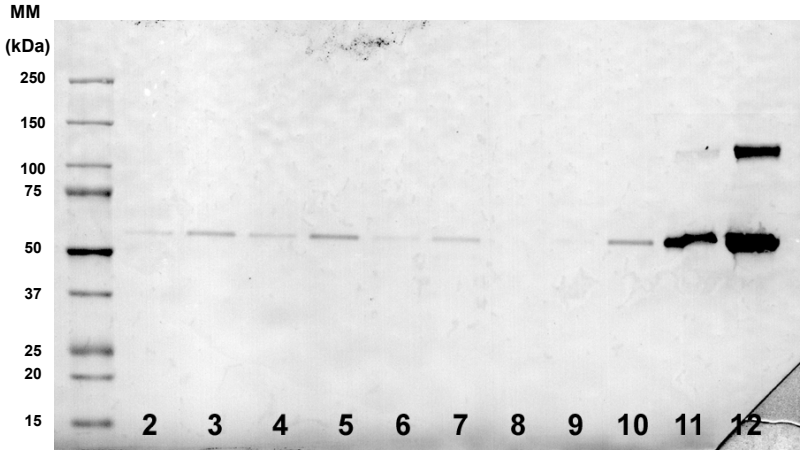

Figure 2. SDS-PAGE (non-reduced, 4-12% gel) of different purified fibrinogen preparations and purified A1AT followed by Western blot analyses using anti-A1AT IgG-HRP and BM blue POD substrate. Lanes 2 and 3: 1 and 2 μg fibrinogen from Chromogenix, resp., lanes 4 and 5: 1 and 2 μg fibrinogen from Enzyme Research Laboratories, resp., lanes 6 and 7: 1 and 2 μg fibrinogen prepared in house, resp., lanes 8-12: 0, 0.1, 1.0, 10 and 100 ng purified A1AT, resp. The band between 100 and 150 kDa in lanes 11 and 12 probably represented A1AT dimer.

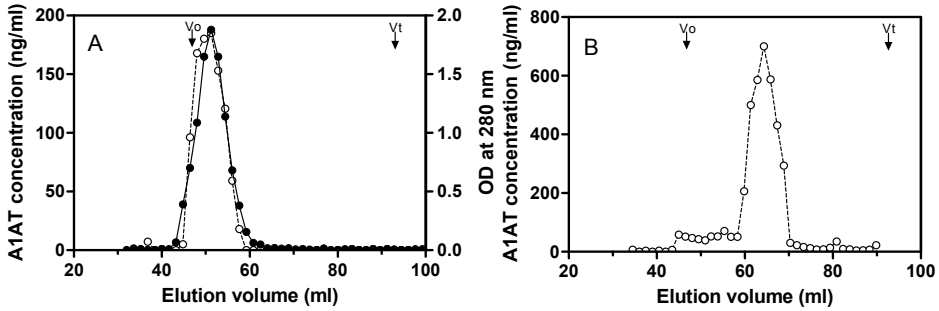

Figure 3. Gel filtration of purified fibrinogen (panel A) or purified A1AT (panel B) on Superdex 200 in 0.05 M Tris-HCl buffer pH 7.5 containing 0.15 M NaCl and 0.01 % (v/v) Tween 20. Closed circles: optical density at 280 nm; open circles: A1AT concentration.

M NaCl as the eluent. The fractions were tested with an A1AT ELISA and the optical density was determined at 280 nm, which reflected primarily the fibrinogen concentration, because the fibrinogen preparation was >95% pure. Fig. 3A shows that both fibrinogen (340 kDa) and A1AT (52 kDa) migrated with an elution volume of about 51 ml just behind the void volume (V0) of the column. By contrast, purified A1AT in a parallel experiment migrated more slowly with an elution volume of about 64 ml, in line with a molecular mass of 52 kDa (Fig. 3B). These experiments indicated that A1AT present in purified fibrinogen was fully bound to fibrinogen in a Tris-HCl buffer with a physiological ionic strength. Gel filtration in a buffer containing 1.0 M NaCl instead of 0.15 M NaCl yielded similar results. However, buffers containing 1M KSCN or 6 M urea resulted in a partial separation of fibrinogen and more slowly migrating A1AT, suggesting that under these conditions the A1AT-fibrinogen complex at least partially dissociated (not shown), which is in line with the observed dissociation of the complex during SDS-PAGE (Fig. 2).

A1AT-fibrinogen complexes in plasma

To further investigate the presence of A1AT in plasma clots and purified fibrinogen we set up a sandwich ELISA for A1AT-fibrinogen complexes, which were captured with immobilized antibodies against A1AT and detected with HRP-labeled antibodies against fibrinogen. Purified fibrinogen containing A1AT-fibrinogen complexes, as demonstrated by gel filtration, showed a strong signal, whereas partially A1AT-depleted fibrinogen showed a reduced signal and purified A1AT no signal (Fig. 4A). These experiments confirmed the existence of A1AT-fibrinogen complexes in purified fibrinogen and illustrated the specificity of the ELISA. Pooled normal plasma showed a moderate response in the ELISA, which tended to decrease at a high (40%) plasma concentration (Fig. 4B). Because the decrease might be due to competition by free A1AT in plasma, the A1AT-fibrinogen complexes were separated from free A1AT in plasma by ammonium sulfate precipitation. Indeed, the signal of plasma after ammonium sulfate precipitation strongly increased (Fig. 4B). These

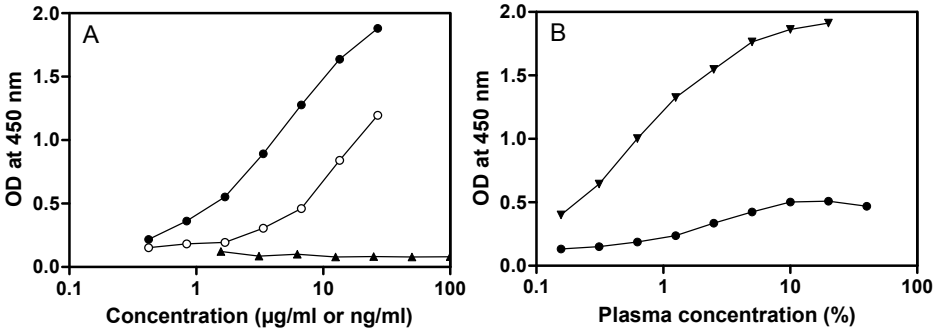

Figure 4. ELISA for A1AT-fibrinogen complex. The left panel (A) shows the response of varying concentrations of purified fibrinogen in µg/ml (closed circles), partially A1AT-depleted fibrinogen in µg/ml (open circles) and purified A1AT in ng/ml (closed triangles). The right panel (B) shows the response of varying concentrations of pooled normal plasma, before (closed circles) and after (closed triangles) eliminating free A1AT by ammonium sulfate precipitation.

experiments suggested that plasma contained A1AT-fibrinogen complexes. Comparison of the signal of plasma after ammonium sulfate precipitation (Fig. 4B) with the signal of purified fibrinogen (Fig. 4A) containing 0.5 µg A1AT per mg of fibrinogen (estimated from the Western blot in Fig. 2) indicated that pooled normal plasma contained 0.4 µg fibrinogen-bound A1AT per ml. This implied that most A1AT found in a washed plasma clot (0.6 µg A1AT/ml) was already bound to fibrinogen before clotting.

Fibrin-Sepharose chromatography of purified A1AT

In order to study the affinity of purified A1AT for fibrin(ogen), affinity chromatography was performed with fibrinogen-Sepharose and fibrin-Sepharose. No difference was found between the two column materials. Fig. 5A shows an experiment in which purified A1AT was applied onto a fibrin-Sepharose column. After washing the column, bound A1AT was eluted with a buffer containing 2M KSCN. The elution profile (closed circles) indicated that >99% of the A1AT did not bind, but a more sensitive assay (open circles) showed that about 0.1% did bind to the fibrin-Sepharose. The A1AT in the breakthrough fractions was rechromatographed, and no A1AT peak was found after elution with KSCN (Fig. 5B). The A1AT in the elution fractions of Fig. 5A was dialyzed to remove KSCN and was also rechromatographed. About 70% was recovered in the breakthrough and 30% was recovered after elution with KSCN (not shown). A control experiment with a similar small quantity of A1AT showed again that >99% of the material did not bind to the fibrin-Sepharose. Altogether, these experiments showed that purified A1AT had no affinity to fibrin-Sepharose, but it contained a small fraction (0.1%) with a high affinity to fibrin-Sepharose. This material only partially kept its affinity after elution with 2M KSCN.

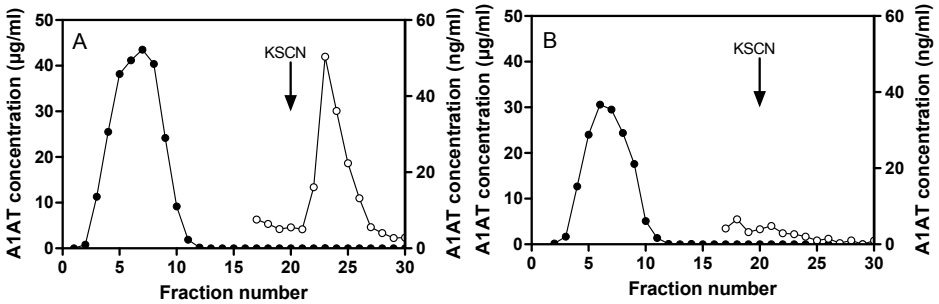

Figure 5. Fibrin-Sepharose chromatography of A1AT. The left panel (A) shows the elution profile of 200 µg purified A1AT, both in µg/ml (closed circles) and in ng/ml (open circles). About 0.1% of the material was bound and eluted with 2 M KSCN. Unbound A1AT in fractions 4-9 was rechromatographed, as shown in the right panel (B). No material was eluted with 2 M KSCN, indicating that bound and eluted A1AT in panel A represented a subfraction of A1AT with a high affinity for fibrin.

Functional characterization of fibrin(ogen)-bound A1AT

To study whether or not fibrin(ogen)-bound A1AT is still active as a serpin, the interaction with elastase was investigated by SDS-PAGE and Western blotting using anti-A1AT IgG. Fig. 6A shows purified A1AT, as a positive control, after incubation with (lane 2–4) or without (lane 1) increasing amounts of elastase. Intact A1AT almost fully disappeared and both A1AT-elastase complex and cleaved A1AT appeared. Purified fibrinogen containing fibrinogen-bound A1AT was also incubated with (lanes 6–8) or without (lane 5) increasing amounts of elastase. As in the previous experiment, intact A1AT almost fully disappeared and both A1AT-elastase complex and cleaved A1AT appeared, indicating that fibrinogen-bound A1AT was able to interact and to form a complex with elastase. However, the proportion of cleaved A1AT was greater than after incubation of purified A1AT with elastase, suggesting that the stability of the complex, possibly during SDS-PAGE, was less than normal.

Fig. 6B shows a similar experiment with fibrin-bound A1AT. Plasma clots were extensively washed with buffer and then permeated with increasing amounts of elastase. After incubation the unbound and clot-bound fractions were analyzed separately. Without elastase (lanes 1 and 5) only clot-bound A1AT was observed, as expected. This A1AT disappeared after incubation with increasing amounts of elastase. At the same time A1AT-elastase complex (tiny amounts in lanes 3 and 4) and cleaved A1AT appeared, however, primarily in the unbound fractions. This experiment showed that: (1) fibrin clot-bound A1AT was accessible to elastase and able to interact and to form a complex with elastase, (2) the A1AT-elastase complex was not very stable, and (3) the A1AT-elastase complex and cleaved A1AT were only present in the unbound fractions, suggesting dissociation from fibrin after formation.

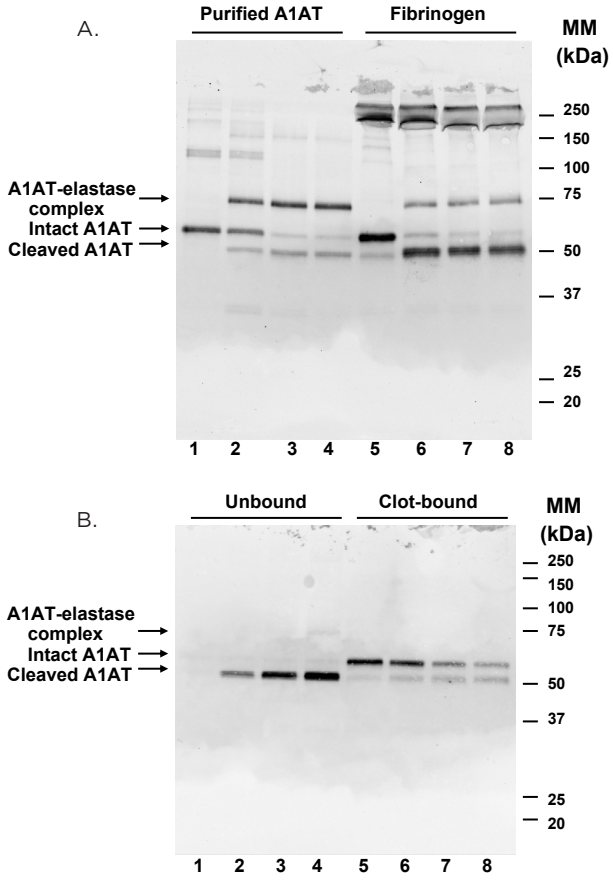

Figure 6. Detection of A1AT-elastase complex by SDS-PAGE and Western blotting with goat anti-A1AT IgG. Upper panel (A): Purified A1AT (17 ng) or purified fibrinogen containing A1AT (34 µg) were incubated with 0 (lanes 1 and 5), 5 (lanes 2 and 6), 10 (lanes 3 and 7) or 20 (lanes 4 and 8) ng elastase and 67% (v/v) of the mixtures was loaded per lane. The bands around 250 kDa in lanes 5-8 were due to non-specific staining of fibrinogen by the secondary antibodies of the Odyssey system. Lower panel (B): washed plasma clots of 200 µl were incubated with 0 (lanes 1 and 5), 36 (lanes 2 and 6), 72 (lanes 3 and 7) or 144 (lanes 4 and 8) ng elastase and 13% (v/v) of both the unbound and clot-bound A1AT was loaded per lane.

Because of the limited stability during SDS-PAGE of the A1AT-elastase complex derived from fibrin(ogen)-bound A1AT the question was raised of whether fibrin(ogen)-bound A1AT is able to inhibit elastase under physiological conditions. This was studied by measuring the inhibition of the degradation of fibrin clots induced by increasing amounts of elastase added before clotting. Clots were prepared from purified fibrinogen containing fibrinogen-bound A1AT or from partially A1AT-depleted fibrinogen. Fig. 7 shows that the clots prepared from partially A1AT-depleted fibrinogen were degraded more extensively, indicating that fibrin-bound A1AT was able to inhibit the fibrinolytic activity of elastase.

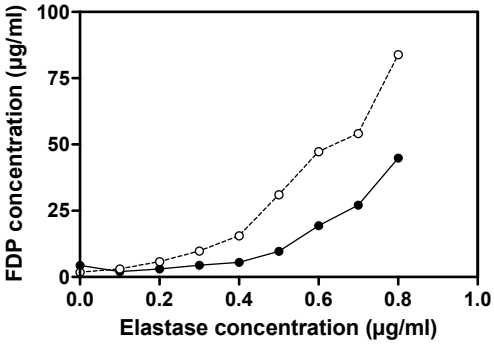

Figure 7. Inhibition of elastase-induced fibrin clot lysis by fibrin(ogen)-bound A1AT. Fibrin clots of 0.4 mg/ml were prepared by mixing purified fibrinogen (closed circles) or partially A1AT-depleted fibrinogen (open circles) with varying concentrations of elastase (0 - 0.8 µg/ml, final concentration) and thrombin. The clots were incubated for 6 hrs at 37°C and centrifuged. The concentration of fibrin degradation products (FDP) was determined in the supernatant by using an ELISA for fibrinogen.

DISCUSSION

Fibrinogen and fibrin are considered as scaffold proteins in hemostasis to which a variety of proteins can bind, thereby influencing clot structure and function [15]. This study identified A1AT as the most abundant protein non-covalently bound to plasma clots. The presence of clot-bound A1AT was explained by the occurrence of strong A1AT-fibrinogen complexes in plasma.

A1AT was identified by 2D gel electrophoresis and mass spectrometry of clot extracts. This approach also revealed other non-covalently bound proteins on clots, which were previously known or unknown as fibrin-binding proteins [9–11]. A recently published study on proteins in plasma clots followed a slightly different approach and detected non-covalently as well as covalently (cross-linked by factor XIII) bound proteins [16]. We found no evidence for cross-linking of A1AT to clots.

While studying the background of our finding of A1AT in plasma clots, we observed that highly purified fibrinogen preparations contained A1AT as a contaminant. This was a crucial finding because this led to the discovery of strong A1AT-fibrinogen complexes, detected by both gel filtration experiments and the use of a specific sandwich ELISA for these complexes. All A1AT in purified fibrinogen occurred presumably as a complex, because no free A1AT was detected by gel filtration. A1AT-fibrinogen complexes were also found in plasma and explained the occurrence of A1AT in plasma clots. In other words, fibrin formation is not essential for A1AT-binding. However, we cannot exclude that fibrin formation increases the binding of A1AT.

A1AT-fibrinogen and A1AT-fibrin were strong complexes, as shown by the following observations: (1) they resisted prolonged washing of plasma clots, even with buffers with an enhanced ionic strength (0.5 M NaCl); (2) A1AT-fibrin complexes only dissociated

when the clots were extracted using denaturing agents such as urea, thiourea and SDS; (3) A1AT-fibrinogen complexes did not dissociate during gel filtration, even not in buffers with an enhanced ionic strength (1.0 M NaCl); and (4) A1AT-fibrinogen complexes only dissociated during gel filtration in buffers containing denaturing agents such as urea and KSCN. These observations suggested a high affinity between A1AT and fibrin(ogen) and raises the question of why a greater proportion of A1AT in plasma is not complexed with fibrinogen. The most likely explanation is that only a small distinct fraction of A1AT shows a high affinity to fibrin(ogen). This notion was supported by the results of fibrin-Sepharose chromatography, showing that a small amount of A1AT (0.1%) could be isolated from purified A1AT and that the remaining A1AT did not show affinity to fibrin. It is not clear why A1AT purified from plasma does contain free fibrin(ogen)-binding A1AT, if we assume that this fraction in plasma is fully bound to fibrinogen. We suggest that free fibrin(ogen)-binding A1AT is formed from either A1AT-fibrinogen or free A1AT during the isolation procedures.

We do not know whether the physico-chemical properties of fibrin(ogen)-binding A1AT differ from those of normal A1AT. The available quantities were too small for extensive analyses. The molecular mass and isoelectric points, as derived from 2D gel electrophoresis, did not substantially differ from normal values, but small differences may not be detectable by this technique. It is known that serpins may adopt a variety of conformations, because the native fold is characterized by being metastable. Examples of additional conformations include the latent form, the cleaved form and polymerized forms [7,8,17]. Some of these conformations were induced in this study by prolonged heating of purified A1AT according to protocols in the literature, but we were unable to induce a fibrin(ogen) binding conformation of A1AT (not shown). In spite of these negative results, it remains possible that a specific conformation of A1AT binds to fibrin(ogen). Our finding that a substantial part (about 70%) of fibrin-bound A1AT isolated by fibrin-Sepharose chromatography with 2M KSCN and subsequent dialysis lost its affinity to fibrin-Sepharose, might be explained by the loss of a specific conformation. Other serpins, including plasminogen activator inhibitor-1 [18] and α_2-antiplasmin [19] also bind non-covalently to fibrin and might possess similar proteins structures and conformations.

A1AT may form stable complexes with non-proteolytic proteins such as immunoglobulin A [20]. The complex between A1AT and immunoglobulin A is mediated by a disulfide bridge. Our results indicated that the A1AT-fibrin(ogen) complex cannot be ascribed to a disulfide bridge between the two proteins.

An important question is whether or not fibrin(ogen)-bound A1AT has proteinase inhibitor activity. This question was positively answered by showing the capability of fibrin(ogen)-bound A1AT to form a complex with leukocyte elastase, the primary target enzyme of A1AT, by using SDS-PAGE. These complexes were, however, less stable during SDS-PAGE than the complex of normal A1AT with elastase, in particular the complex

of fibrin-bound A1AT with elastase appeared to be unstable. The partial dissociation of these A1AT-elastase complexes into free elastase and cleaved A1AT is probably due to the harsh conditions of SDS-PAGE and may have only a limited physiological meaning. This is based on the following considerations: (1) The complex between control A1AT and elastase was also not fully stable (purified A1AT in Fig. 6A) which is in line with other studies [21], whereas this complex is generally considered as stable under physiological conditions; (2) the stability during SDS-PAGE of A1AT-elastase complexes in this study and of the plasmin-α_2-antiplasmin complex in a previous study [14] depended on the buffer composition of the system. Complexes were less stable in Tris buffer than in phosphate buffer and we hypothesize that the amino group in Tris may facilitate the hydrolysis of the ester bond in the acyl enzyme during sample preparation. It is, however, not excluded that the lability of serpin-enzyme complexes during SDS-PAGE might reflect a relatively low stability under physiological conditions, as was shown for instance for the human neuroserpin-tissue plasminogen activator complex [22]. Therefore we studied the functional activity of fibrin(ogen)-bound A1AT also in another model. We found in a fibrin clot lysis assay, that fibrin(ogen)-bound A1AT did inhibit elastase activity.

