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Contents

CHAPTER 1

General Introduction

The hemostatic system

Hemostasis is a complex process to stop bleeding from damaged blood vessels. A disturbance in the hemostatic system may result in either a bleeding or a thrombotic tendency. The hemostatic process can be divided into platelet adhesion and aggregation, coagulation, and fibrinolysis also termed primary, secondary, and tertiary hemostasis. The actual hemostatic process, however, involves a dynamic interplay between these events. Figure 1 shows a schematic representation of hemostasis.

Primary hemostasis

Primary hemostasis comprises the formation of a platelet plug to close a ruptured vessel. After injury to the vessel wall, exposure of collagen and other subendothelial proteins initiates recruitment of platelets. Plasma von Willebrand factor (VWF) binds to the subendothelial collagen and becomes adhesive to platelets. The multimeric size of VWF is regulated by the protease ADAMTS13 (a disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13). Platelets are slowed down by rolling over collagen bound VWF, and eventually adhere by multiple receptors. Then platelets become activated by various triggers, including collagen and thrombin, which results in the activation of the aggregation receptor αIIbβ3. Finally, platelet-platelet interaction, mediated by VWF or fibrinogen binding to αIIbβ3 on two adjacent platelets, results in the formation of a platelet plug.

Secondary hemostasis

In secondary hemostasis, complex reactions of pro- and antihemostatic proteins lead to the generation of thrombin. Thrombin generation is required for both platelet activation and the cleavage of fibrinogen into fibrin. The formation of fibrin is necessary to reinforce the platelet plug.

The classis cascade model of coagulation was in 1964 independently proposed by two groups of scientists [1,2]. This model involves the sequential activation of clotting factors leading to the generation of thrombin. Two coagulation pathways were proposed: the intrinsic pathway initiated via coagulation factor XII, and the extrinsic pathway initiated through the tissue factor-VIIa complex. These two pathways form the basis of the coagulation screening: the intrinsic activation is used in the activated partial thromboplastin time (APTT), and the extrinsic activation in the prothrombin time (PT). However, the more recent cell-based theory better reflects hemostasis in vivo than the classic cascade model of coagulation [3-5]. This theory suggests that coagulation does not occur as a cascade, but in three overlapping stages: 1) initiation occurring on a tissue factor bearing cell, 2) amplification to activate platelets and cofactors for the start of large scale thrombin generation, and 3) propagation with the generation of large amounts of thrombin on the platelet surface.
Coagulation is initiated when vessel wall damage exposes tissue factor (TF) to the bloodstream. TF on TF-bearing cells binds to coagulation factor VII and the FVIIa/TF complex activates small amounts of factors IX and X. Activated factor X is able to convert small amounts of prothrombin (factor II) into thrombin (activated factor II; IIa). In the amplification phase, thrombin activates platelets, factor V (cofactor for factor X), factor VIII (cofactor for factor IX), and factor XI (which can also activate factor IX). During the propagation phase, occurring on the surface of the activated platelets, factor IXa binds to factor VIIIa to activate factor X. Factor Xa combines with factor Va to convert large amounts of prothrombin into thrombin. Finally, thrombin will convert fibrinogen into fibrin to stabilize the clot.

Thrombin generation is regulated by several anticoagulant proteins. For instance, tissue factor pathway inhibitor (TFPI) inactivates factor VIIa and Xa. Antithrombin mostly inhibits thrombin, but also inhibits factor IXa, factor Xa, factor XIa, and factor VIIa. Furthermore, thrombin binding to the endothelial receptor thrombomodulin activates protein C, which together with its cofactor protein S inactivates cofactors Va and VIIIa.

Fibrinolysis

Since minor injuries are common, there are constantly clots produced to stop bleeding. If clots remained after the tissue is healed, the vascular bed would become obstructed with clots. Therefore, clots need to be broken down by fibrinolysis, also termed tertiary hemostasis. Plasmin is responsible for clot lysis by degrading insoluble fibrin to soluble fibrin degradation products. Plasmin is formed by activation of plasminogen by tissue-type plasminogen activator (t-PA). Plasmin generation is regulated by plasminogen activator inhibitor-1 (PAI-1), which is an inhibitor of t-PA, and plasmin inhibitor, which inactivates plasmin. Furthermore, activated factor XIII and activated thrombin activatable fibrinolysis inhibitor (TAFI), both of which are activated by thrombin generated in the coagulation cascade, make the fibrin clot more resistant to plasmin. Specifically, factor XIIIa cross-links fibrin fibers to enhance the stability of the fibrin clot, and TAFI interferes with plasminogen and t-PA binding to the clot by removing the lysine binding sites on fibrin.