The experiments of elastase permeation through a washed plasma clot followed by SDS-PAGE analysis of the unbound and clot-bound fractions (Fig. 6B) showed two additional phenomena: (1) the A1AT-elastase complex and cleaved A1AT were mainly detected in the unbound fraction, indicating that strongly bound A1AT easily dissociated from the clot after complex formation with elastase. This mechanism is comparable to the dissociation of antithrombin bound to heparin after complex formation with thrombin [23,24], a fundamental mechanism behind the therapeutic use of heparin as an anticoagulant; (2) nearly all clot-bound A1AT was converted into A1AT-elastase complex and cleaved A1AT, indicating not only that all clot-bound A1AT was active, but also that all clot-bound A1AT, incorporated during clot formation, was accessible to exogenous elastase. This is in line with a recent observation that the interiors of the fibers in a fibrin clot are accessible to outside proteins [25].

The function of fibrin(ogen)-bound A1AT is still unclear. The amounts in a plasma clot are relatively small, but exceed the amounts of other fibrin-bound proteins with proven significance, such as plasminogen, thrombin and activated Factor XIII (Fig. 1 and [11]). Fibrin(ogen)-bound A1AT might play a role in the anti-inflammatory properties of A1AT. Fibrin(ogen) binding of A1AT might also be connected to thrombosis and hemostasis, because A1AT inhibits *in vitro* numerous enzymes of the coagulation and fibrinolysis systems [26–31]. In addition, A1AT inhibits leukocyte elastase and cathepsin G, which are released during coagulation of blood and represent the major enzymes of the alternative pathway of fibrinolysis [32–34]. Recent studies demonstrated *in vivo* that these enzymes may also promote clot formation, coupling thereby coagulation and innate immunity [35]. It is therefore easily conceivable that fibrin-bound A1AT is involved in the local regulation

of these enzymes in coagulation and fibrinolysis. Finally, other functions of fibrin-bound A1AT might include the promotion of cell spreading in wound healing [36].

The following questions still remain to be answered. What distinguishes fibrin(ogen)-bound A1AT from normal A1AT? What type of binding is responsible for the tight A1AT-fibrin(ogen) complex? What are the binding characteristics (Kd and stoichiometry)? Which sites in A1AT and fibrinogen are involved in the binding? Do the activity and specificity of A1AT substantially change upon binding and is there a relation between binding and disease? However, in spite of these questions this study provides novel information about the biochemistry of the serpin A1AT and its potential role in thrombosis and hemostasis.

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Chapter 4

Binding of carboxypeptidase N to fibrinogen and fibrin

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ABSTRACT

The ultimate step in the blood coagulation cascade is the formation of fibrin. Several proteins are known to bind to fibrin and may thereby change clot properties or clot function. Our previous studies identified carboxypeptidase N (CPN) as a novel plasma clot component. CPN cleaves C-terminal lysine and arginine residues from several proteins. The activity of CPN is increased upon its proteolysis by several proteases. The aim of this study is to investigate the presence of CPN in a plasma clot in more detail. Plasma clots were formed by adding thrombin, $CaCl_2$ and aprotinin to citrated plasma. Unbound proteins were washed away and non-covalently bound proteins were extracted and analyzed with 2D gel electrophoresis and mass spectrometry. The identification of CPN as a fibrin clot-bound protein was verified using Western blotting. Clot-bound CPN consisted of the same molecular forms as CPN in plasma and its content was approximately 30 ng/ml plasma clot. Using surface plasmon resonance we showed that CPN can bind to fibrinogen as well as to fibrin. In conclusion, CPN binds to fibrinogen and is present in a fibrin clot prepared from plasma. Because CPN binds to a fibrin clot, there could be a possible role for CPN as a fibrinolysis inhibitor.

INTRODUCTION

The final step in blood coagulation is the conversion of fibrinogen into fibrin monomers by the proteolytic cleavage of fibrinogen by thrombin. Polymerization of fibrin monomers and cross-linking by activated factor XIII result in a stable fibrin network [1,2]. Several proteins can bind to fibrinogen and/or fibrin and can thereby influence clot formation, clot structure and function as well as clot degradation [3]. The fibrin clot is degraded by the fibrinolytic system and the main enzyme of the fibrinolytic system is plasmin, which is formed from plasminogen after cleavage by tissue-type plasminogen activator (t-PA). New C-terminal lysine residues are generated by partial degradation of fibrin by plasmin. These lysine residues form new binding sites for plasminogen and t-PA, which results in increased plasmin generation. This positive feedback loop of plasminogen activation is inhibited by activated thrombin activatable fibrinolysis inhibitor (TAFIa). TAFI is a procarboxypeptidase B of the zinc metallocarboxypeptidase family and TAFIa cleaves C-terminal lysine and arginine residues from partially degraded fibrin, thereby removing binding sites for plasminogen and t-PA [4].

Carboxypeptidase N (CPN) belongs to the same family of zinc metallocarboxypeptidases as TAFI and was discovered as an enzyme that cleaves C-terminal arginine residues from bradykinin; it was therefore originally called kininase I [5]. CPN can also inactivate the complement anaphylatoxins C3a, C4a and C5a [6]. CPN exists as a tetramer with a mass of about 280 kDa, consisting of two heterodimers each with a catalytic subunit (CPN1) and a regulatory subunit (CPN2). The catalytic subunit is 55 kDa in its native form, but is *in vivo* proteolytically cleaved at the C-terminus to form a 48 kDa subunit. The two forms are present in blood in a 1:1 ratio [7]. The regulatory subunit of 83 kDa is not active, but stabilizes the protein and inhibits the clearance of the catalytic subunit from the circulation. CPN is sensitive to proteolysis by plasmin, trypsin and plasma or urinary kallikrein. Proteolysis of CPN finally results in a more active protein [8]. Hydrolysis of the 83 kDa regulatory subunit into a 72 kDa and a 13 kDa fragment results in the dissociation of the tetramer into two heterodimers. The hydrolysis of the catalytic subunits yields fragments of 27 and 21 kDa with increased CPN activity [9].

In a previous study we identified CPN as a plasma clot component [10] with possible antifibrinolytic properties [11]. However, detailed information about the presence of CPN in the plasma clot could not be derived from that study. Therefore the aim of this study was to investigate the binding of CPN to a fibrin clot in more detail. We determined the amount of protein present in the plasma clot, that the protein is present in the forms that are also present in plasma and that CPN is able to bind directly to fibrin and fibrinogen.

MATERIALS AND METHODS

Materials

Human thrombin and albumin from bovine serum were obtained from Sigma-Aldrich (St. Louis, MO, USA). Aprotinin (Trasylol) was from Bayer (Leverkusen, Germany). Human fibrinogen (plasminogen, von Willebrand factor and fibronectin depleted) and human CPN purified from plasma were obtained from Enzyme Research Laboratories (South Bend, IN, USA). Bis-Tris (10% and 12%) Criterion XT precast gels and XT MOPS buffer were from Bio-Rad (Hercules, CA, USA). Sypro Ruby was obtained from Invitrogen (Paisley, UK) and 0.45 µm nitrocellulose transfer membrane from Whatman (Dassel, Germany). DeStreak, IPG buffer pH 3–10, immobiline strips, Ettan Spot Picker, IPGphore and Typhoon Trio apparatus were from GE Healthcare (Little Chalfont, Buckinghamshire, UK). The Biacore T100, CM5 sensor chips and amine coupling kit were obtained from GE Healthcare (Uppsala, Sweden). The rabbit polyclonal IgG to human CPN1 was obtained from Abbiotec (San Diego, CA, USA) and the mouse monoclonal IgG to human CPN2 was from Abfrontier (Seoul, Korea). The Odyssey apparatus and IRDye® 800CW secondary donkey-anti-mouse and donkey-anti-rabbit antibodies were obtained from Li-Cor Bioscience (Lincoln, NE, USA).

Plasma clot preparation

In vitro clots of 500 µl citrated platelet-poor plasma (from a pool of 10 healthy volunteers, Sanquin, Leiden, the Netherlands) were prepared by adding calcium chloride (final concentration 20 mM), thrombin (final concentration 1 NIH U/ml) and aprotinin (final concentration 100 KIU/ml) [12]. After 2 hours of incubation at room temperature, the clots were extensively washed by perfusing them with 10 ml Tris-buffered saline (50 mM Tris-HCl, 100 mM NaCl, pH 7.4) containing aprotinin (100 KIU/ml) at 4°C. The clots were compacted by centrifugation, washed with deionized water and non-covalently clot-bound proteins were extracted for 2D gel electrophoresis with 150 µl rehydration buffer (7M urea, 2M thiourea, 4% (w/v) CHAPS, 0.5% (v/v) IPG 3–10 buffer) for 1 hour at room temperature. For optimal 2D gel electrophoresis, 1% (v/v) DeStreak was added to the extract. The non-covalently clot-bound proteins were extracted for SDS-PAGE with sample buffer (11% (v/v) glycerol, 3% (w/v) SDS, 50 mM Tris-HCl, pH 8.5) for 1 hour at room temperature. To reduce samples, 5% (v/v) β-mercaptoethanol was added to the sample buffer when indicated.

2D gel electrophoresis and mass spectrometry

Plasma clot extract was analyzed with 2D gel electrophoresis. The proteins in the 150 µl extract were separated in the first dimension using a 11 cm immobiline drystrip with a 3–10NL pH range by isoelectric focusing on the IPGphor with the following running protocol: 30V for 12 hours (rehydration), 1000V for 4 hours (gradient), 8000V for 5 hours (step-n-hold), with a 50 µA limit per gel. After isoelectric focusing, the gel strip was equilibrated in buffer (6 M urea, 50 mM Tris-HCl pH 8.8, 20% (v/v) glycerol, 2% (w/v) SDS) with 1% (w/v) DTT for 15 minutes followed by a second equilibration step with equilibration buffer containing 1% (w/v) iodoacetamide for 15 minutes. For the second dimension the gel strip was placed on a 12% Bis-Tris gel and run for 1 hour at 200V constant, using XT MOPS buffer as running buffer. The proteins in the gel were visualized by Sypro Ruby staining according to the manufacturer's instructions and scanned on a Typhoon Trio at an excitation wavelength of 532 nm and an emission wavelength of 610 nm. Protein spots were excised from gel and analyzed using nanoflow LC-MS/MS as described before [10].

Western blot analysis

To confirm the identification of CPN as a plasma clot component, a clot extract was made and analyzed by 2D gel electrophoresis as described above. The proteins were transferred from a 12% Bis-Tris precast gel to a nitrocellulose membrane by blotting at 100V constant for 1 hour. The membrane was incubated with block buffer (PBS, 1% BSA, pH 7.4). CPN1 was analyzed by incubating the blot after the blocking step with the rabbit polyclonal CPN1 antibody (1µg/ml) and CPN2 was analyzed with the mouse monoclonal CPN2 antibody (1:2000), all diluted in 5% (w/v) milk and 0.1% (v/v) Tween 20 in PBS, pH7.4 and incubated overnight at 4°C. The membrane was then incubated for 1 hour at room temperature with the secondary antibodies IRDye® 800CW donkey-anti-mouse or IRDye® 800CW donkey-anti-rabbit diluted 10000 times in 5% (w/v) milk and 0.1% (v/v) Tween 20 in PBS, pH 7.4. To visualize the protein, the membrane was scanned on an Odyssey scanner.

To investigate further which CPN subunits or fragments were bound to a washed plasma clot and the amount of protein present, a plasma clot was made as described above. CPN1 was analyzed by SDS-PAGE using a 12.5% Tris-HCl gel at 0.02 A constant. CPN2 was analyzed using a 10% Criterion XT Bis-Tris gel with MOPS running buffer at 150 V constant. The proteins were transferred from the gels to a nitrocellulose membrane by blotting at 100V constant for 1 hour and CPN1 and CPN2 were analyzed as described above.

Surface plasmon resonance

Binding of CPN to fibrinogen and fibrin was analyzed using a Biacore T100. Fibrinogen, additionally purified using Superdex 200, or bovine serum albumin (BSA) in 10 mM sodium acetate pH 4.0, was immobilized on a CM5 sensor chip using the amine coupling kit according to manufacturer's instructions. Approximately 10000 resonance units (RU) of fibrinogen and BSA were covalently attached to the chip. Immobilized fibrinogen on one flow cell was converted to fibrin monomer by treatment with thrombin (1 U/ml) in running buffer (20 mM HEPES buffer, pH 7.4 with 150 mM NaCl, 2.5 mM $CaCl_2$ and 0.1% (v/v) Tween 20) for 20 minutes at 2 µl/min. Thrombin was removed by injecting 2 M NaCl for 60 seconds at 30 µl/min. Binding experiments were performed in running buffer at a flow rate of 30 µl/min and 25°C. CPN (0 – 450 nM) in running buffer was injected for 11 minutes and the dissociation was monitored for 10 minutes. BSA (100 nM) in running buffer was tested as a control. To regenerate the surface, 0.5 M potassium thiocyanate (KSCN) was injected twice for 60 seconds. The data was visualized using Biacore T100 evaluation software (version 2.0.3). Before data analysis, all data were corrected for the response obtained from the flow cell with immobilized BSA (which did not exceed 21% of the uncorrected response of CPN) and for buffer injection. Due to high response levels and non-steady-state behavior, data were not analyzed quantitatively.

RESULTS AND DISCUSSION

In line with previous results [10,13], 2D gel electrophoresis and mass spectrometry analysis revealed that CPN1 and CPN2 were present in a washed fibrin clot made from plasma (figure 1A). The apparent molecular masses on the 2D gel were comparable to what was published for CPN1 (55 kDa) and CPN2 (83 kDa). The theoretical isoelectric point (pI) of CPN1 is around 6.9 and of CPN2 around 5.5 (www.expasy.org). The apparent pI on the 2D gel of CPN2 differed from the theoretical pI, probably due to glycosylation of the protein. We confirmed by Western blot analysis that the protein identified with mass spectrometry was CPN2 (figure 1B). The protein spot identified as CPN1 was flanked by at least two other proteins spots, which most probably were post-translationally modified CPN1 subunits. Unfortunately we could not detect CPN1 by Western blot analysis, possibly due to the low amount of protein on the 2D gel in combination with a low affinity antibody.

Both CPN1 and CPN2 are sensitive to proteolytic cleavage by e.g. plasmin which results in a more active enzyme when the catalytic subunit of 48 kDa is cleaved into fragments of 27 and 21 kDa [9]. We investigated which forms of CPN are present in a fibrin clot using mass spectrometry data [10] and Western blot analysis. The peptides obtained from mass spectrometry analysis that were matched to the CPN2 sequence included the

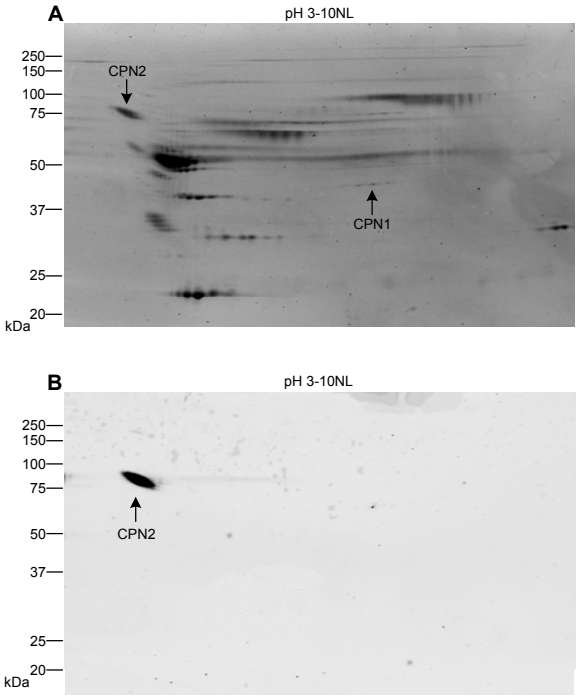

Figure 1: An overview of plasma clot components
Plasma clots were made by adding $CaCl_2$, thrombin and aprotinin to platelet-poor citrated normal plasma, unbound proteins were washed away and bound proteins were extracted. A) Proteins in the clot extract were separated with 2D gel electrophoresis and visualized by staining with Sypro Ruby. CPN1 and CPN2 were identified using mass spectrometry and are indicated by arrows. B) Western blot analysis of a 2D gel using specific CPN antibodies. CPN2 is indicated by an arrow. CPN1 was not detected on this Western blot using a specific antibody to CPN1.

peptides Ser^{479}-Arg^{500} and Trp^{501}-Arg^{509} in the C-terminal region of the protein located after the plasmin cleave site at the arginine residue on position 478 [9], suggesting that the intact CPN2 subunit is present in the plasma clot. In addition, SDS-PAGE, in combination with Western blot analysis using a specific CPN2 antibody, showed only one broad band of the highly glycosylated CPN2 and did not show the cleaved subunit of around 73 kDa (figure 2A). Using SDS-PAGE followed by Western blot analysis with a specific CPN1 antibody, the presence of CPN1 in a plasma clot was confirmed (figure 2B). In addition, both the intact CPN1 subunit of 55 kDa and the cleaved 48 kDa subunit were present as plasma clot components. The cleaved fragments of 27 and 21 kDa were not detected, suggesting that the catalytic subunit present in a fibrin clot is not a further cleaved, more active enzyme. The cleaved CPN1 subunit of 48 kDa is present in plasma in a 1:1 ratio with the intact CPN1 subunit of 55 kDa and this cleavage occurs most likely upon its excretion into the circulation directly after synthesis [7]. The formation of the 48 kDa subunit has no effect on the activity of the enzyme. Taken together, we concluded that the forms of

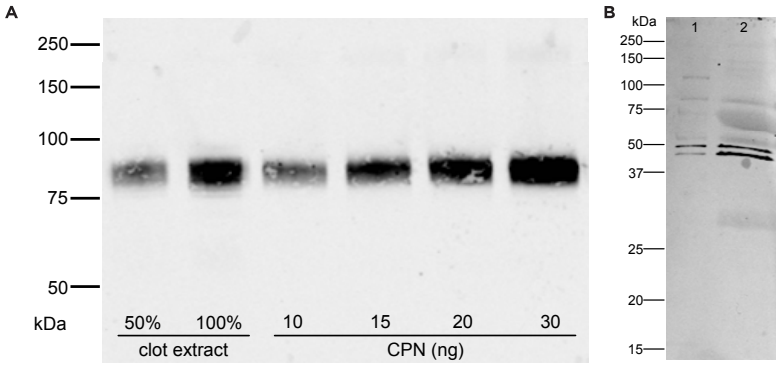

Figure 2: SDS-PAGE and Western blot analysis of CPN
Plasma clots were made by adding $CaCl_2$, thrombin and aprotinin to platelet-poor citrated normal plasma, unbound proteins were washed away and bound proteins were extracted. A) The clot extract (50% and 100%) and different amounts of purified CPN (10 – 30 ng) were analyzed with SDS-PAGE and Western blot analysis using a specific CPN2 antibody. A calibration curve was made from the different purified CPN concentrations and the amount of CPN in the plasma clot extract was calculated using the calibration curve. B) The plasma clot extract (1) and 100 ng purified CPN (2) were analyzed by using SDS-PAGE and Western blot with a specific CPN1 antibody.

CPN that are normally present in plasma were also found within the fibrin clot. We did not find any evidence of the presence of substantially cleaved CPN forms within fibrin clots.

The amount of CPN present in the plasma clot extract was estimated with Western blot analysis using the specific CPN2 antibody. Different amounts of purified CPN and two different concentrations of the plasma clot extract were run on SDS-PAGE and Western blot (figure 2A). The estimated amount of CPN per ml plasma clot was approximately 30 ng.

The binding of CPN to fibrinogen and fibrin was investigated using surface plasmon resonance. CPN solutions (0 - 450 nM) were run over immobilized fibrinogen and fibrin. Increasing concentrations of CPN showed increasing amounts of protein bound to the fibrinogen surface (figure 3). Similar results were found for a fibrin surface (data not shown). BSA (100 nM) did not bind to immobilized fibrinogen or fibrin (data not shown). These results imply that the binding of CPN to the fibrin clot is specific and does not require another plasma component. It was not possible to perform kinetic analysis because data could not be fitted according to standard binding models due to the high amounts of protein that were bound to the fibrinogen and fibrin immobilized on the chip. Although equilibrium binding was not reached with the CPN concentrations used, we estimated an apparent Kd for complexes with fibrinogen and fibrin around 100 nM.

The physiological function of the binding of CPN to fibrinogen and a fibrin clot is not yet clear. CPN is known to inactivate bradykinin and the complement anaphylatoxins C3a, C4a and C5a [5,6]. Two case reports suggest that a CPN deficiency causes angioedema. In these cases no predisposition for bleeding was reported [14,15]. CPN cleaves C-terminal lysines and arginines from similar target proteins as activated TAFI [16,17]. However, while TAFIa is a clear fibrinolysis inhibitor the role of CPN in the inhibition of fibrinolysis has

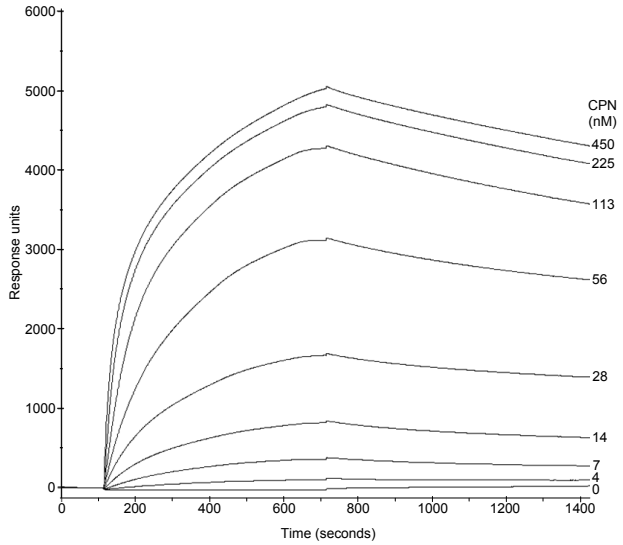

Figure 3: Binding of CPN to fibrinogen
Surface plasmon resonance sensorgrams of CPN binding to fibrinogen obtained using Biacore T100. Different amounts of CPN (0 – 450 nM) were injected over a CM5 chip containing immobilized fibrinogen. Equilibrium analysis was performed using Biacore T100 evaluation software to estimate the apparent Kd (100 nM).

not been fully established. Walker *et al*. [11] investigated the antifibrinolytic properties of CPN extensively. They concluded that CPN may be a long-term antifibrinolytic enzyme, in particular when it is proteolytically cleaved. Activated TAFI seems to be the main inhibitor of fibrinolysis but has a half-life of only 10 minutes [18]. Therefore CPN present in a fibrin clot may prolong the inhibition of fibrinolysis after TAFIa has been inactivated.

In conclusion, the data presented here suggests that the forms of CPN that are normally present in plasma bind to fibrinogen and fibrin and are present in a fibrin clot and may therefore play a role in the inhibition of fibrinolysis.

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Chapter 5

Oxidized high-density lipoprotein reduces blood clot firmness

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Submitted

Several high-density lipoprotein (HDL)-associated proteins are present in a clot prepared from plasma, suggesting that HDL particles are bound to fibrin [1]. Proteomic studies with purified HDL reveal up to 75 HDL-associated proteins which are involved in processes such as lipid metabolism, hemostasis, protease inhibition, the immune system and the complement system [2,3]. These proteins are differentially distributed over HDL subspecies that vary in size and composition [4,5]. The diverse biological functions described for HDL [6] may be mediated by the distinct HDL subspecies with their specific associated proteins. Low plasma levels of HDL are a risk factor for arterial and venous thrombosis [7–9] and thrombosis patients could have a low quantity of HDL-associated proteins in their thrombi [10]. Since it is not yet clear how HDL affects hemostasis and thrombosis the aim of this study was to further investigate this function of HDL. The role of HDL in reverse cholesterol transport is regarded as the most important function that contributes to the negative association between HDL level and arterial thrombosis. However, HDL has additional properties including anti-oxidant properties, anti-inflammatory properties, anticoagulant properties and favorable effects on endothelial function [6]. HDL is thought to be anticoagulant by acting as a cofactor for the activated protein C pathway together with protein S [11]. Another mechanism by which HDL could be anticoagulant is that phosphatidyl serine loses its procoagulant properties when incorporated into HDL particles, because the surface area of HDL is too small to accommodate the prothrombinase complex [12].

The possible function of HDL in coagulation and fibrinolysis was investigated by thromboelastometry using ROTEM® analysis. Additional methodological details and results are provided as supplementary information. We added different amounts (1–8 mg/ml) of commercially available HDL (comHDL) to the whole blood of healthy volunteers. Figure 1A shows a typical ROTEM® curve in the absence and presence of 4 mg/ml comHDL. There was no effect of comHDL on the coagulation parameters (figure 1B). The maximum clot firmness (MCF, figure 1C) and the amplitude at 30 minutes, 45 minutes and 60 minutes (figure 1D) were decreased in the presence of 4 mg/ml comHDL (n=8), indicating decreased blood clot firmness and apparent lysis. The decrease in clot firmness and the apparent lysis were concentration-dependent (data not shown).

The data presented in figure 1 were obtained with one batch of comHDL. When we tested more batches, not every batch of comHDL had a similar effect on the clot firmness. SDS-PAGE analysis of two comHDL batches that showed an effect on clot firmness and two batches that did not show any effect on clot firmness using ROTEM® analysis revealed that the effect was correlated with an apparent smear in the gels above 25 kDa (figure S1A). This smear on SDS-PAGE of comHDL was similar to the smear of oxidized HDL (oxHDL) that was prepared from native HDL (nHDL) isolated in house (figure S1B).

We also investigated the effect of nHDL and oxHDL on clot firmness using ROTEM® analysis. The clot firmness reduced during the thromboelastometry by the addition of

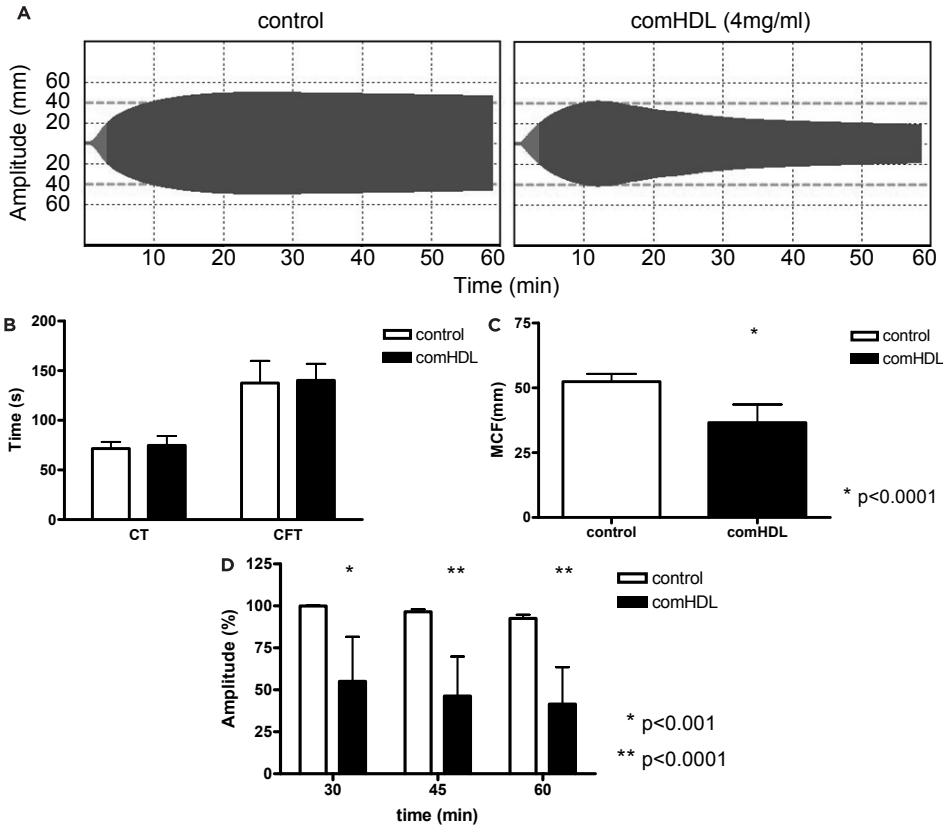

Figure 1. Thromboelastogmetry analysis of whole blood from 8 healthy subjects in the absence and presence of 4 mg/ml comHDL.

A) A typical thromboelastogram from whole blood with added comHDL (4 mg/ml) or 150 mM NaCl/0.2 mM EDTA, pH 7.4 as control. B) The mean (±SD) clotting time (CT) and clot formation time (CFT). C) The mean (±SD) maximum clot firmness (MCF). D) The mean (±SD) amplitude at 30 minutes, 45 minutes and 60 minutes.

oxHDL, but not by nHDL. The mean amplitude (±SD) at 30 minutes, at 45 minutes, and at 60 minutes decreased from 100 (±0)%, 96 (±2)% and 91 (±3)% to 63 (±25)%, 51 (±24)% and 45 (±24)%, respectively ($p<0.01$) by the addition of 4 mg/ml oxHDL (figure S2A). This decrease in clot firmness was specific for oxHDL since oxLDL did not show any effect (figure S2B). The effect on clot firmness was not inhibited by ε-aminocaproic acid (EACA) (figure S3A) and we did not measure increased fibrin degradation products after ROTEM® analysis in the presence of oxHDL (figure S3B). In addition, in the presence of cytochalasin B, which inhibits platelet function and decreases the MCF, the effect of ox-HDL was not seen (data not shown). Taken together this suggested that the decrease in clot firmness induced by oxHDL was not caused by fibrin breakdown but predominantly involved platelets.

ROTEM® analysis suggested a time-dependent inhibitory effect of oxHDL on blood clot firmness (figure 1A). During thromboelastometry platelets are activated and aggregate, as shown by the increase in clot firmness at the beginning. In the presence of oxHDL the clot firmness started to decline after about 10 minutes suggesting that platelet aggregates fall apart. This decline stopped at an amplitude of around 9 mm, which was similar to the amplitude reached when the effect of platelets was eliminated by cytochalasin B. These data suggested that the platelet contribution to the clot firmness was lost completely when oxHDL was added to the blood. Studies in the past observed similar thromboelastographic patterns and suggested either the relaxation of the retracted clot [13] or the release of the clot from the wall of the cup due to increased clot retraction [14].

HDL can inhibit platelet aggregation via binding to the scavenger receptor B type I (SR-BI) on platelets. However whether this is true for nHDL, specific HDL subfractions or oxidatively-modified HDL is still controversial [15–18]. The possible involvement of platelets was studied by investigating the effect of oxHDL on both plasma clot retraction and platelet aggregation. OxHDL (2–4 mg/ml) did not have any effect on plasma clot retraction using plasma with 150 * 10^9 platelets/l (figure S4A). Only at a low platelet concentration (75 * 10^9 platelets/l) and a high oxHDL concentration (4 mg/ml) was a minimal decrease in plasma clot retraction observed (data not shown). OxHDL as well as nHDL (2 mg/ml) slightly decreased platelet aggregation induced by thrombin, however there was no difference between nHDL and oxHDL (figure S4B). These data are in line with Valiyaveettil *et al.* [15], though other studies concluded that nHDL had no effect at all and that oxHDL can even activate platelets [16,19]. The reasons for these discrepancies are not clear. Differences in lipoprotein isolation methods, platelet agonist and oxidation conditions may play a part in the inconsistent results.

In conclusion, we did not observe any effect of nHDL on coagulation and fibrinolysis using thromboelastometry. However, oxHDL diminished the blood clot firmness by an unknown mechanism involving platelets. The small decreases in clot retraction at low platelet concentrations and in platelet aggregation could not explain the diminished clot firmness. We rejected the hypothesis that in the presence of oxHDL the clot retraction becomes stronger and results in the release of the clot from the cup wall and in that way decreases the clot firmness [14]. Unfortunately, using standard plasma clot retraction assays we could not determine the effect of oxHDL on clot relaxation. More studies are needed to elucidate the mechanism by which oxHDL diminishes the blood clot firmness.

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SUPPLEMENTARY DATA: MATERIALS AND METHODS

Blood collection

Blood was collected from healthy volunteers by venipuncture in the antecubital vein using the Vacutainer system (Becton Dickinson, Plymouth, UK) containing sodium citrate (final concentration 0.105 M). Platelet-rich plasma (PRP) was obtained by centrifugation of citrated blood for 15 minutes at 150 x g and room temperature. Platelet-poor plasma (PPP) was obtained by centrifugation of citrated blood for 15 minutes at 1500 x g and room temperature.

Lipoprotein isolation

Platelet-poor plasma was obtained by centrifugation of citrated blood for 15 minutes at 1500 x g and 4°C. Lipoprotein fractions were obtained by density-gradient ultracentrifugation in a Beckman SW41 rotor (41.000 rpm, 24 h, 12°C) according to Redgrave *et al.* [1]. The lipoprotein fractions were collected by tube slicing and dialyzed against 150 mM NaCl/ 0.2 mM EDTA, pH 7.4 and stored in the dark at 4°C.

Lipoprotein oxidation

Before oxidation, lipoprotein solutions were dialyzed against 10 mM sodium phosphate/150 mM NaCl, pH 7.4. High-density lipoprotein (HDL) was oxidized by incubation with $CuSO_4$ as described before [2] for 8 h at 37°C and the oxidation status was checked using SDS-PAGE (15%) and protein staining with a colloidal blue staining kit (Invitrogen, Paisley, UK). Low-density lipoprotein (LDL, 14–17 mg/ml) was oxidized by incubation with $CuSO_4$ at a ratio of 10 µM $CuSO_4$ to 1 mg/ml protein for 90 minutes at 37°C. Oxidation status of LDL was checked by measuring conjugated diene formation at 234 nm [3]. Oxidation was stopped by the addition of EDTA (EDTA:$CuSO_4$ ratio 50:1, mol/mol) and oxidized lipoproteins were directly dialyzed against 150 mM NaCl/0.2 mM EDTA, pH 7.4 and stored at 4°C.

ROTEM

Thromboelastometry analysis was performed using ROTEM® (TEM International GmbH, Munich, Germany) according to the manufacturer's recommendations. We examined the effect of commercially available HDL (comHDL, Merck Chemicals, Darmstadt, Germany), native HDL (nHDL), and oxidized HDL (oxHDL) by the addition of the lipoprotein (0.3 vol) to whole citrated blood (0.7 vol) using EXTEM analysis. Native LDL (nLDL) and oxidized

LDL (oxLDL) were used as a control. For other control experiments, a similar volume of 150 mM NaCl/0.2 mM EDTA, pH 7.4 was added to the blood. The following standard ROTEM® parameters were analyzed; clotting time (CT), clot formation time (CFT), maximum clot firmness (MCF), lysis index at 30 minutes (LI30), lysis index at 45 minutes (LI45) and lysis index at 60 minutes (LI60). ε-Aminocaproic acid (EACA, 1 mM final concentration) was added to investigate plasmin-mediated fibrinolysis. Cytochalasin D (2.5 µg/ml final concentration) was added to inhibit platelet function.

Detection of fibrin degradation products

Thrombi that were analyzed using thromboelastometry for approximately one hour were centrifuged for 15 minutes at 1500 x g and the obtained serum was stored at -20°C until further analysis. Fibrin degradation products were measured in these samples using a fibrinogen ELISA (Affinity Biologicals, Ancaster, ON, Canada) according to manufacturer's instructions.

Clot retraction

Different platelet concentrations were obtained by mixing PRP and PPP. A low platelet concentration of 75 * 10^9 platelets/l and a high platelet concentration of 150 * 10^9 platelets/l were used in the analysis. OxHDL and nHDL were added to plasma at concentrations of 2 mg/ml and 4 mg/ml. Plasma was incubated at 37 °C and clot retraction was analyzed in triplicate and documented by photographic images at 0, 5, 10, 15, 20, 25, 30, 40, 50, 60 and 90 minutes after the initiation of coagulation by tissue factor (Innovin, 1000 times diluted, Dade Behring, Marburg, Germany). The percentage of retraction was determined by measurement of the clot area at the different time points using Adobe Photoshop CS3.

Platelet aggregation

The platelet concentration in PRP was adjusted to 200 * 10^9 platelets/l by mixing PRP and PPP and plasma was preheated at 37 °C before analysis. Platelets were activated by bovine thrombin (1 U/ml, Siemens, Munich, Germany) and fibrin clot formation was inhibited by Pefabloc (1 mg/ml, Pentapharm, Basel, Switzerland). OxHDL and nHDL were added to plasma at a concentration of 2 mg/ml. Aggregation was measured in a dual channel Chrono-log aggregometer (Havertown, PA, USA). Maximal aggregation of the control samples was set on 100% and aggregation percentage of the samples with added oxHDL and nHDL was calculated relative to the 100% aggregation of control samples.

Statistical methods

The effect of lipoproteins on the parameters of thromboelastometry, plasma clot retraction and platelet aggregation were evaluated using the Mann-Whitney U test. All statistical analyses were performed with SPSS for Windows, version 20 (SPSS Inc, Chicago, IL, USA).

SUPPLEMENTARY REFERENCES

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SUPPLEMENTARY FIGURES

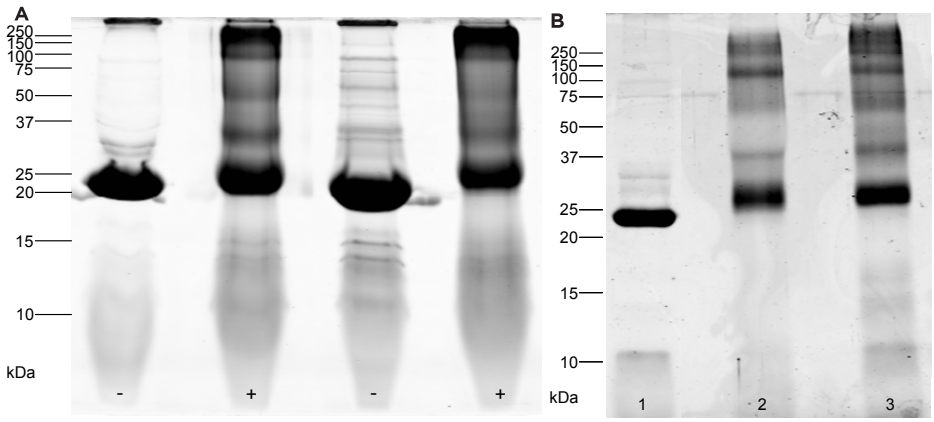

Figure S1: SDS-PAGE analysis of different HDL preparations after staining with colloidal blue.
A) Different batches of comHDL were analyzed. The comHDL batches that diminished blood clot firmness in ROTEM® analysis are indicated with a plus sign. The minus sign indicates the batches that did not show any effect. **B)** Native HDL isolated from plasma (lane 1), native HDL oxidized with $CuSO_4$ (lane 2) and comHDL (lane 3).

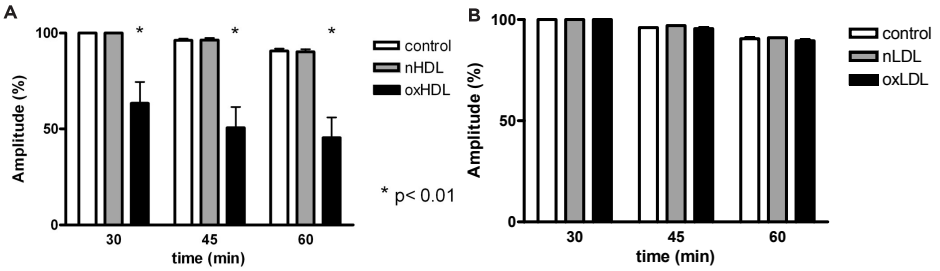

Figure S2: Effect of different HDL and LDL preparations on blood clot firmness, investigated using thromboelastometry analysis.

A) nHDL and oxHDL (4 mg/ml) were added to whole blood of 5 healthy volunteers. **B)** nLDL and oxLDL (4 mg/ml) were added to whole blood of 2 healthy volunteers. The mean amplitude (%, ±SD) at 30 minutes, 45 minutes and 60 minutes is shown. The p-value indicates the significance of the difference with the control at the same time point.

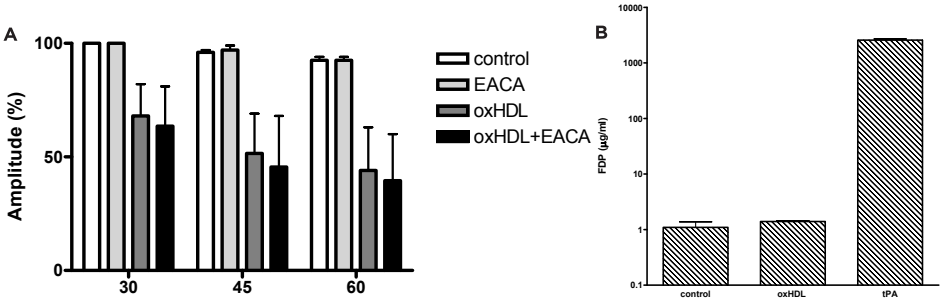

Figure S3: Effect of oxHDL on blood clot firmness and fibrinolysis.

A) Thromboelastometry analysis was performed in the presence and absence of 1 mM EACA. EACA inhibits plasmin-mediated fibrinolysis. OxHDL (4 mg/ml) was added to whole blood and compared to control in the absence and presence of EACA. The mean amplitude (%, ±SD) at 30 minutes, 45 minutes and 60 minutes is shown of 2 healthy volunteers. **B)** Fibrin degradation products (FDP) in serum were measured in duplicate using a fibrinogen ELISA. ROTEM® samples analyzed for one hour were used to measure FDP. A blood sample with added tPA (50 ng/ml) was taken as a positive control. Mean values ± SD are shown.

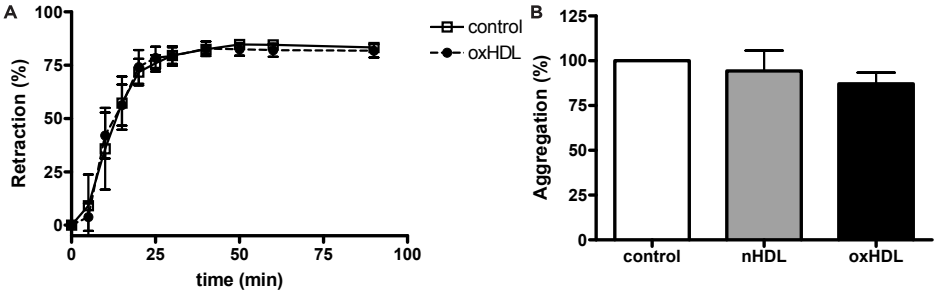

Figure S4: The effect of oxHDL on clot retraction and platelet aggregation.

A) The clot retraction of plasma with 150 * 10^9 platelets/l was analyzed in the presence and absence of 4 mg/ml oxHDL (n=2). **B)** Platelet aggregation was investigated in plasma with 200 * 10^9 platelets/l in the absence and presence of 2 mg/ml nHDL (n=7) or oxHDL (n=9). The maximal aggregation of the control sample was set on 100%. Mean values ± SD are shown.

Chapter 6

Proteomic analysis reveals that apolipoprotein AI levels are decreased in patients with Budd-Chiari syndrome

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ABSTRACT

Background and aims: Budd-Chiari syndrome (BCS) is a rare vascular liver disorder caused by thrombosis of the hepatic veins. In some patients no known thrombophilic factor can be identified. This study aims to identify novel factors that might play a role in thrombosis in BCS-patients by using a proteomic approach. *Methods:* The abundance of plasma clot-bound proteins was compared between 9 BCS-patients and 9 controls by two-dimensional difference gel electrophoresis. The protein with the most significant decrease in patients was identified with mass spectrometry. Plasma levels of this protein were measured and the results were validated in a large cohort of BCS-patients. *Results:* A total of 26 protein spots significantly differed ($p<0.001$). The spot with the most significant decrease in patients was identified by mass spectrometry as apolipoprotein AI (apo AI). The mean level of apo AI in plasma of these BCS-patients (0.74 g/l) was also significantly lower than in controls (1.45 g/l, $p= 0.002$). This finding was validated in a large cohort of 101 BCS-patients and 101 controls (0.97 g/l vs. 1.32 g/l, $p<0.0001$). There was no major correlation between plasma levels of apo AI and various liver function tests. *Conclusions:* BCS-patients show decreased clot-bound protein abundance and plasma levels of Apo AI. Decreased levels of apo AI may play a role in the etiology of thrombosis in BCS-patients and possibly in other patients with venous thrombosis.

INTRODUCTION

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

Fibrin clot formation is the final step of a complex cascade of reactions that represents the coagulation system. Dysregulation of any of the numerous components of the clotting cascade and the fibrinolytic system can potentially disrupt the hemostatic balance, leading to an increased tendency of either bleeding or thrombosis. Alterations in the plasma concentration of certain proteins influencing blood coagulation (e.g. elevated levels of factor VIII or a protein C deficiency) can therefore be involved in the onset of venous thrombosis [10,11]. Several proteins bind to fibrin as an essential step in their mechanism of action (e.g. thrombin, factor XIII, plasminogen and tissue-type plasminogen activator) [12]. Consequently, some prothrombotic abnormalities may also be reflected by changes in the concentration of proteins that bind to a plasma clot.

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

PATIENTS AND METHODS

Materials

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

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

To examine the plasma clot composition of patients with BCS and healthy controls, blood samples were collected from 9 consecutive patients with BCS admitted to the Department of Gastroenterology and Hepatology of the Erasmus Medical Center in Rotterdam, the Netherlands. For each patient a healthy, non-related control person was recruited from department personnel. Controls were of the same sex, ethnicity and age (with a range of five years) as the patient. Furthermore, controls did not have a previous history of thrombosis or malignancy and were not using oral contraceptives. Peripheral blood samples were obtained from both patients and controls by means of venapuncture and collected in tubes containing 0.11 M trisodium citrate. Platelet-free plasma was acquired by a two-step centrifugation method (10 minutes at 2,000g and 10 minutes at 20,000g) at 4°C and subsequently stored at –70 °C until further analysis.

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

Sample preparation for 2D-DIGE

Clots of 500 µl citrated plasma were prepared by adding calcium chloride (20 mM) and thrombin (1 NIH U/ml) for the initiation of coagulation and aprotinin (100 KIU/ml) to prevent proteolytic degradation [14]. After an incubation period of 2 hours at room temperature, the clots were extensively washed by permeating them with 10 ml Tris-buffered saline (50 mM Tris-HCl, 100 mM NaCl, pH 7.4) containing aprotinin (100 KIU/ml) overnight at 4°C. The clots were compacted by centrifugation, washed with deionized water and

noncovalently clot-bound proteins were extracted with 150 µl rehydration buffer (7M urea, 2M thiourea, 4% (w/v) CHAPS, 30 mM Tris-HCl, pH 8.5) for 1 hour at room temperature. Samples of 50 µl were labeled with the N-hydroxysuccinimide esters of Cy3 or Cy5 minimal fluorescent cyanine dyes. Five patient samples were labeled with Cy3 minimal dye and the other 4 patients with Cy5 minimal dye. The matched controls were labeled with Cy5 and Cy3 minimal dye, respectively. The samples were randomized to Cy3 and Cy5 labeling to minimize dye-based artifacts. Labeling was performed using 400 pmol dye per 50 µl sample, containing about 1.5 µg protein. Samples were labeled on ice for 30 minutes and quenched with 0.2 mM lysine. A pool of all 9 patient and 9 control samples was prepared and 50 µl was labeled with Cy2 minimal dye. This sample was used as an internal standard. For 2D-gel electrophoresis, each labeled patient sample was pooled with the labeled matched control sample and the labeled internal standard and analyzed simultaneously to reduce gel-to-gel variations. The total volume of sample was adjusted to 185 µl with rehydration buffer, 0.9% IPG-buffer 3–10 pH range and 1.2% (v/v) DeStreak.

2D-DIGE

The 9 pools containing the labeled samples of the BCS-patients, their matched controls and the internal standard were run on 9 different gels. The presence of the internal standard in each pool facilitated gel-to-gel matching. The proteins were separated in the first dimension with a 17 cm immobiline drystrip with a 3–10NL pH range. The isoelectric focusing was carried out at 20°C on the IPGphore II with the following running protocol: 12 h at 30 V (rehydration), 1 h at 500 V, 1 h at 1000 V, and 60000 Vh at 8000 V, with a 50 µA limit per gel.

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

Image acquisition and analysis

After 2D-DIGE separation, gels were scanned using Typhoon 9410 (Amersham Pharmacia Biotech) at 100-micron resolution. 2-D images from the Cy2-, Cy3-, and Cy5-labeled protein fractions were scanned using a 488, 532 and 633 nm laser, respectively. Gel images were cropped using ImageQuant TL software and image analysis was performed with Decyder V6.5 software (Amersham Pharmacia Biotech). Spot detection was performed using the differential in-gel analysis (DIA) module by setting 7500 as estimated number of spots. The Cy2, Cy3, and Cy5 images of each gel were merged and spot boundaries

were detected. Spots resulting from non-protein source, like dust particles, were filtered out by removing spots with a slope greater than 1.3. The gel with the highest spot-count was assigned as the master gel, which was used as a template. Gel-to-gel matching of the standard spot maps of each gel was performed using the biological variation analysis (BVA) software module to ensure that the same protein spots were compared between gels. Normalized Cy3 and Cy5 spot volumes were compared to the corresponding Cy2 standard spot volume within each gel, which gave a standardized abundance.

Mass spectrometry

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

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

Plasma samples from large case-control study

To validate findings from the proteomic study in a larger case-control population, we used plasma samples from the EN-Vie Study. The EN-Vie Study, as described previously, is a prospective multicenter observational study of patients with BCS [8,15]. During the study period of two years a total of 163 newly diagnosed patients with BCS were included from nine European countries. Apart from recorded data on clinical parameters, underlying etiology and treatment outcome, blood samples were also collected. Furthermore, each enrolled

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

The EN-Vie Study was conducted with approval from all national and, if necessary, local ethical committees, in accordance with the nation-specific rules. All patients and controls agreed to participate in the study by means of a written informed consent.

For this case-control study, only patients for whom stored plasma samples and a matched control person were available were considered eligible.

Measurement of apolipoprotein AI and HDL cholesterol levels in plasma

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

Miscellaneous methods

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

Statistical analyses

The protein abundance in plasma clots of patients with BCS and controls was compared using the Student's T-Test within the Decyder software. Differences in apo AI levels in plasma were tested using the non-parametric Mann-Whitney U Test for the 9 BCS pa-

tients or the Student's T-Test for the larger cohort of BCS-patients from the EN-Vie study. Spearman's Rho correlation coefficients were calculated as a non-parametric measure of correlation between apo AI levels and liver function tests. A p-value of <0.05 was considered statistically significant. All statistical analyses were performed with the Statistical Package for Social Sciences for Windows, version 15.0 (SPSS, Chicago, IL).

RESULTS

Plasma samples used for *in vitro* clot formation were collected from nine patients with BCS, 7 females and 2 males, with a median age at diagnosis of 35 years (range 16–54 years). Underlying etiologic factors were myeloproliferative disorders (n=4), homozygous Factor V Leiden mutation (n=1), antiphospholipid antibodies (n=3) and oral contraceptives (n=2). In two patients no risk factors could be identified and in three patients two prothrombotic factors were present. The median age of the controls (7 females and 2 males) was 31 years (range 22–49 years).

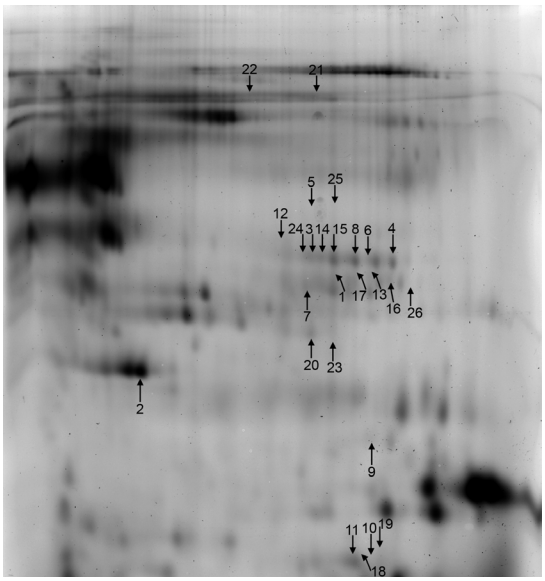

Figure 1. Image of the master gel of 2D-DIGE, displaying proteins that significantly differed in abundance between BCS-patients and controls, as analyzed with Decyder software.
The arrows on the 10.5% polyacrylamide gel indicate the 26 protein spots that are statistically significantly (p<0.001) different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

Differences in plasma clot composition between BCS-patients and controls

After *in vitro* plasma clot formation, the protein composition of clots from patients with BCS was compared to that of plasma clots from controls. On the master gel, 1369 different protein spots were detected after 2D-DIGE analysis. All other gels of case-control pairs were matched and compared with the master gel and this resulted in the detection of 26 protein spots that statistically significantly ($p<0.001$) differed in abundance between patients and controls (Fig. 1). The protein spot with the most significant decrease ($p=6.6 \times 10^{-5}$) was spot number 2 (Table 1). The mean value for the standardized abundance of patient samples for this spot was 0.70 as compared to 1.65 for the controls, corre-

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

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

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

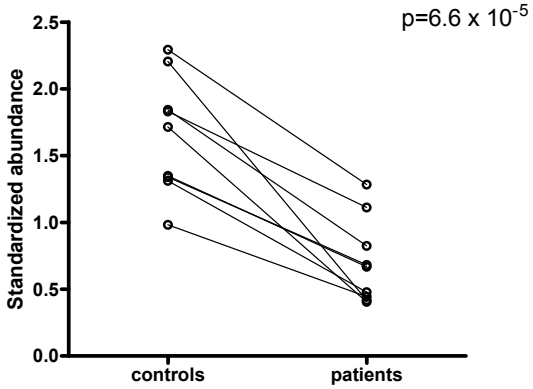

Figure 2. Protein abundance of apolipoprotein AI of BCS-patients and their matched controls. Statistical analysis was performed using the BVA module of DeCyder software and shows on average a 2.4 fold lower abundance for apolipoprotein AI in the patient plasma clots (mean abundance of 0.70) compared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

sponding to a 2.4-fold lower abundance in patients samples (Fig. 2). This protein spot was excised from preparative gels and analyzed using MALDI-TOF-MS. The protein was identified as apolipoprotein AI (apo AI) with the following identification details; NCBI gi|90108664, mass 28061Da, Mascot score 76–237, number of peptides matched 18–24, sequence coverage (%) 54–81, with a p-value between 3e-19 and 0.0036 (n=4). The other 25 spots with a statistically significant difference in protein abundance between patients and controls have not yet been identified.

Plasma levels of apo AI and HDL cholesterol

The low abundance of apo AI in plasma clots of BCS-patients was also seen in plasma levels. Mean (± SD) plasma level of apo AI in these 9 cases was significantly lower than in the controls, 0.74 (± 0.21) g/l vs. 1.45 (± 0.31) g/l, respectively (p=0.002), corresponding to a 2.0 fold difference in plasma levels.

To validate these findings we used plasma samples from the EN-Vie Study cohort. From this study, plasma samples for measurement of apo AI levels were available for 107 patients. However, for six patients there was no control person and these patients were excluded from the analysis, leaving 101 eligible case-control pairs. Mean age of the patient population was 38 years (range 16–83) and 42% were males. Results from the apo AI assay are shown in Fig. 3A. Patients with BCS had significantly lower apo AI levels in plasma as compared to the controls (mean ± SD 0.97 ± 0.36 g/l vs. 1.32 ± 0.24 g/l, p<0.0001). The pattern of the patient values might suggest the existence of a subgroup with low levels of apo AI. When the group of patients with apo AI levels below 0.85 g/l were compared to those with higher apo AI levels, it became clear that the subgroup of

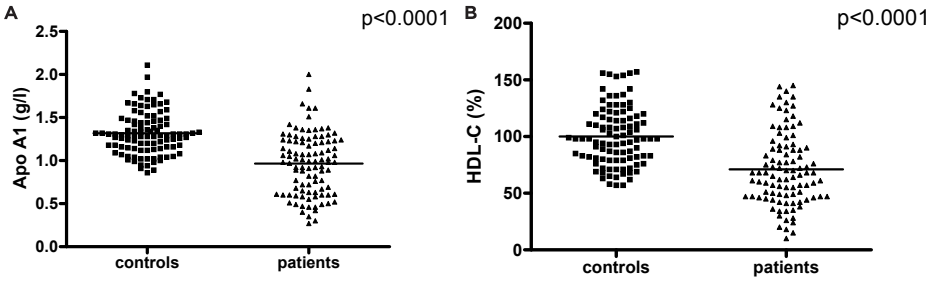

Figure 3. Apolipoprotein AI levels (A) and HDL cholesterol levels (B) in plasma of patients with Budd-Chiari syndrome and healthy controls.
Individual data points and mean values are given.

cases with lower apo AI levels included relatively more patients with a myeloproliferative disorder (20 of 36 (56%) vs. 15 of 63 (24%), respectively, p=0.001). No other differences in underlying etiology of thrombosis or characteristics at diagnosis were found between these two apparent subgroups.

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

Apo AI levels and liver function tests

Because apo AI is synthesized by the liver, an impaired liver function may influence the plasma levels. To estimate the effect of decreased hepatic synthetic function on apo AI levels in patients with BCS, we determined the correlation between apo AI and different parameters of liver function in the EN-Vie study cohort. As shown in Table 2, there was an association between apo AI levels and several parameters of liver function. However, the correlations were only weak, with correlation coefficients lower than 0.35. Furthermore, when we compared the apo AI levels of cases with normal albumin levels (>35 g/l, n=34) to their matched controls, BCS-patients still had significantly lower apo AI levels than their healthy controls (1.08 ± 0.36 g/l vs. 1.31 ± 0.23 g/l, p=0.002) (Fig. 4). To assure that

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

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

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

albumin levels were not artificially elevated, we assessed whether patients had received prior albumin infusion. None of the cases with albumin levels in the normal range had been treated with albumin.

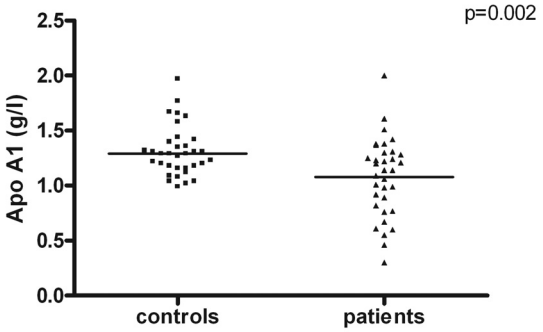

Figure 4. Apolipoprotein AI levels in plasma of cases with normal albumin levels (>35g/l; n=34) compared to their healthy controls.
Individual data points and mean values are given

DISCUSSION

In this study we have used a proteomic approach to investigate differences in proteins bound to a plasma clot in order to detect novel players in the regulation of hemostasis that may be associated with the development of venous thrombosis in BCS. To our knowledge this is the first report on analysis of plasma clot proteins in patient samples using proteomics as a detection method. Previously we have shown that this technique could be used to identify new fibrin-binding proteins in plasma [14]. In 2D-DIGE an internal standard is run on each gel together with a patient sample and a control sample. This allows a direct comparison of protein abundance between series of samples without interference by gel-to-gel variation. Using 2D-DIGE, 9 samples of patients with BCS were compared with samples of their matched controls. A total of 26 protein spots significantly differed ($p<0.001$) in abundance between cases and controls and were either increased or decreased in the patient samples. The protein spot displaying one of the most significant differences between both groups was identified with mass spectrometry as apo AI. The other 25 protein spots with a different abundance in cases and controls still need to be identified. The standardized protein abundance of apo AI was reduced by a factor 2.4 in BCS-patient samples. A similar reduction was found in the plasma concentration of apo AI in these 9 patients. The findings of the first part of this study were confirmed in a validation study by measuring the concentration of apo AI in plasma in a large cohort of 101 BCS-patients. Apo AI was found to be significantly decreased compared to the healthy,

matched controls. HDL cholesterol levels were also found to be decreased in patients compared to controls, and correlated well with Apo AI plasma levels as seen before [16].

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

A low apo AI level has previously been reported as a marker of liver fibrosis [18,19]. Therefore, we cannot exclude that fibrosis might partially explain the low levels of apo AI found in BCS-patients. Nevertheless, we found no association between apo AI levels and the presence of fibrosis and/or cirrhosis in a subgroup of 26 BCS-patients of whom a liver biopsy sample was available (data not shown). Hence, we believe that low levels of apo AI, next to other risk factors, might play a causal role in the development of venous thrombosis in BCS, in addition to being a consequence of venous thrombosis. It is of interest that apo AI levels appeared to be particularly low in BCS-patients with an underlying myeloproliverative disorder. It has been shown that in myeloproliferative disorders, which represent a major risk factor for BCS, HDL levels can be markedly decreased due to an increased catabolism of apo AI [20]. Further studies are needed to clarify this association and its potential role in the development of venous thrombosis.

The mechanism through which decreased levels of apo AI could result in venous thrombosis in patients with BCS is not yet clear. Apo AI is the main protein component of the reverse cholesterol transporter HDL. For arterial thrombosis plasma levels of HDL are known to be inversely related with risk [21]. For venous thrombosis the role of HDL and apo AI is less clear. However, there are strong indications that the same inverse relation

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

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

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APPENDIX

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Chapter 7

Evidence for an enhanced fibrinolytic capacity in cirrhosis as measured with two different global fibrinolysis tests

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ABSTRACT

Background & Objectives: It has been known for a long time that cirrhosis is associated with hyperfibrinolysis, which might contribute to an increased risk and severity of bleeding. However, recent papers have questioned the presence of a hyperfibrinolytic state in cirrhotic patients and postulated a rebalanced system due to concomitant changes in both pro- and anti-fibrinolytic factors. Therefore we re-investigated the fibrinolytic state of cirrhotic patients using two different overall tests including a recently developed test for global fibrinolytic capacity (GFC) using whole blood. *Patients and Methods:* Blood was collected from 30 healthy controls and 75 patients with cirrhosis of varying severity (34 Child-Pugh A, 28 Child-Pugh B and 13 Child-Pugh C). The plasma clot lysis time (CLT), which is inversely correlated with fibrinolysis, was determined as well as the GFC. *Results:* The mean CLT was 74.5 min in the controls and decreased significantly to 66.9 min in Child-Pugh class A patients, 59.3 min in class B patients and 61.0 min in class C patients, and hyperfibrinolysis existed in 40% of the patients. The median GFC was 1.7 μg/ml in the controls and increased significantly to 4.0 μg/ml in Child-Pugh class A patients, 11.1 μg/ml in class B patients and 22.5 μg/ml in class C patients, and hyperfibrinolysis existed in 43% of the patients. Taken together, 60% of the patients showed hyperfibrinolysis in at least one of the two global assays. *Conclusion:* A rebalanced fibrinolytic system may occur, but hyperfibrinolysis is found in the majority of patients with cirrhosis.

INTRODUCTION

Liver cirrhosis is associated with an increased bleeding tendency. One of the underlying mechanisms is an impaired hemostatic system, because the liver is responsible for the synthesis as well as for the clearance of several hemostatic factors. An important component of hemostasis is the fibrinolytic system [1]. It has been known for a long time that this system may be disturbed in cirrhosis leading to the enhancement of fibrinolysis [2] by a shift in balance between pro-fibrinolytic and anti-fibrinolytic factors [3]. This shift in balance is observed at the level of plasminogen activation, i.e. the balance between tissue-type plasminogen activator (tPA) and plasminogen activator inhibitor–1 (PAI-1), as well as at the level of plasmin, i.e. the balance between plasmin(ogen) and α_2-antiplasmin. A more recently discovered major regulator of the fibrinolytic system is thrombin-activatable fibrinolysis inhibitor (TAFI) which is also synthesized in the liver [4]. Lisman *et al*. [5] found that this inhibitor is significantly decreased in cirrhosis. However, global fibrinolysis, as observed in a plasma clot lysis system, appeared not to be increased in their study. This was explained by strongly reduced antithrombin levels associated with a relatively high thrombin generation in the assay and a concomitant higher proportion of TAFI activation, which might compensate for the reduced TAFI levels. Because the plasma clot lysis assay is considered to be superior to the older fibrinolysis assays used for the detection of accelerated fibrinolysis, the existence of hyperfibrinolysis in cirrhosis was questioned [5] and it was postulated that the fibrinolytic system is rebalanced in patients with cirrhosis by concomitant changes in both pro- and antifibrinolytic factors [6]. Colucci *et al*. [7] confirmed the reduced TAFI levels in cirrhotic patients, but these authors did find increased fibrinolysis in a similar plasma clot lysis assay. The background to this controversy is still unclear. New global tests are needed to establish whether or not a true hyperfibrinolytic state exists in cirrhosis. [8,9].

The status of the fibrinolytic system cannot be satisfactorily assessed by measuring levels of individual components, because the system, as indicated above, strongly depends on the balance of various pro-fibrinolytic and anti-fibrinolytic factors. The system also depends on the balance of various pro-coagulant and anti-coagulant factors that regulate amongst others the activation of TAFI. We recently developed a new test for the global fibrinolytic capacity in undiluted whole blood [10]. In this test, in contrast to the plasma clot lysis assay, no exogenous tPA is added, and platelets as well as other blood cells are present to mimic better the *in vivo* situation in circulating blood. To address the question of whether hyperfibrinolysis is present in cirrhotic patients, we used this test, as well as the plasma clot lysis assay, to investigate the fibrinolytic system in 75 patients with cirrhosis of varying severity and in 30 healthy controls. This study was performed within the framework of our studies on the hemostatic and thrombotic complications of liver diseases [11–13].

PATIENTS AND METHODS

Patients

We studied 75 consecutive ambulatory and hospitalized patients with different types of etiology of cirrhosis and 30 healthy controls. The diagnosis of cirrhosis was histologically proven or clinically proven by a radiological examination of the liver. Patients were classified into 3 groups of increasing severity (A to C) according to the Child-Pugh's score [14]. In primary sclerosing cholangitis (PSC) and primary biliary cirrhosis (PBC), the bilirubin references were changed to reflect the fact that these diseases feature high conjugated bilirubin levels. The upper limit for 1 point was 70 μmol/l and the upper limit for 2 points was 170 μmol/l in the calculation of the Child-Pugh scores of these patients [14]. The severity of the disease was also assessed by calculating the MELD score according to the formula 9.57 x ln(creatinine) + 3.78 x ln(bilirubin) + 11.2 x ln(INR) + 6.43 [15].

In order to further characterize the patients with respect to bleeding, esophageal varices were classified retrospectively as small-sized (n=12 patients), moderate-sized (n=9), large-sized (n=11) and varices with red signs (n=1). A number of patients (n=18) had no varices and information of 24 patients was not available. Four patients developed a gastrointestinal bleeding, as defined by Violi *et al*. [16], in a follow-up period of one year.

Exclusion criteria were malignancy, anticoagulant treatment or the use of antiplatelet drugs, pre-existing bleeding disorders, a history of venous thrombosis, pregnancy, recent platelet substitution or plasma substitution (<1 week prior to inclusion), or recent bleeding episodes (<1 week prior to inclusion). The control group consisted of 30 healthy subjects with no clinical evidence of any history of liver disease. They were recruited from friends, neighbours or partners of the patients.

This study was performed in accordance with the guidelines for Good Clinical Practice/ ICH, the principles of the Declaration of Helsinki 1964, as modified by the 52nd WMA General Assembly, Edinburgh, Scotland, October 2000, and the local national laws governing the conduct of clinical research studies. The Medical Ethical Committee of the Erasmus MC approved the study protocol. All participants gave written informed consent.

Blood collection

Blood was collected by venipuncture after minimal stasis in the antecubital vein in standard citrated tubes (Vacutainer, Becton Dickinson, Franklin Lakes, NJ, USA) or Stabilyte tubes (0.45 M citrate pH 4.3; Trinity Biotech, Bray, Ireland) and centrifuged at 2500 g for

15 minutes at 4° C. Plasma was collected and stored in aliquots at -80° C. Blood for the global fibrinolytic capacity test [10] was collected in 3 ml Vacutainer tubes containing 1.4 NIH units of thrombin (Stat chemistry tubes, Becton Dickinson) supplemented with or without 60 µl 10,000 KIU/ml aprotinin (Bayer, Leverkusen, Germany) before blood collection.

Plasma clot lysis assay

The plasma clot lysis assay was performed essentially as described previously [17]. Citrated plasma was diluted 1.7 times in assay buffer (25 mM Hepes, 137 mM NaCl, 3.5 mM KCl, 1% (w/v) BSA, pH 7.4). The diluted plasma (85 µl) was added to wells of a microtitre plate containing 15 µl of a reaction mixture. The reaction mixture contained the following components with final concentrations in the clot: tissue factor (Innovin, 1000 times diluted; Dade Behring, Liederbach, Germany), $CaCl_2$ (17 mM), tPA (25 ng/ml, Actilyse, Boehringer Ingelheim, Germany) and phospholipid vesicles (10 µM). After mixing the diluted plasma with the reaction mixture, each well was covered with 50 µl paraffin oil and the microtitre plate was placed into the preheated chamber of the microtitre plate reader. The optical density at 405 nm was measured every minute for 300 minutes at 37°C. The clot lysis time (CLT) was the time from the midpoint of clear to maximum turbidity, which represents clot formation, to the midpoint of maximum turbidity to clear transition, which represents clot lysis. The assay was performed in duplicate. Results of three patients (one of each Child-Pugh class) were missing.

Global fibrinolytic capacity test

The test of the global fibrinolytic capacity (GFC) was performed essentially as described previously [10]. The thrombin-containing blood collection tubes were immediately incubated for 3 hours at 37°C. After this incubation period the clots were released from the tube wall with a plastic spatula. The tubes were centrifuged at 4°C at 2500 g for 15 minutes. An aliquot of 500 µl serum was collected and mixed with 10 µl of aprotinin (10,000 KIU/ml) to block plasmin activity. Serum samples were stored at -80°C. The fibrin degradation products (FnDPs) were measured using a latex agglutination assay (Auto Dimer, Biopool) on a Sysmex CA-1500 Analyzer. The GFC was calculated by subtracting the FnDP concentration in the thrombin-containing Vacutainer tubes with aprotinin from the FnDP concentration in the thrombin-containing Vacutainer tubes without aprotinin. The assay was performed in duplicate and the results were expressed in µg/ml.

Other hemostasis assays

The fibrinogen concentration was determined with a clotting rate assay according to Clauss [18] using thrombin from Dade Behring (Dade Thrombin Reagent). The plasminogen concentration was determined by a chromogenic substrate method using streptokinase (Kabikinase from Pharmacia, Woerden, the Netherlands) and the substrate S-2251 (Chromogenix, Instrumentation Laboratory, Milano, Italy). The α_2-antiplasmin concentration was determined by a chromogenic substrate method using the COAMATIC Plasmin Inhibitor kit (Chromogenix). The antithrombin concentration was determined by a chromogenic substrate method using the COAMATIC Antithrombin kit (Chromogenix). The results of the latter three assays were expressed in U/ml (pooled normal plasma contained 1 U/ml). Levels of tPA antigen in plasma were assayed by a slightly modified commercially available enzyme-linked immunosorbent assay (t-PA Antigen Elisa Reagent Kit, Technoclone, Vienna, Austria). The functional assay of TAFI was performed using a clot lysis assay as described previously and expressed in minutes [19]. The activity levels of t-PA and PAI-1 were determined in Stabilyte plasma using the bioimmunoassays Chromolize t-PA activity and Chromolize PAI-1 activity (Trinity Biotech). All other assays were performed in citrated plasma.

Statistics

Statistical analysis was performed using the GraphPad Prism version 4 software package (GraphPad Software, San Diego, Ca, USA). Parametric analysis was performed using a one-way ANOVA with Bonferroni's post test and non-parametric analysis using the Kruskall-Wallis ANOVA test with Dunn's post test. Correlations were determined by calculation Spearman's rho. P-values of <0.05 were considered statistically significant.

RESULTS

The existence of hyperfibrinolysis was studied in a group of 75 cirrhotic patients and 30 healthy controls. As shown in Table 1, the patients had different types of etiology and were divided into Child-Pugh class A (N=34), class B (N=28) and class C (N=13). The MELD score increased in accordance with the Child-Pugh classification. Table 2 summarizes the results of a series of hemostatic factor assays performed in plasma samples. In agreement with the literature, levels of fibrinogen, antithrombin and plasminogen decreased in cirrhosis, whereas tPA activity and tPA antigen increased. In addition, levels of α_2-antiplasmin activity and TAFI activity decreased, whereas PAI-1 activity did not change significantly. All changes correlated with the severity of the disease.

Table 1. Demographic characteristics of the study population. The cirrhotic patients are grouped according to Child-Pugh class A, B or C.

	Controls	Class A	Class B	Class C
N	30	34	28	13
Male:Female	15:15	19:15	22:6	7:6
Age in years (mean ± SD)	50 ± 13	50 ± 13	51 ± 11	55 ± 10
Etiology				
Hepatitis B		6	4	1
Hepatitis C		10	0	1
Autoimmune		4	3	1
PSC[1]		5	2	0
PBC[2]		1	1	2
Hemochromatosis		1	2	0
Alcohol		7	14	6
Cryptogenic		0	2	2
MELD score (median and range)		5.9 (0.7-14.1)	12.1 (7.6-21.1)	16.4 (9.7-41.9)

[1]PSC=primary sclerosing cholangitis
[2]PBC=primary biliary cirrhosis

Table 2. Hemostatic parameters of the study population. The cirrhotic patients are grouped according to Child-Pugh class A, B or C.

	Controls (N=30)	Class A (N=34)	Class B (N=28)	Class C (N=13)	P-value
Factor assays					
Fibrinogen (mg/ml)	3.10 (2.75-3.40)	3.30 (2.60-3.75)	2.70 (2.10-3.35)	2.00 (1.55-2.70)**	0.0010
Antithrombin (U/ml)	1.07 (1.00-1.15)	0.84 (0.63-0.92)***	0.46 (0.40-0.60)***	0.28 (0.19-0.41)***	<0.0001
Plasminogen (U/ml)	1.01 ± 0.16	0.81 ± 0.18***	0.57 ± 0.13***	0.44 ± 0.12***	<0.0001
tPA activity (IU/ml)	0.72 (0.51-1.04)	0.94 (0.70-1.24)	1.52 (0.88-2.00)**	1.93 (0.79-2.02)**	0.0010
tPA antigen (ng/ml)	4.6 (3.4-6.8)	7.8 (4.4-14.7)	18.7 (10.7-30.6)***	36.3 (20.7-39.0)***	<0.0001
α_2-Antiplasmin activity (U/ml)	1.09 ± 0.09	0.91 ± 0.13***	0.70 ± 0.13***	0.50 ± 0.16***	<0.0001
PAI-1 activity (IU/ml)	6.59 (2.18-11.59)	4.21 (1.98-9.90)	3.06 (1.67-8.61)	7.26 (1.63-16.40)	>0.05
TAFI activity (min)	11.2 ± 2.2	10.0 ± 2.4	6.5 ± 3.2***	5.2 ± 2.0***	<0.0001
Global assays					
Plasma clot lysis time (min)	74.5 ± 9.6	66.9 ± 8.3*	59.3 ± 10.6***	61.0 ± 14.9**	<0.0001
Global fibrinolytic capacity (µg/ml)	1.7 (0.8-3.5)	4.0 (1.6-13.0)	11.1 (2.3-31.9)***	22.5 (3.1-89.6)**	<0.0001

Data are presented as mean ± SD (normal distributions) or median with 25th and 75th percentile (non-normal distributions). The P-values were calculated using one-way ANOVA or by Kruskal-Wallis test. The P-values for the comparisons with controls were calculated using Bonferroni's multiple comparison test or with Dunn's multiple comparison test, respectively. * P<0.05; ** P<0.01; *** P<0.001

Table 2 and Fig. 1 show the results of the plasma clot lysis assay in our study population. The clot lysis time (CLT) decreases as fibrinolysis increases. The CLT (mean ± SD) was 74.5 ± 9.6 min in the controls and decreased significantly to 66.9 ± 8.3 min in Child-Pugh class A patients, 59.3 ± 10.6 min in class B patients and 61.0 ± 14.9 min in class C patients. The CLT also correlated significantly with the MELD score of the patients (rho -0.40, P=0.001). Hyperfibrinolysis, defined as a fibrinolytic state with a CLT below the lowest CLT value of the 30 controls (i.e. below 61.0 min) existed in 21% of class A patients, in 52% of class B

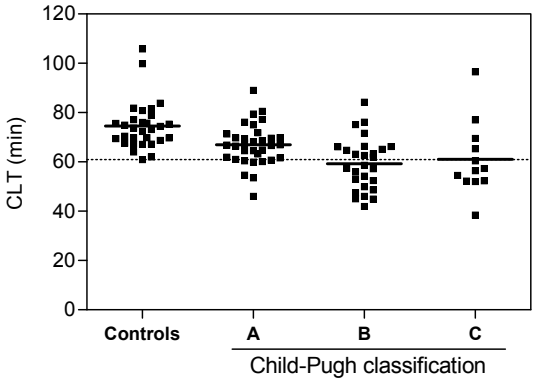

Figure 1. The plasma clot lysis time (CLT) of the study population.
The cirrhotic patients are grouped according to Child-Pugh class A, B or C. Mean values are indicated by horizontal lines and the lowest CLT of the controls (61.0 min) by a dotted line. Patients below this dotted line are considered as hyperfibrinolytic in this assay.

patients and in 67% of class C patients (Fig.1). Our results showed that hyperfibrinolysis, as measured with a plasma clot lysis assay, existed in 40% of patients with cirrhosis.

The existence of hyperfibrinolysis in cirrhosis was also studied using a new global fibrinolysis assay in whole blood (Table 2 and Fig. 2). The global fibrinolytic capacity (GFC) increases with intensity of fibrinolysis. The GFC (median with 25th and 75th percentile) was 1.7 (0.8–3.5) μg/ml in the controls and increased gradually to 4.0 (1.6–13.0) μg/ml in Child-Pugh class A patients, 11.1 (2.3–31.9) μg/ml in class B patients and 22.5 (3.1–89.6) μg/ml in class C patients. The GFC also correlated significantly with the MELD score of the patients (rho 0.41, P<0.001). Hyperfibrinolysis, defined as a fibrinolytic state with a GFC above the highest GFC value of the 30 controls (i.e. above 11.4 μg/ml) existed in 29% of class A patients, in 50% of class B patients and in 62% of class C patients (Fig. 2). These results show that hyperfibrinolysis, as measured with the new global fibrinolysis assay, existed in 43% of patients with cirrhosis.

Male and female controls did not significantly differ in CLT and GFC (p=0.35 and 0.14, respectively, Mann-Whitney test). Fibrinolytic differences between patients and controls could therefore not be ascribed to the overrepresentation of male subjects in the patient groups (Table 1).

Table 3 show correlations between the global fibrinolytic capacity (GFC) values and other hemostatic parameters in plasma of both controls and cirrhotic patients. In controls, the GFC was positively correlated with tPA activity, which is in line with our previous work [10]. The correlation coefficients were negative for the fibrinolysis inhibitors a_2-antiplasmin activity, PAI-1 activity and TAFI activity, but these correlations were not statistically signifi-

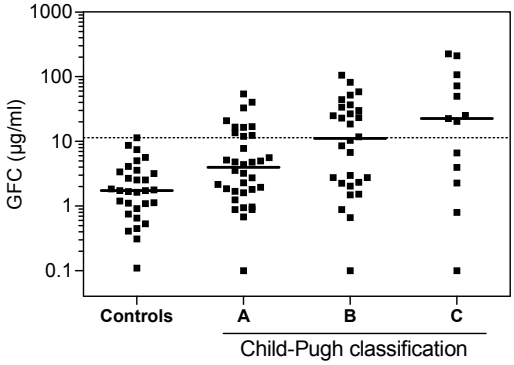

Figure 2. The global fibrinolytic capacity (GFC) of the study population, as measured in whole blood.
The cirrhotic patients are grouped according to Child-Pugh class A, B or C. Median values are indicated by horizontal lines and the highest GFC of the controls (11.4 µg/ml) by a dotted line. Patients above this dotted line are considered as hyperfibrinolytic in this assay.

cant. The correlation between the GFC and tPA activity in patients was even stronger than in the controls suggesting a causal role of increased tPA activity in hyperfibrinolysis. The GFC in patients was also strongly correlated, in a negative manner, with PAI-1 activity. This relationship might also be causal, because PAI-1 was not dependent on cirrhosis (Table 2).

The results of the two global fibrinolysis assays did not correlate in the controls (Table 3). They did, however, moderately correlate in the patients ($r = -0.46$, $P<0.001$). The scatter diagrams in Fig. 3 further demonstrate this correlation. This figure also illustrates that in the patient group 29/72 (40%) showed no hyperfibrinolysis in any of the two global assays, 11/72 (15%) showed hyperfibrinolysis only in the plasma clot lysis assay, 14/72 (19%) showed hyperfibrinolysis only in the global fibrinolysis assay in whole blood and 18/72 (25%) showed hyperfibrinolysis in both assays. This implies that 43/72 (60%) of the patients exhibited hyperfibrinolysis in at least one of the two global assays. The group of

Table 3. Correlations between the global fibrinolytic capacity (GFC) values and other hemostatic parameters in controls and cirrhotic patients. Spearman's rho is given along with the P-value.

	Controls (N=30)		**Patients (N=75)**	
Factor assays	rho	P-value	rho	P-value
Fibrinogen	0.04	0.85	-0.07	0.55
Antithrombin	0.09	0.65	-0.33	0.004
Plasminogen	0.09	0.64	-0.29	0.01
tPA activity	0.54	0.002	0.79	<0.001
tPA antigen	0.33	0.07	0.19	0.11
α_2-Antiplasmin activity	-0.25	0.18	-0.32	0.005
PAI-1 activity	-0.32	0.09	-0.60	<0.001
TAFI activity	-0.07	0.73	-0.32	0.006
Global assay				
Plasma clot lysis time	-0.25	0.18	-0.46	<0.001

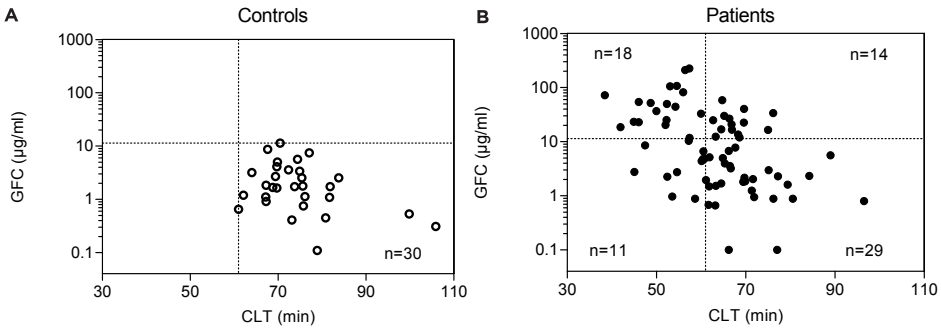

Figure 3. Scatter diagrams showing correlations between the global fibrinolytic capacity (GFC) and plasma clot lysis time (CLT) in the controls (upper panel) and the cirrhotic patients (lower panel) of the study population.
The dotted lines indicate the borders of the control group in the two assays. Correlation coefficients are given in Table 3.

29 patients not showing hyperfibrinolysis in any of the global assays, even included three severely ill patients with Child-Pugh C. The absence of hyperfibrinolysis in two of them (etiology: cryptogenic and hepatitis C, respectively), could be explained by their strongly increased PAI-1 levels (36 and 46 IU/ml, respectively) and correspondingly decreased tPA activities (0.19 and 0.25 IU/ml, respectively). As mentioned previously, high PAI-1 levels are not typical of cirrhosis, but they may occasionally occur (Table 2).

DISCUSSION

Only a limited number of studies have so far been performed on the relationship between abnormalities in fibrinolysis and bleeding in cirrhosis. Violi *et al.* observed that high D-dimer levels and high t-PA activity were associated with an increased risk of gastrointestinal bleeding [16]. However the association between hyperfibrinolysis and bleeding remains controversial. There is, in general, a relatively poor correlation between bleeding in cirrhosis and the outcome of conventional hemostasis tests [8,20]. One reason is that these tests, including the commonly used fibrinolysis tests, might not sufficiently reflect the complex balance between hemostatic factors that exists *in vivo* [8,21,22]. Although hyperfibrinolysis in cirrhosis seems to be a well established phenomenon, serious concern exists about the reliability of the fibrinolysis tests used [5]. In this study we confirmed, however, the existence of hyperfibrinolysis in a significant proportion (60%) of patients with cirrhosis by using both the plasma clot lysis assay and a new global fibrinolysis assay in undiluted whole blood [10].

In order to obtain a deeper insight into the hemostatic characteristics of the patients in our study and to compare them with other populations described in the literature, we

tested the most relevant fibrinolytic factors in plasma. In agreement with the literature, both pro-fibrinolytic (plasminogen) and anti-fibrinolytic (α_2-antiplasmin, TAFI) factors synthesized by the liver, strongly decreased in cirrhosis. The activity of PAI-1, synthesized by a variety of hepatic and non-hepatic cell types, did not change, while tPA activity and tPA antigen, primarily synthesized by the vascular endothelium, significantly increased. The increase in tPA levels is usually ascribed to decreased liver clearance [23]. Clearance of tPA involves both liver endothelial cells and liver parenchymal cells after recognition by the mannose receptor and low-density lipoprotein receptor-related protein, respectively [24–26]. Urokinase-type plasminogen activator, not specifically measured in this study but potentially involved in the global tests, might also be increased in cirrhosis [27].

To measure the overall effect of the changes in plasma levels of known and possibly unknown fibrinolysis factors we applied the global plasma clot lysis assay. In contrast to Lisman *et al*. [5], but in agreement with Colucci *et al*. [7], a significant decrease in clot lysis time was observed pointing to an increase in plasma fibrinolytic potential in cirrhotic patients. The antithrombin levels were significantly reduced in our cirrhotic patients, which might have resulted in more thrombin and more TAFI activation during the assay. However, this anti-fibrinolytic effect was apparently overruled by the pro-fibrinolytic effects in cirrhosis. In addition, more thrombin might also promote fibrinolysis by the so-called coagulation-associated enhancement of fibrinolysis based on thrombin-dependent inactivation of PAI-1 [28]. As Colucci *et al*. [7] also noted, the possibility cannot be excluded that minor methodological differences in the plasma clot lysis assay and/or differences in the selection of patients may account for the discrepancy with the results of Lisman *et al*. [5]. Our results are in line with those obtained in a small group of 14 cirrhotic patients tested using a new commercially available global fibrinolytic capacity test in citrated plasma [29].

One disadvantage of the plasma clot lysis assay is that a relatively high concentration (25 ng/ml) of exogenous tPA is added to induce clot lysis. This makes the assay less sensitive to the balance of endogenous levels of tPA and PAI-1 in the circulation. We agree with Lisman *et al*. [5] that the plasma clot lysis assay might reflect local conditions where vessel wall injury leads to massive release of tPA from the endothelial cells. However, it is presently unclear whether systemic or local fibrinolytic conditions prevail during the regulation of the hemostatic system in cirrhosis. Because systemic tPA levels are strongly increased in the cirrhotic patients, it seems plausible that they do affect local hemostasis and that a global assay sensitive to endogenous levels of tPA is required to reflect local conditions. We therefore applied the global fibrinolytic capacity test in whole blood, which does depend fully on the endogenous levels of tPA and PAI-1 and should be considered as a different test, complementary to the plasma clot lysis assay. Using this assay, 32/72

patients showed hyperfibrinolysis; 18 of these patients also showed hyperfibrinolysis in the plasma clot lysis assay and 14 patients did not. The latter patients illustrate that the global fibrinolytic capacity test in whole blood is able to identify a subgroup of cirrhotic patients with hyperfibrinolysis that is not identified by the plasma clot lysis assay.

In conclusion, the hypothesis of a rebalanced fibrinolytic system in cirrhosis with no increased fibrinolysis [6] seems to be true in at most 40% of our patients. Hyperfibrinolysis in at least one of the two global fibrinolysis assays is detectable in the majority of the patients (60%). These findings remain an *in vitro* observation without direct implications for the clinical practice. However, they should be taken into account in current discussions on the coagulopathy of chronic liver disease [9,30]. In addition, the new global fibrinolytic tests may make it possible in future clinical studies to investigate whether hyperfibrinolysis is associated with bleeding problems in cirrhosis. The number of bleedings documented in the present study (n=4) was too small for such an investigation. Larger studies are required to establish if and how these fibrinolysis assays could be used in the clinic to estimate the risk of bleeding.

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Chapter 8

Biological variation in tPA-induced plasma clot lysis time

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ABSTRACT

Hypofibrinolysis is a risk factor for venous and arterial thrombosis, and can be assessed by using a turbidimetric tPA-induced clot lysis time (CLT) assay. Biological variation in clot lysis time may affect the interpretation and usefulness of CLT as a risk factor for thrombosis. Sufficient information about assay variation and biological variation in CLT is not yet available. Thus, this study aimed to determine the analytical, within-subject and between-subject variation in CLT. We collected blood samples from 40 healthy individuals throughout a period of 1 year (average 11.8 visits) and determined the CLT of each plasma sample in duplicate. The mean (±SD) CLT was 83.8 (±11.1) minutes. The coefficients of variation for total variation, analytical variation, within-subject variation and between-subject variation were 13.4%, 2.6%, 8.2% and 10.2%, respectively. One measurement can estimate the CLT that does not deviate more than 20% from its true value. The contribution of analytical variation to the within-subject variation was 5.0%, the index of individuality was 0.84 and the reference change value was 23.8%. The CLT was longer in the morning compared to the afternoon and was slightly longer in older individuals (>40 yr) compared to younger (≤ 40 yr) individuals. There was no seasonal variation in CLT and no association with air pollution. CLT correlated weakly with fibrinogen, C-reactive protein, prothrombin time and thrombin generation. This study provides insight into the biological variation of CLT, which can be used in future studies testing CLT as a potential risk factor for thrombosis.

INTRODUCTION

Arterial and venous thrombosis are multifactorial disorders which are associated with several risk factors [1,2]. However, these risk factors together do not explain the total thrombosis risk and there are even thrombosis patients without any known risk factors [1,3]. Much research is thus focused on identifying novel risk factors [4–6]. Hypofibrinolysis is one such novel factor found to be a potential risk factor for arterial and venous thrombosis [7,8]. Hypofibrinolysis can be examined by using a clot lysis assay [9], which provides a global overview of coagulation and fibrinolysis in plasma and is occasionally considered as a measure of the fibrinolytic potential. In the assay, coagulation is induced by tissue factor and fibrinolysis is stimulated by exogenously added tissue-type plasminogen activator (tPA). The time between coagulation and fibrinolysis, as followed by turbidity measurement, is the clot lysis time (CLT). Because the added tPA concentrations are much higher than the endogenous plasminogen activator concentrations, it might be more appropriate to consider the CLT as a measure of fibrin resistance to exogenous tPA than as fibrinolytic potential. Although this assay was used to test hypofibrinolysis as a risk factor in several studies, it was not completely conducted in the same way. For example, blood sampling and centrifugation conditions during plasma preparation differ between laboratories. Another important point to consider is that all studies that established hypofibrinolysis as a risk factor tested only one blood sample and then considered this as the true value. Information about the biological variation in CLT is needed to be able to determine the best study design for testing hypofibrinolysis as a risk factor for thrombosis. This information is not sufficiently available. Therefore, in this study we assess the biological variation in CLT. We also investigated whether preanalytical variations such as centrifugation conditions to obtain plasma and freeze-thaw cycles of samples have an influence on the CLT. In addition, we determined the analytical, within-subject and between-subject variation of CLT in a group of healthy individuals. With information about the different variations we are able to calculate how many blood samples are needed to measure the homeostatic setting point of a subject, the analytical imprecision, and the reference change value. The biological variation can be the result of several factors. Firstly, plasma components may be determined by diurnal or seasonal variation [10–15]. Therefore we assessed the diurnal and seasonal variations of CLT. Secondly, inflammation can have an effect on the biological variation and air pollution can result in an inflammatory response [16]. Therefore, we determined the correlation of air pollution, inflammatory markers and coagulation factors with CLT.

MATERIALS AND METHODS

Pre-analytical variations

To obtain information about pre-analytical influences on the CLT we collected citrated blood from 5 healthy volunteers, between 9 and 11 am. The blood was centrifuged at different speeds and for different time periods; A: 265 x g for 10 minutes; B: 2000 x g for 5 minutes; C: 2000 x g for 10 minutes; D: 2000 x g for 30 minutes; E: 2000 x g for 10 minutes and recentrifugation of the plasma at 20,000 x g for 10 minutes. Platelets were counted with an ABX micros 60 hematology analyser (ABX diagnostics, Montpellier, France). We tested the CLT of the plasma samples obtained under the different centrifugation conditions with or without the addition of 30 µg/ml potato carboxypeptidase inhibitor (PCI, Sigma-Aldrich, St. Louis, MO, USA) and before and after several freeze-thaw cycles with a maximum of 5 cycles.

Study population

The study population of the biological variation study consisted of forty healthy individuals, as described previously [15]. From each individual blood was collected at maximally 13 visits throughout 1 year, with a mean of 11.8 visits. From twelve participants two extra blood samples were collected on one of the 13 collection days, to be able to determine the diurnal variation. Before each blood collection, the participants were asked to fill out a questionnaire about daily habits, stating whether they had recently used any medication or whether they had suffered from any recent illness. Exclusion criteria were symptoms of chronic infectious diseases, acute infections or any surgical procedure within the past 3 months. The total study period was from January 2005 till December 2006. The study protocol was in accordance with the Declaration of Helsinki and was approved by the Medical Ethics Committee of the Erasmus Medical Center Rotterdam. Written informed consent was obtained from each participant.

Blood collection

Blood was collected between 9 and 10 am, while the study participant was sitting down and resting. From twelve participants, two extra blood samples for the diurnal variation testing were taken around noon between 12 am and 1.30 pm and in the afternoon between 3 and 4 pm. These samples were excluded from all other calculations. The study participants were allowed to have a light breakfast on the morning of the blood collection. Blood was drawn by venipuncture in the antecubital vein using the Vacutainer system (Becton Dickinson, Plymouth, UK) containing sodium citrate (final concentration

of 0.105 mol/l). Platelet-poor plasma was obtained by centrifugation twice at 3500 x g for 15 minutes at 4°C and stored at -80°C for further analysis.

Plasma clot lysis assay

To study the CLT, the plasma clot lysis assay was used essentially as described previously [7]. Platelet-poor plasma was diluted 1.7 times in buffer (25 mM Hepes, 137 mM NaCl, 3.5 mM KCl, 1% (w/v) BSA, pH 7.4) at room temperature. The diluted plasma (85 µl) was added to wells of a microtiter plate containing 15 µl of a reaction mixture. The reaction mixture contained the following components (with the final concentrations in the assay): tissue factor (Innovin, 1000 times diluted; Dade Behring, Marburg, Germany), $CaCl_2$ (17 mM), tPA (30 ng/ml, Actilyse, Boehringer Ingelheim, Ingelheim am Rhein, Germany), phospholipid vesicles (10 µM, Rossix, Mölndal, Sweden) and PCI (30 µg/ml) when indicated. After mixing the diluted plasma with the reaction mixture on a plate shaker, each well was covered with 50 µl paraffin oil (Merck, Darmstadt, Germany) and the microtiter plate was placed into the preheated chamber of the microplate reader (Victor^3, PerkinElmer, Waltham, USA). The optical density at 405 nm was measured every minute for 300 minutes at 37°C. The CLT was the time from the midpoint of minimum turbidity to maximum turbidity, which represents clot formation, to the midpoint of maximum turbidity to minimum turbidity, which represents clot lysis. These midpoints were calculated by sigmoidal curve fitting using Origin7 software (OriginLab, Northampton, USA). CLTs were measured in duplicate. Two control samples consisting of 2 different pools of normal plasma were measured in duplicate on each microtiter plate and the CLT of the 2 control samples were used as a reference. The mean CLT of the control samples on all microtiter plates of the study (n=14) was used to normalize the CLTs on each microtiter plate. The inter-assay variations were 7.8% and 8.0% for the 2 control plasmas. These normalized CLTs were used in all the calculations and statistical analyses.

Measurement of hemostatic markers

PAI-1 levels were measured using a PAI-1 antigen ELISA reagent kit (Technoclone, Vienna, Austria). Fibrinogen concentration was measured according to the von Clauss method on an ACL-300 (HemosIL(Fibrinogen-C), Instrumentation Laboratory, Breda, the Netherlands). C-reactive protein (CRP) was measured using a high-sensitivity in-house enzyme immunoassay using rabbit anti-human CRP IgG as capture and tagging antibody (DAKO A/S, Glostrup, Denmark). Human CRP Standard (Dade Behring, Marburg, Germany) was used as calibrator. The prothrombin time was measured on a Sysmex CA-1500 (Dade Thrombin reagent, Siemens Diagnostics, Leusden, the Netherlands) and thrombin generation in tissue factor (TF)-triggered platelet-poor plasma was measured with the

CAT method using a thrombin calibrator [17]. Thrombin generation measurements were performed with 80 µl plasma with 1 pM TF (PPP Reagent Low) and 4 µM phospholipids. Fluorescence was measured with a Fluoroskan Ascent reader (Thermo Labsystems OY, Helsinki, Finland) equipped with a 390/460 nm filter set. All reagents and software used for thrombin generation were obtained from Thrombinoscope BV (Maastricht, the Netherlands). Four parameters were derived from the thrombin generation curve: lag time (defined as the time to reach 1/6 of the peak height), time to peak (ttpeak), endogenous thrombin potential (ETP), and peak height.

Air pollution monitoring data

We obtained concentrations of particulate matter (PM) with aerodynamic diameter <10 µm (PM_{10}), carbon monoxide, nitrogen monoxide, nitrogen dioxide, and ozone from the Dutch National Air Quality Monitoring Network (National Institute for Public Health and the Environment 2009), which measured these air pollutants hourly at monitoring station number 418 (Schiedamse Vest, Rotterdam, the Netherlands). For data analysis, 6h, 12h and 24h means were calculated, as described previously [18].

Statistical methods

Pre-analytical variations were evaluated using the Wilcoxon Signed ranks test. The CLT data of the 40 healthy volunteers was tested for outliers using the extreme studentized deviate method (Grubb's test). The sources of variation, analytical, within-subject and between-subject variation were calculated using a nested random effect analysis with SAS software (version 9.2). We calculated the number of repeated measurements that were needed to estimate the homeostatic setting point which deviates not more than 10%, 15% or 20% from its true value with a probability of 95%, the contribution of the analytical imprecision to the within-subject variation, the index of individuality, the reference change value and the seasonal variation as described before [15]. We divided the year into four times three months, December to February was winter, March to May was spring, June to August was summer and September to November was autumn. To check for diurnal variation the CLTs and PAI-1 levels in the morning, around noon and in the afternoon were compared using the Wilcoxon Signed ranks test. For air pollution measurements, linear regression analysis was performed between CLT and mean air pollution concentration at different periods before each blood sampling with R software as described before [18]. Correlations coefficients between fibrinogen, prothrombin time and thrombin generation parameters and CLT were analyzed using Pearson's correlation test. Correlation coefficient of CRP and CLT was analyzed using Spearman's correlation test because CRP was not normally distributed. All statistical analysis were performed with SPSS for Windows,

version 15.0 (SPSS Inc, Chicago, IL, USA) unless stated otherwise. A p-value of <0.05 was considered statistically significant.

RESULTS

Pre-analytical variations

We collected blood from 5 healthy volunteers in order to investigate pre-analytical variation in CLT as a result of variation in plasma preparation. The CLT of fresh platelet-rich plasma (A) was significantly longer compared to four different fresh platelet-poor plasma preparations (B-E) ($p<0.05$, table 1). In the presence of PCI, an inhibitor of activated thrombin activatable fibrinolysis inhibitor (TAFIa), the CLT of fresh platelet-rich plasma (A) was still longer than the CLT of the platelet-poor plasmas (B-E), probably due to the presence of plasminogen activator inhibitor-1 (PAI-1) in platelets. However, the TAFI-effect defined as the CLT in the absence of PCI divided by the CLT in the presence of PCI was higher in platelet-rich plasma than in the platelet-poor plasmas (1.9 versus 1.5), indicating that not only PAI-1 but also TAFI contributed to the longer CLT of platelet-rich plasma (table 1).

The CLT of frozen platelet-rich plasma (A) was also longer compared to platelet-poor plasma (B) (table 1). However, the TAFI-effect was hardly higher in platelet-rich plasma than in the platelet-poor plasmas (1.5 versus 1.4), indicating that TAFI did not contribute more to the CLT of frozen platelet-rich plasma compared to frozen platelet-poor plasma.

Table 1. Effect of pre-analytical conditions on the plasma clot lysis assay

	A	B	C	D	E
Centrifugation conditions	265 x g for 10 min	2000 x g for 5 min	2000 x g for 10 min	2000 x g for 30 min	2000 x g for 10 min + 20000 x g for 10 min
Platelet count (10^9/L)	482 ± 181	148 ± 92	77 ± 55	6 ± 4	8 ± 7
CLT fresh plasma (min)	145.8 ± 64.0*	94.0 ± 22.3	87.7 ± 13.3	90.6 ± 14.7	93.0 ± 13.5
CLT fresh plasma + PCI (min)	73.4 ± 17.3*	62.3 ± 12.3	60.7 ± 10.8	61.3 ± 10.3	61.2 ± 10.3
TAFI-effect fresh plasma	1.9 ± 0.4	1.5 ± 0.2	1.5 ± 0.1	1.5 ± 0.1	1.5 ± 0.2
CLT frozen plasma (min)	109.3 ± 36.5**	91.9 ± 22.0	84.6 ± 17.8	95.7 ± 18.0	91.7 ± 16.6
CLT frozen plasma +PCI (min)	71.2 ± 17.2***	64.4 ± 14.6	61.9 ± 9.9	67.7 ± 12.2	66.7 ± 10.2
TAFI-effect frozen plasma	1.5 ± 0.1	1.4 ± 0.1	1.4 ± 0.1	1.4 ± 0.1	1.4 ± 0.2

Citrated blood from 5 healthy volunteers was centrifuged under varying conditions. The CLT (mean ± SD) of the plasma samples was determined with and without PCI before and after freezing and thawing. The TAFI-effect is defined as the CLT in the absence of PCI divided by the CLT in the presence of PCI. * CLT significantly different from the corresponding platelet-poor plasmas (B-E) ($p<0.05$), ** CLT significantly different from B ($p<0.05$). *** CLT significantly different from B-C ($p<0.05$). PCI, potato carboxypeptidase inhibitor

Altogether, the mean CLT of platelet-rich plasma was shorter after freezing and thawing (145.8 ± 64.0 versus 109.3 ± 36.5 min, $p<0.05$), whereas the mean CLT of platelet-poor plasma did not change after freezing and thawing (about 90 min, table 1). Four additional freeze-thaw cycles of platelet-poor plasma did not influence the CLT (data not shown).

Biological variation of CLT

Blood samples used to determine the biological variation in CLT were collected from 40 healthy volunteers over the course of one year. Analysis of CLT data identified one volunteer as an outlier and this volunteer was excluded from analysis. Characteristics of the study population are shown in table 2. The mean age of the volunteers was 41 years (range 21–70), 64% were female (n=25) and 15% were smokers (n=6). Nine women were using oral contraceptives (36% of women) throughout the study period.

Table 2. Characteristics of the study population

Variable	Study population (n=39)
Age (years)	41 (21-70)
Females	25 (64%)
BMI ($kg.m^{-2}$)	22.5 (18.3-27.2)
Smokers	6 (15%)
Oral contraceptives	9 (36% of women)

Values are given as n (%) for categorical variables and as means (range) for age and BMI. BMI, body mass index.

A total of 459 blood samples were used in this study and were collected on 197 different days spread throughout the study period. CLTs were measured in duplicate using the plasma clot lysis assay (figure 1). The mean CLT (±SD) of the 39 individuals was 83.8 (±11.1) minutes with a CV for the total, analytical, within-subject and between-subject variation of 13.4%, 2.6%, 8.2%, and 10.2%, respectively (table 3). In order to estimate the homeostatic setting point that does not deviate more than 10% from its true value, three repeated

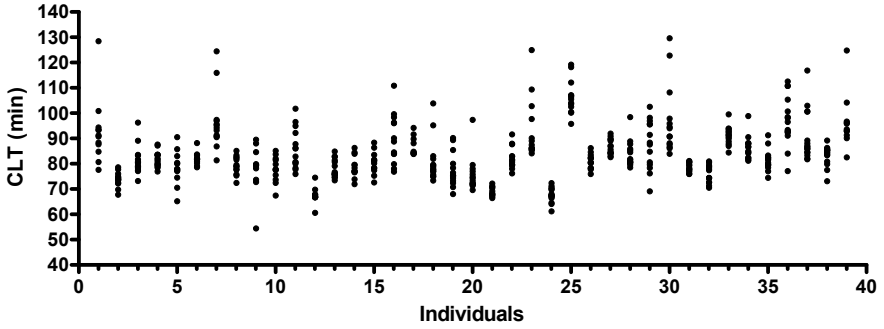

Figure 1: Plasma clot lysis time of healthy individuals
CLTs in minutes of all blood samples from the 39 healthy individuals.

measurements of CLT are needed. If only one sample is measured, which is more common in daily practice, we estimate a CLT that does not deviate more that 20% from its true value. For two measurements, a CLT that does not deviate more than 15% is estimated. The index of individuality, or the ratio of the within-subject variation to between-subject variation, was calculated to be 0.84 and the reference change value, which indicates how much a test result of an individual needs to deviate from the homeostatic setting point to be statistically different with a 95% probability, was 23.8% for CLT (table 3).

Table 3. Study of the biological variation in plasma clot lysis time

Individuals (n)	39
Observations (n)	459
Mean CLT ± SD (min)	83.8 ± 11.1
Total CV (%)	13.4
Analytical CV (%)	2.6
Within-subject CV (%)	8.2
Between-subject CV (%)	10.2
Contribution of imprecision (%)	5.0
Index of individuality	0.84
Reference change value (%)	23.8
Repeated measurements (10%)	3
Repeated measurements (15%)	2
Repeated measurements (20%)	1

Repeated measurements (%), the number of repeated measurements that was needed to estimate the CLT that does not deviate more than 10%, 15% or 20% from its true value, with a probability of 95%. CV, coefficient of variation

Determinants of biological variation

The CLT was significantly longer in the morning compared to around noon and afternoon (p=0.004, figure 2), but was comparable around noon and afternoon. PAI-1 levels were also higher in the morning compared to around noon and afternoon (p=0.002) with median (range) PAI-1 levels in the morning of 18.1 (1.6–77.4) ng/ml, around noon of 9.1 (0.9–32.3) ng/ml and in the afternoon of 3.8 (0.7–14.1) ng/ml. In contrast to the CLT the PAI-1 levels differed significantly between noon and afternoon (p= 0.006). No seasonal variation was observed in CLT throughout the year (figure 3). None of the pollutants analyzed, PM_{10}, CO, NO, NO_2 and O_3, showed a significant correlation with CLT (data not shown).

Older individuals (>40 yr, n=20) had longer CLTs than younger individuals (≤ 40 yr, n=19) (86.7 ± 12.0 min vs. 80.7 ± 8.7 min respectively, p<0.0001). There was no significant difference between men (n=14) and women (n=25) (84.7 ± 12.1 min vs. 83.2 ± 10.2 min, respectively, p=0.11). Women (≤ 40 yr) who used oral contraceptives (n=8) had shorter CLTs than women who did not use oral contraceptives (n=6) (77.1 ± 6.5 min vs. 84.0 ± 9.4 min respectively, p<0.02).

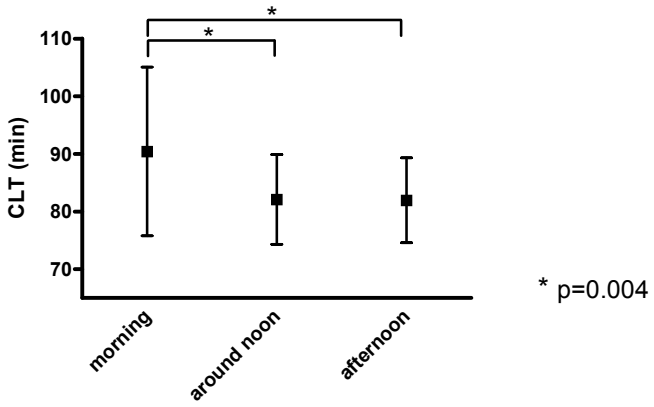

Figure 2: Diurnal variation in plasma clot lysis time
In the morning CLT was higher than around noon and in the afternoon (p=0.004) (n=12). Morning time points were between 9 and 10 am, around noon between 12 am and 1.30 pm and in the afternoon between 3 and 4 pm. The CLT between noon and afternoon did not differ. Mean CLT with standard deviation are displayed. The CLT was compared using the Wilcoxon Signed Ranks test.

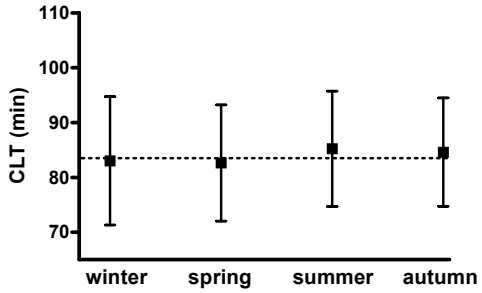

Figure 3: Seasonal variation in clot lysis time
The CLT was similar in the four seasons of the year. Mean CLTs with standard deviation are displayed. The dotted line is the seasonal variation throughout the year calculated using an additive-variance component model in ANOVA.

The CLT was correlated with fibrinogen (R=0.119, p=0.01), CRP (R=-0.155, p<0.05), prothrombin time (R=-0.282, p<0.0001), thrombin generation lag time (R=0.305, p<0.0001) and thrombin generation time to peak (R=0.188, p<0.0001). There was no correlation between CLT and thrombin generation peak height or endogenous thrombin potential (table 4). Prothrombin time explained about 8%, thrombin generation lag time about 9% and thrombin generation time to peak about 4% of the variation in CLT. Fibrinogen and CRP explained only about 1% of the variation in CLT.

Table 4. Correlation of inflammation markers and hemostatic factors with CLT

	Median (95% range)	R	p-value
Fibrinogen (mg/ml)	2.7 (1.9-3.7)	0.119	0.01
CRP (mg/l)	0.30 (0.06-3.6)	-0.155	<0.05
PT (sec)	12.2 (11.1-13.9)	-0.282	<0.0001
Thrombin generation			
lag time (min)	4.0 (3.0-6.3)	0.305	<0.0001
ttpeak (min)	8.0 (5.6-11.3)	0.188	<0.0001
peak height (nM)	138 (52-276)	0.001	0.977
ETP (nM.min)	1001 (523-1550)	-0.005	0.914

Correlations coefficients were analyzed using Pearson's correlation test except for the correlation coefficient of CRP which was analyzed using Spearman's correlation test. CRP, C-reactive protein, PT, prothrombin time, ttpeak, time to peak, ETP, endogenous thrombin potential.

DISCUSSION

In this study we determined the variation in CLT. Variation in pre-analytical factors such as centrifugation conditions to obtain platelet-poor plasma and freeze-thaw cycles did not influence the CLT. The coefficients of variation of analytical, within-subject and between-subject variation were 2.6%, 8.2% and 10.2%, respectively. The time point of blood sampling during the day is important and with one measurement we can estimate the CLT that does not deviate more than 20% from its true value. This information on biological variation in CLT was absent in the literature but is important to design a study properly to test hypofibrinolysis as a risk factor for thrombosis and also hyperfibrinolysis as a risk factor for bleeding.

Many different assays were used in the past years to assess the fibrinolytic activity of individuals. Although assays in whole blood are preferable [19], they are not always possible. Nowadays the fibrinolytic state of plasma samples is frequently determined by using the turbidimetric tPA-induced plasma clot lysis assay. A short clot lysis time (CLT) indicates a high sensitivity to lysis and a long CLT indicates a low sensitivity to lysis. The assay thus reflects the sensitivity of the fibrinolytic system (including the clot itself) to exogenous tPA and is therefore less affected by endogenous plasminogen activator levels. In a large group of healthy individuals 77% of the variation in CLT was explained by plasma levels of number of fibrinolysis and coagulation factors, including PAI-1, plasminogen, TAFI, prothrombin and α_2-antiplasmin [20]. The test is simple and feasible [21], but is still poorly standardized between research groups. Variations include for example the final plasma concentration, the type and concentration of clotting agent (tissue factor vs. thrombin), and the type and concentration of fibrinolytic agent (tPA vs. urokinase).

Pre-analytical variation was studied by measuring the effect of different centrifugation conditions during plasma preparation, because residual platelets might affect fibrinolysis via various mechanisms and there is no consensus between different laboratories on how to obtain plasma. The CLT of fresh platelet-rich plasma was indeed significantly longer

than that of platelet-poor plasma, but once the platelet count was below 150x 10^9/L (obtained by centrifugation at 2000 x g for 5 min) the CLT did not further decrease. This implies that the CLT is not sensitive to the procedure of platelet-poor plasma preparation. The lower fibrinolytic potential of platelet-rich plasma could partially, but not completely be ascribed to TAFIa because in the presence of PCI there was still a difference in CLT between fresh platelet-rich plasma and fresh platelet-poor plasma. This difference in CLT is probably due to more PAI-1 in platelet-rich plasma. After a freeze-thaw cycle the difference in CLT between platelet-rich plasma and platelet-poor plasma was still present, but less pronounced because the effect of TAFI was smaller in frozen platelet-rich plasma. Platelets are probably destroyed during a freeze-thaw cycle of plasma. The activation of TAFI is apparently favored by intact platelets, in spite of the formation of procoagulant microparticles during the destruction of platelets. Freeze-thaw cycles of platelet-poor plasma did not affect the CLT, which implies that the fibrinolytic potential can be accurately determined in stored plasma samples, at least from healthy volunteers.

Our data show that when the CLT is determined in only one sample, we have to keep in mind that this CLT can deviate, but not more than 20% with a probability of 95%, from its true value. If the estimated CLT needs to deviate not more than 15% or 10%, the CLT needs to be measured in at least 2 or 3 samples, respectively. Changes in the serial results from one individual can be due to the biological variation. With the reference change value the significance of differences in serial results from an individual can be assessed. The reference change value for CLT was 23.8%, which means that the CLT obtained in one measurement needs to deviate more than 23.8% from the previous measurement to be considered as significantly different and not as a result of biological variation.

It is important to take the blood samples for CLT at the same time of day, due to the diurnal variation that we observed. The CLT was longer in the morning compared to the afternoon. Several circulating plasma components have variations throughout the day and could therefore contribute to a diurnal variation in CLT, including PAI-1, FVIIa, tPA, protein C, and protein S [10,11,22]. The variation of PAI-1 is probably the strongest determinant of the diurnal variation of CLT. Indeed, we observed higher PAI-1 levels in the morning compared to around noon and in the afternoon. FVII activity, prothrombin fraction fragments F1+2 and PAI-1 activity are the highest and tPA activity is the lowest in the morning, suggesting a hypercoagulable and hypofibrinolytic state in the morning [10–12]. However, protein C, protein S and TFPI levels in the morning are significantly higher than levels at noon [13,14], which may reduce the hypercoagulable state in the morning.

In contrast to a diurnal variation, we did not observe any seasonal variation, such as was found for fibrinogen and thrombin generation [15]. We also did not observe any association of CLT with air pollution parameters, including PM_{10}, CO, NO, NO_2 and O_3, which affected thrombin generation in the same individuals [18]. The effect of thrombin generation on CLT, as measured in the present study, was apparently not sufficiently

strong to induce an indirect association between CLT and air pollution. Direct positive correlation between thrombin generation and CLT was only seen with the parameters lag time and time to peak but not with the peak height and endogenous thrombin potential. Prothrombin time was negatively correlated with CLT. Fibrinogen was positively correlated with CLT and may be a confounder for the associations of CLT with thrombin generation test and the prothrombin time, because fibrinogen is positively correlated with thrombin generation lag time [17] and negatively associated with prothrombin time ($R=-0.359$, $p<0.001$, this study). Fibrinogen and CRP showed only a weak correlation with CLT, probably because the healthy volunteers in this study had fibrinogen and CRP levels in a normal range. CLT increased with increasing age, which is in line with previous studies [7,21]. Aging is associated with increased plasma levels of fibrinogen, factor VII, factor VIII and PAI-1[23,24]. A reduced sensitivity to fibrinolysis may contribute to the prothrombotic state of the elderly.

We concluded from this study that the clot lysis assay is a useful assay to study the fibrinolytic state of (stored) platelet-poor plasma. The existence of a diurnal variation should be taken into account when obtaining blood samples. This study provides data to determine how the CLT can be measured and how fibrinolysis can be tested in the future as a risk factor for thrombosis and bleeding disorders.

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Chapter 9

Summary and general discussion

SUMMARY

In chapter 1 the subject of this thesis is introduced. Several risk factors for thrombosis are known, however there are still thrombotic patients without any known risk factor. This suggests that the hemostatic system has not been completely elucidated. The central component of a thrombus is fibrin. The aim of this thesis was to identify novel fibrin clot-bound proteins to get one step closer to identifying novel players in hemostasis. Identification of novel players in hemostasis could help in determining new risk factors and additionally in understanding the pathogenesis of thrombotic disorders.

In chapter 2 we identified 18 fibrin-clot bound plasma proteins using 2D gel electrophoresis and mass spectrometry. Nine out of the 18 proteins were not known before to be present in a fibrin clot. Most proteins that we identified can be classified into three groups related to their function: blood coagulation, protease inhibition and high-density lipoprotein (HDL) metabolism. Six out of the 9 novel plasma clot components are known to be associated with HDL, suggesting that a HDL particle binds to the fibrin clot.

In chapter 3 we investigated the binding of one of the 9 novel fibrin clot-bound proteins, α_1-antitrypsin (A1AT), to fibrin in more detail. A1AT was the most abundant protein spot on the 2D gel and the amount was estimated to be about 0.6 µg A1AT per ml of plasma clot. We investigated the affinity of A1AT for fibrin using fibrin-sepharose. Only a small amount of purified A1AT (0.1%) bound to the fibrin-sepharose. However, when we eluted this bound A1AT from the column, dialyzed the elution buffer containing potassium thiocyanate out and applied this A1AT sample again on the fibrin-sepharose column, around 30% of the A1AT was bound. This suggests that the A1AT bound to the fibrin clot is a distinct form of A1AT with a high affinity for fibrin. This A1AT that is bound to a fibrin clot inhibited the fibrinolytic activity of elastase. Leukocytes that infiltrate the fibrin clot secrete elastase and A1AT may therefore act locally as an antifibrinolytic protein.

Carboxypeptidase N (CPN) is another novel plasma clot component that has been shown to have antifibrinolytic activity. The presence of CPN in a plasma clot was investigated in more detail in chapter 4. CPN occurred at a concentration of about 30 ng per ml plasma clot and the forms of CPN present in a clot were similar to the forms that circulate in plasma. We did not detect proteolytically cleaved forms of CPN that might show higher activity in the clot. Using plasmon surface resonance we showed that CPN can directly bind to fibrinogen as well as fibrin with an apparent Kd of around 100 nM.

Two-thirds of the novel plasma clot components were HDL-associated proteins. Therefore we investigated the role of HDL in hemostasis using thromboelastometry in chapter 5. Native HDL (nHDL) did not show any effect, though we observed that oxidized HDL (oxHDL) diminished blood clot firmness without interfering with coagulation. This diminished clot firmness did not involve fibrin breakdown, suggesting that platelets play a role. However, the effects of oxHDL on plasma clot retraction and platelet aggregation were

small and could not explain the diminished blood clot firmness. More studies are needed to elucidate the mechanism.

Establishing differences in plasma clot components between healthy controls and thrombotic patients could determine novel risk factors for thrombosis. In chapter 6 we compared the abundance of the plasma clot components of Budd-Chiari syndrome (BCS) patients with hepatic vein thrombosis and controls using two-dimensional fluorescence-based difference gel electrophoresis (2D-DIGE). BCS patients had a lower plasma clot abundance of apolipoprotein AI (apo AI). Apo AI is the main protein component of HDL. To validate these findings we studied plasma levels of apo AI and HDL cholesterol in patients with BCS and controls. BSC patients without liver protein synthesis insufficiency also had lower plasma apo AI levels, indicating that the lower apo AI plasma levels were not completely due to possible liver insufficiency and may play a role in the development of BCS.

Bleeding complications are common in patients with cirrhosis, possibly due to a disturbed balance in hemostatic factors. Fibrinolysis can be investigated using different assays, but there is still controversy about whether patients with cirrhosis show increased fibrinolysis. Therefore we investigated fibrinolysis in a group of cirrhotic patients using a plasma clot lysis assay as well as a global fibrinolytic capacity assay using whole blood in chapter 7. We observed that 40% of the patients displayed hyperfibrinolysis in the plasma clot lysis assay and 43% in the global fibrinolytic capacity assay. Of all patients, 60% showed hyperfibrinolysis in at least one of the two assays. A correlation between observed hyperfibrinolysis and bleeding complications could not be established because of the limited number of patients who displayed bleeding complications during follow up in our study.

In chapter 7 we observed that 40% of the cirrhosis patients displayed hyperfibrinolysis, as measured with the plasma clot lysis assay. However, not all previous studies had detected hyperfibrinolysis in their group of cirrhosis patients using the same assay. Therefore we investigated the biological variation in clot lysis time (CLT), measured with the plasma clot lysis assay, in chapter 8. This might in part reveal the cause of the different results of the studies. In addition, information on the biological variation of CLT is needed to optimally design new studies on fibrinolysis. The total, analytical, within-subject and between-subject variation was 13.4%, 2.6%, 8.2% and 10.2%, respectively. Centrifugation conditions to obtain platelet-poor plasma did not influence the CLT. The CLT was longer in the morning than for the rest of the day, but did not show a seasonal variation. When CLT is determined in one sample, it can deviate, but not more than 20% with a probability of 95%, from its true value. If the measurement of CLT needs to be more accurate, more samples need to be measured.

GENERAL DISCUSSION AND FUTURE DIRECTIONS

Identification of fibrin clot-bound proteins

We used 2D gel electrophoresis to identify novel fibrin clot-bound plasma proteins. The intensity of the stained spots in the 2D gel suggests that the proteins that we identified represent nearly the entire protein material non-covalently bound to a fibrin clot in static conditions. Some plasma clot components could be lost or gained as fibrin clot-bound protein by the absence of blood flow and cells when compared to *in vivo* formed thrombi.

We did not visualize the very low abundant proteins using Sypro Ruby staining, such as tissue-type plasminogen activator (t-PA), which is known to bind to a fibrin clot. Another limitation of this approach is that proteins can be lost during rehydration by adsorption to the rehydration chamber wall and that the very high molecular weight (>150,000) and very low molecular weight proteins (<10,000) are underrepresented [1,2]. However, we did observe the high molecular weight proteins fibronectin and α_2-macroglobulin with 2D gel electrophoresis. In addition, using 1D gel electrophoresis (SDS-PAGE) and protein staining we did not observe any additional high or low molecular weight protein in a plasma clot extract. We observed fairly good reproducibility using various 2D gels. Spots that were identified using mass spectrometry were reproducibly detected in at least 7 out of 10 2D gels. Most of the protein spots that were not visualized in all gels were the very high molecular weight proteins. In the study design followed in this thesis, fibrinolysis was inhibited by aprotinin and hence proteins that specifically bind to sites on fibrin that are exposed upon partial cleavage of fibrin by plasmin are not detected. Plasminogen is a protein that binds to interchain lysine residues in intact fibrin as well as to C-terminal lysine residues on partially degraded fibrin. We did observe plasminogen as a fibrin clot-bound protein in our study, but the abundance will be higher when fibrin is partially degraded by plasmin [3]. Howes *et al.* [4] also performed a study using proteomics to identify novel plasma clot components. Most proteins identified were related to coagulation and inflammation, while we identified mainly proteins that were related to coagulation, protease inhibition and HDL metabolism. There are some clear differences between the two proteomic approaches. The most important difference is that Howes *et al.* [4] analyzed the total protein composition of the clot, thereby also identifying proteins that are cross-linked via activated factor XIII, while we focused on non-covalently plasma clot-bound proteins. Identifying proteins by examining the whole clot is technically more challenging because of the high abundance of fibrin compared to the other plasma clot components. High-abundant proteins may overwhelm low-abundant proteins. Different sampling procedures and particularly differences in anticoagulants used, could also influence the identification of proteins with mass spectrometry [5]. Although we cannot exclude the possibility that we may have missed non-covalently fibrin clot-bound

proteins we did observe 9 proteins that were not described before as binding to a fibrin clot. To get a complete picture of a fibrin clot, it would be interesting to investigate not only non-covalently bound proteins but also the proteins that are cross-linked in more detail in the future. Other mass spectrometry methods may be more appropriate than 2D gel electrophoresis, e.g. the non-gel-based chromatographic multidimensional protein identification technology (MudPIT). Analysis of the yeast proteome using MudPIT shows that this technique can be used to identify large numbers of proteins, including low (<10,000) and high (>190,000) molecular weight proteins [6]. Nevertheless, multiple analytical approaches are needed to detect all clot-bound proteins. Inclusion of cells and flow to the model system would be necessary to get a complete overview of all fibrin clot components that are present in the *in vivo* situation.

Characterization of fibrin clot-bound proteins

From the presence in the fibrin clot prepared from plasma we cannot conclude whether a protein is directly or indirectly bound to fibrin. The two proteins that we investigated, A1AT and CPN, both directly bind to fibrin. However, for other novel plasma clot components the binding characteristics still need to be determined. Fibrin-bound A1AT proved to be a distinct form of A1AT, however how this form differs from the unbound form is not known. Serpins, like A1AT, are known to exist in different conformations including the native form with the reactive center loop (RCL) expelled, the native form with a partially inserted RCL, the inactive latent form with a fully inserted RCL and the polymeric form [7]. However, other conformations cannot be excluded. We did prepare latent and polymeric A1AT [8], though we did not succeed in creating a fibrin-binding form of A1AT. The characteristics of the A1AT with high affinity for fibrin that differ from A1AT without affinity for fibrin still need to be determined. For example conformation studies using crystallography, post-translational modification analysis with mass spectrometry [9] and sequence analysis to detect possible genetic differences could be performed. In plasma samples we clearly detected A1AT-fibrinogen complexes, indicating that A1AT with high affinity for fibrinogen and fibrin circulates *in vivo* in complex with fibrinogen. We cannot exclude the possibility that the change in A1AT towards a fibrin binding form in purified A1AT is partially induced by the isolation procedure.

Both CPN subunit 1 and CPN subunit 2 are sensitive to proteolytic cleavage by e.g. plasmin. Proteolysis of CPN results in a more active enzyme when the catalytic subunit of 48 kDa is cleaved into fragments of 27 and 21 kDa [10]. From data presented in this thesis we concluded that the subunits that are present in a plasma clot represent the subunits that are normally present in plasma. The 27 and 21 kDa fragments that are obtained from cleavage of CPN1 were not detected, but their occurrence in a fibrin clot could not be excluded. Our study design included inhibition of fibrinolysis by the addition of aprotinin.

Therefore, we could not determine whether fibrin clot-bound CPN can be cleaved by plasmin. Studies investigating the possibility of plasmin cleaving fibrin clot-bound CPN should be performed in the future. In addition, properties and functionality of plasmin-cleaved CPN have been investigated *in vitro* [10]. Therefore, it is necessary to determine whether CPN can be cleaved by plasmin *in vivo* in the circulation or bound to a thrombus.

The presence of several HDL-associated proteins in a fibrin clot suggested that whole HDL particles bind to fibrin. However, HDL particles are heterogeneous, both in size and composition. The relatively high abundance of apolipoprotein J (apo J) on the 2D gel was remarkable. Apo J is predominantly found in the small and dense HDL3c subspecies besides other proteins that we also identified as fibrin clot-bound proteins including A1AT, apo AIV and thrombin. In addition, fibrinogen is also identified as being associated with the HDL3c subfraction [11]. The HDL particles that contain apo J may account for only 2% of the total HDL [12]. The abundant presence of apo J in the clot suggests that one or a few specific subfractions of HDL may bind to a fibrin clot and whether these indeed include the HDL3c subfraction needs to be elucidated. Further studies using mass spectrometry could determine the protein composition of the HDL subfraction(s) that are bound to a fibrin clot in a purified system. Data from other studies that investigated the different HDL subspecies in detail [11] can be used to compare the protein composition of the fibrin clot-bound HDL. Binding studies with different HDL subspecies could be performed to investigate whether certain subspecies have a high affinity for fibrin.

Functionality of fibrin clot-bound proteins

In addition to binding characteristics, the functions of the novel fibrin clot-bound proteins need to be elucidated. We showed that fibrin clot-bound A1AT inhibits the fibrinolytic function of elastase *in vitro*. We used a purified system with fibrinogen, A1AT and elastase. Additional studies performed in a plasma system containing other pro- and anti-fibrinolytic proteins and blood cells might help to determine the relative contribution of elastase and A1AT to fibrinolysis in the future. Infants with A1AT deficiency have been reported to have bleeding complications, but these seem to be largely due to a vitamin K-deficiency [13].

The function of fibrin clot-bound CPN is not completely clear either. Native CPN exhibits low, but long-term anti-fibrinolytic activity and this activity is increased approximately 8-fold upon cleavage of the 48 kDa catalytic subunit [14]. CPN may function as a long-term anti-fibrinolytic enzyme when active thrombin activatable fibrinolysis inhibitor (TAFI) loses its activity. From our studies we did not find any evidence of the presence of substantially cleaved CPN forms within fibrin clots. As discussed above, further studies might provide more insight into whether fibrin clot-bound CPN is cleaved by plasmin *in vivo*. Other studies that investigate the relative contribution of CPN to the inhibition of

fibrinolysis are needed to obtain more information on whether fibrin clot-bound CPN is involved in fibrinolysis or in other systems e.g. the complement system.

Studies in the past have indicated that HDL has anticoagulant properties [15,16]. We did not observe any effect of nHDL on coagulation and fibrinolysis using thromboelastometry. However, oxHDL diminished the blood clot firmness by a yet unknown mechanism. We observed small effects of oxHDL on clot retraction and platelet aggregation that were too small to explain the phenomenon. Platelet aggregation occurs in two phases, the primary reversible phase followed by a secondary irreversible phase [17]. These two phases of platelet aggregation might possibly play a role in the decline in clot firmness. The pattern of the tromboelastrogram obtained in the presence of oxHDL gives the impression that the platelets become activated and aggregate but then fall apart again during thromboelastometry analysis, suggesting that the reversible platelet aggregates fail to turn into the irreversible macroaggregates in the presence of oxHDL. More studies need to be performed to unravel the mechanism by which oxHDL decreases the clot firmness of a blood clot and to study whether fibrin clot binding is involved. These studies could include different oxidation methods and varying oxidation phases. Different platelet assays might provide more insight, including measuring the contractile force of platelets in an isometric setup.

Identification of novel fibrin clot-bound proteins may lead to the detection of novel risk factors of thrombosis. We described that low levels of apo AI, one of the novel plasma clot components, is a possible risk factor for BCS. Although we cannot fully exclude the possibility that the liver impairment may contribute to the lower apo AI levels in BCS patients, the decrease was not completely due to liver impairment. This suggests that apo AI may play a role in the development of thrombus formation leading to BCS. How apo AI, which is the main protein component of HDL, could play a role in the pathogenesis of thrombosis is not completely clear. Multiple roles of HDL in coagulation are known that could be protective in the development of thrombosis [18]. Low HDL cholesterol levels are an independent risk factor for thrombosis, even though LDL cholesterol levels may be low. Therefore an interesting therapeutic goal is to increase the plasma levels of HDL. However, there is still some debate about whether raising total HDL cholesterol results in a clinical benefit [19,20]. Statins lower LDL cholesterol levels and moderately increase HDL cholesterol levels [21]. However, there is not a substantial protective effect of statins on venous thrombosis although very small effects cannot be ruled out [22]. Another way to increase HDL cholesterol levels and lower LDL cholesterol levels is via the inhibition of cholesteryl ester transfer protein (CETP). CETP inhibitors are currently in phase III trials to test their clinical benefit. A large meta-analysis indicates that genetically mediated CETP inhibition results in a modest increase in HDL cholesterol and a weak clinical benefit [23]. Questions remain about the functionality of the raised HDL. Studies with gemfibrozil, a

fibric acid derivative, suggest that raising specific HDL subspecies could lead to beneficial clinical results [24].

Fibrinolysis

Patients with liver failure due to cirrhosis generally have an increased bleeding tendency. It is not clear whether differences in hemostatic factors may contribute to the increased bleeding tendency in addition to other conditions such as portal hypertension and renal dysfunction [25]. Our data obtained using two different global fibrinolysis assays showed that patients with cirrhosis display hyperfibrinolysis. This is in line with other studies that investigated hyperfibrinolysis in cirrhosis samples with the plasma clot lysis assays [26,27]. However, Lisman *et al.* [28] did not find evidence of increased fibrinolysis. The reason for this discrepancy is not clear, however small differences in patient selection or assay characteristics cannot be ruled out. For the future it would be of interest to determine whether hyperfibrinolysis in cirrhosis patients predicts bleeding risk. In addition, this can be used to determine which patients are in need of blood products or antifibrinolytic agents during invasive surgery. Biological variation in CLT may have an influence on the outcome of the clot lysis assay and possibly provide an explanation for the different outcomes of studies that used the same clot lysis assay. Therefore we determined the biological variation in CLT in chapter 8. We concluded that storage of platelet-poor plasma does not influence the variation in CLT. The measurement of one sample is sufficient if a maximum of 20% deviation of the homeostatic setting point with a probability of 95% is accurate enough. If the measurement of CLT needs to be more accurate, more samples need to be measured. The biological variation in CLT is possibly too large to accurately estimate the homeostatic setting point using one sample and this may have contributed to the discrepancy between the studies. Measuring more samples from more individuals might give a better insight into the question of whether cirrhosis patients display hyperfibrinolysis.

Conclusions

Overall, several novel fibrin clot-bound plasma proteins were identified in the studies described in this thesis. Although we did not unravel the mechanism of each protein individually, the biochemical studies with A1AT and the proteomic studies with HDL suggest that these proteins might play a role in hemostasis and thrombosis. A complete overview of the fibrin clot components and their functions in hemostasis would help to unravel further the pathogenesis of bleeding and thrombosis.

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SAMENVATTING

In hoofdstuk 1 wordt het onderwerp van dit proefschrift geïntroduceerd. Verschillende risico factoren voor trombose zijn bekend, maar er zijn nog steeds patiënten met trombose die geen bekende risico factor hebben. Dit suggereert dat het stollingssysteem niet volledig ontrafeld is. Het doel van dit proefschrift is daarom om nieuwe eiwitten te identificeren die aan een fibrine stolsel binden om zo een stap dichter bij de identificatie te komen van nieuwe eiwitten die een rol in de hemostase spelen. Informatie over mogelijk nieuwe eiwitten kan helpen om nieuwe risico factoren vast te stellen en uiteindelijk de pathogenese van trombose beter te begrijpen.

In hoofdstuk 2 hebben we 18 fibrine stolsel bindende eiwitten geïdentificeerd met behulp van 2D gel elektroforese en massaspectrometrie. Van de helft van de eiwitten was nog niet bekend dat ze voorkwamen in een fibrine stolsel. De meeste eiwitten die geïdentificeerd zijn kunnen in 3 groepen verdeeld worden op basis van hun functie: bloed stolling, remming van proteases en high-density lipoprotein (HDL) metabolisme. Zes van de 9 nieuwe plasma stolsel componenten zijn geassocieerd met HDL, wat suggereert dat een compleet HDL deeltje aan het fibrine stolsel bindt.

In hoofdstuk 3 hebben we de binding van een van de 9 nieuwe plasma stolsel componenten, α_1-antitrypsine (A1AT), aan fibrine in detail bekeken. A1AT was de meest intense eiwit spot op de 2D gel en de hoeveelheid eiwit was geschat op ongeveer 0.6 µg A1AT per ml plasma stolsel. We hebben de affiniteit van A1AT voor fibrine bekeken met fibrine-sepharose. Er bond maar een kleine hoeveelheid A1AT (0.1%) aan de fibrine-sepharose. Maar wanneer we dit gebonden A1AT van de kolom elueerde, de elutiebuffer met kalium thiocyanaat eruit dialyseerde en dat A1AT weer opbrachten op de kolom zagen we dat rond de 30% van de A1AT was gebonden. Dit suggereert dat de A1AT gebonden aan het fibrine stolsel een aparte vorm van A1AT is met een hoge affiniteit voor fibrine. Gebonden A1AT inhibeerde de fibrinolytische activiteit van elastase. Leukocyten die in een fibrine stolsel infiltreren scheiden elastase uit en A1AT kan daardoor lokaal als een anti-fibrinolytisch eiwit fungeren.

Carboxypeptidase N (CPN) is een ander nieuw plasma stolsel component dat wellicht een anti-fibrinolytische werking heeft. De aanwezigheid van CPN in een plasma stolsel was in detail onderzocht in hoofdstuk 4. De hoeveelheid CPN in een plasma stolsel was ongeveer 30 ng per ml en de vormen van CPN in een stolsel waren dezelfde vormen die in plasma circuleren. We konden niet de proteolytisch geknipte vormen van CPN die wellicht een hogere activiteit hebben detecteren. Met plasmon surface resonance hebben we aangetoond dat CPN direct aan fibrinogeen en fibrine kan binden met een Kd rond 100 nM.

Twee derde van de nieuwe plasma stolsel componenten waren HDL eiwitten. Daarom hebben we de rol van HDL in hemostase onderzocht met tromboelastometrie in hoofdstuk 5. Normaal HDL (nHDL) had geen effect maar we zagen wel dat geoxideerd HDL

(oxHDL) de sterkte van een bloed stolsel verminderde zonder invloed uit te oefenen op de stolling. Afbraak van fibrine was niet betrokken bij de verminderde sterkte van het stolsel wat suggereert dat plaatjes een rol spelen. Maar het effect van oxHDL op de retractie van een plasma stolsel en plaatjes aggregatie was klein en kon niet het effect op de clot sterkte verklaren. Meer onderzoek is nodig om het mechanisme te verklaren.

Het vaststellen van verschillen in aanwezigheid van eiwitten in een plasma stolsel van gezonde controles en trombose patiënten zou nieuwe risico factoren voor trombose kunnen opleveren. In hoofdstuk 6 hebben we de hoeveelheid van eiwitten in een plasma stolsel vergeleken tussen Budd-Chiari syndroom (BCS) patiënten en controles met 2D-fluorescence-based difference gel electrophoresis (2D-DIGE). BCS patiënten hadden minder apolipoprotein AI (apo AI) aanwezig in hun stolsels. Apo AI is het voornaamste eiwit in HDL en de plasma concentratie van Apo AI en HDL cholesterol was ook lager bij een grote groep BCS patiënten in vergelijking met controle personen. BSC patiënten zonder insufficiëntie in hun eiwit synthese in de lever hadden ook lager Apo AI plasma concentratie dat aangeeft dat de verlaagde plasma concentratie van apo AI niet alleen komt door de mogelijke lever insufficiëntie en dus mogelijk een rol speelt in de ontwikkeling van veneuze trombose in BCS patiënten.

Bloedings complicaties zijn veel voorkomend in patiënten met cirrose, mogelijk door een verstoorde balans in stollings- en fibrinolytische factoren. Fibrinolyse kan onderzocht worden met verschillende assay's, maar het is nog niet geheel duidelijk of patiënten met cirrose verhoogde fibrinolyse hebben. Daarom hebben wij de fibrinolyse in een groep cirrose patiënten met een plasma clot lysis assay onderzocht en ook met een globale fibrinolyse capaciteit assay in hoofdstuk 7. We hebben geconstateerd dat 40% van de patiënten hyperfibrinolyse hadden in de plasma clot lysis assay en 43% in de globale fibrinolytische capaciteit assay. Van alle patiënten had 60% hyperfibrinolyse in op z'n minst een van de twee assay's. De correlatie tussen de hyperfibrinolyse en bloeding complicaties konden we niet vaststellen omdat er weinig patiënten waren met bloedingen in onze studie gedurende de studieperiode.

In hoofdstuk 7 hebben we gezien dat 40% van de cirrose patiënten hyperfibrinolyse hebben als het gemeten wordt met de plasma clot lysis assay. Niet alle studies detecteren hyperfibrinolyse als ze cirrose patiënten meten met dezelfde assay. Daarom hebben we in hoofdstuk 8 de biologische variatie in clot lysis tijd (CLT), gemeten met de clot lysis assay bepaald. Deze informatie kan wellicht inzicht geven waarom er verschillende resultaten zijn beschreven in de verschillende studies. Informatie over de biologische variatie van de CLT was niet beschikbaar en deze informatie is nodig voor een optimale studie opzet. De totale, analytische, binnen-persoon en tussen-persoon variatie was 13.4%, 2.6%, 8.2% en 10.2%, respectievelijk. Centrifugatie condities om plaatjes arm plasma te verkrijgen had geen invloed op de CLT. De CLT was 's ochtends langer dan in de rest van de dag maar had geen seizoen's variatie. Wanneer de CLT wordt bepaald in 1 monster kan het afwijken,

maar niet meer dan 20%, met een kans van 95% van de echte waarde. Wanneer de meting van CLT nauwkeuriger moet zijn dan moeten er meer monsters gemeten worden.

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Bedankt!
Simone

PUBLICATIONS

Talens S, Lebbink JH, Malfliet JJ, Demmers JA; Uitte de Willige S, Leebeek FW, Rijken DC (2012). Binding of carboxypeptidase N to fibrinogen and fibrin. Biochem Biophys Res Commun 427(2): 421–425

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Van Zwam M, Samsom JN, Nieuwenhuis EE, Melief MJ, Wierenga-Wolf AF, Dijke IE, **Talens S**, van Meurs M, Voerman JS, Boven LA, Laman JD (2011). Myelin ingestion alters macrophage antigen-presenting function in vitro and in vivo. J Leukoc Biol 90(1): 123–132.

Talens S, Hoekstra J, Dirkx SP, Murad SD, Trebicka J, Elias E, Primignani M, García-Pagán JC, Valla DC, Janssen HL, Leebeek FW, Rijken DC; EN-Vie Study Group (2011). Proteomic analysis reveals that apolipoprotein AI levels are decreased in patients with Budd-Chiari syndrome. J Hepatol 54(5): 908–914.

Talens S, Malfliet JJ, Leebeek FW, Rijken DC (2012). Identification and characterization of α_1-antitrypsin in fibrin clots. Submitted.

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CURRICULUM VITEA

Simone Talens werd geboren op 29 november 1981 in Rhenen. Na het behalen van haar VWO diploma in 2000 aan het Rembrandt College te Veenendaal ging zij Biomedische wetenschappen studeren aan de Universiteit van Utrecht. In 2005 behaalde zij haar master diploma met als afstudeerrichting biomoleculaire wetenschappen. Tijdens haar studie liep ze stage bij de afdeling Biochemie van Membranen van het Biomembraan instituut van de Universiteit van Utrecht onder begeleiding van Dr. T. de Kroon en Dr. P.J. Rijken. Tijdens deze stage deed ze onderzoek naar de expressie en zuivering van het gist eiwit Gut2p. Haar tweede stage met als onderwerp de functies van het eiwit FIC1 deed ze onder begeleiding van Dr. J. Koedam en Dr. L Klomp bij de afdeling endocriene en metabole ziekten in het Universitair Medisch Centrum Utrecht. In 2007 begon ze aan haar promotie onderzoek bij de afdeling Hematologie in het Erasmus Medisch Centrum te Rotterdam onder begeleiding van Dr. D.C. Rijken en Prof. dr. F.W.G. Leebeek. De resultaten van dat promotieonderzoek zijn beschreven in dit proefschrift. Sinds februari 2013 is ze werkzaam als post-doctoral research fellow op de afdeling Laboratory Medicine van de Universiteit van Lund in Malmö, Zweden, in de groep van Prof. Dr. Anna Blom.

PhD PORTFOLIO SUMMARY

Summary of PhD training and teaching activities

Name PhD student: Simone Talens	PhD period: September 2007 – September 2012
Erasmus MC Department: Hematology	Promotor: Prof. Dr. F.W.G. Leebeek
Research School: COEUR	Supervisor: Dr. D.C. Rijken

1. PhD training

	Year	ECTS
Courses		
- Vascular medicine (COEUR)	2007	1.5
- Biomedical research techniques (MolMed)	2008	1.5
- Biomolecular mass spectrometry course	2008	1.5
- Molecular Diagnostics (MolMed)	2008	0.6
- Workshop on bioinformatic analysis, tools and services	2008	0.3
- Molecular biology in cardiovascular research (COEUR)	2009	1.5
- NVTH PhD course on arterial thrombosis, venous thrombosis and bleeding disorders (3x)	2008-2010	2.7
- ETRO advanced teaching course on thrombosis (2x)	2009-2011	3
- Scientific writing in english for publication	2010-2011	2
Seminars		
- COEUR research seminars (8x)	2007-2010	3.2
National conferences		
- Proteomics platform meeting	2007	0.3
- NVTH symposium (5x)	2008-2012	2
- AMSTOL symposium	2009	0.3
- Nederlandse lipiden club seminar	2009	0.3
Presentations		
- oral national conference (2x)	2009-2011	3
- oral international conference (2x)	2010-2012	3
- poster international conference (4x)	2009-2012	6
International conferences		
- International fibrinogen workshop (3x)	2008-2012	3
- ISTH conference (2x)	2009-2011	3
- ISFP conference	2010	1
- ATVB conference	2012	1

2. Teaching activities

Supervision		
- supervision of technical student (2x)	2009-2011	4
Total		34.5