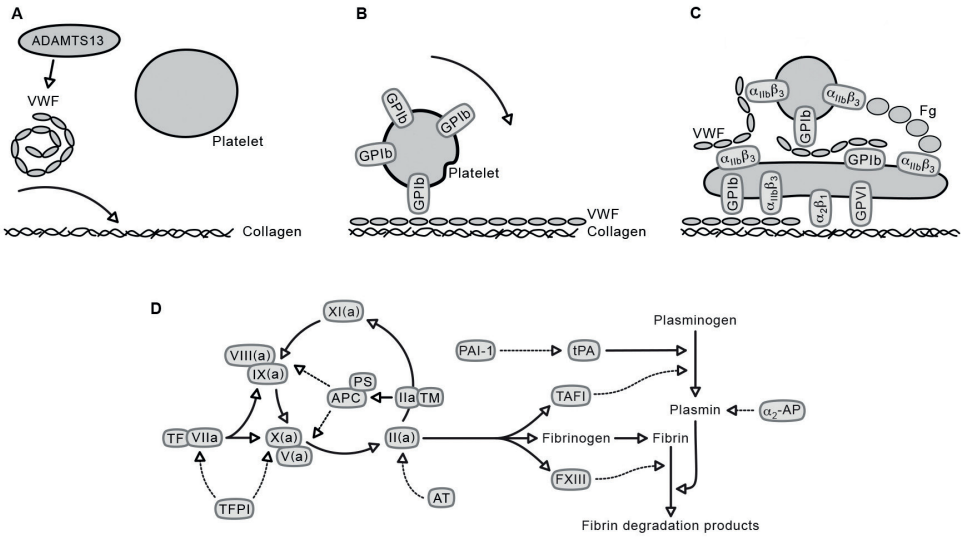

Figure 1 Schematic representation of the hemostatic system. Section A-C represents primary hemostasis with the formation of a platelet plug. Section D shows the coagulation cascade with the formation of fibrin and the subsequent degradation by the fibrinolytic system. Activation steps are indicated by the uninterrupted lines, whereas regulatory or inhibitory steps are indicated by interrupted lines. ADAMTS13: a disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13, VWF: von Willebrand factor, GP: glycoprotein, Fg: fibrinogen, TF: tissue factor, TFPI: tissue factor pathway inhibitor, APC: activated protein C, PS: protein S, TM: thrombomodulin, AT: antithrombin, PAI-1: plasminogen activator inhibitor-1, tPA: tissue-type plasminogen activator, TAFI: thrombin activatable fibrinolysis inhibitor, α2-AP: α2-antiplasmin = plasmin inhibitor.

Hemostatic alterations in liver disease

The liver plays a central role in the hemostatic system as it synthesizes the majority of coagulation factors, anticoagulant proteins, and proteins involved in fibrinolysis. Furthermore, the liver regulates primary hemostasis mediated by platelets, VWF, and ADAMTS13. Consequently, acute or chronic liver diseases frequently have a profound impact on the hemostatic system [6]. The hemostatic changes associated with chronic liver disease will be extensively reviewed in Chapter 2.

In short, the hemostatic changes in a patient with liver disease include thrombocytopenia, decreased levels of pro- and anticoagulant proteins, reduced levels of fibrinolytic proteins, and increased plasma levels of coagulation factor VIII and VWF. Historically, these changes were interpreted as predisposing for a bleeding tendency due to abnormal routine diagnostic tests of hemostasis, such as a prolonged PT and APTT. However, in recent years the concept of a "rebalanced hemostasis" [6] has become widely accepted (Figure 2). This balance is the result of concomitant changes in both pro- and antihemostatic pathways. First, the reduced platelet count and functional platelet defects are accompanied by high plasma levels of the platelet adhesive protein VWF [7]. Furthermore, the VWF-cleaving protease ADAMTS13 is reduced in chronic liver disease, which may also promote platelet function [8]. Second, the decreased production of procoagulant proteins is accompanied by a decreased production of anticoagulant proteins, such as protein C, protein S, and antithrombin. Finally, the balance of fibrinolysis is probably also restored in patients with chronic liver disease due to the concomitant decrease of antifibrinolytics (PI, TAFI) and plasminogen [9]. However, some authors have also reported hyperfibrinolysis in patients with cirrhosis [10]. Clinically, the rebalanced hemostasis is reflected by the large proportion of patients with liver disease who can undergo major surgery without any requirement for blood transfusion [11]. In addition, more sophisticated laboratory tests have suggested a hemostatic balance in patients with liver disease. For example, several studies showed normal to increased thrombin generation in patients with cirrhosis compared to that of healthy volunteers, as a result of a concomitant decline in both pro- and anticoagulant proteins [12-15].

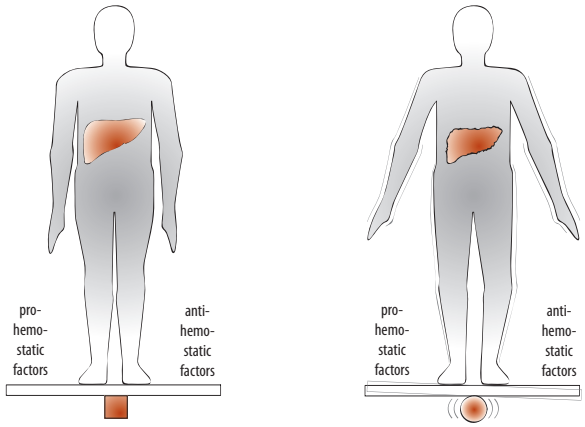

Figure 2 The hemostatic balance in patients with liver disease compared to that in healthy individuals. This figure depicts the stable hemostatic balance in healthy individuals and the rebalanced hemostatic system in patients with liver disease. Although the hemostatic system is in balance, the balance is fragile and may easily tip to either a hypo- or hypercoagulable status with the risk of bleeding or thrombosis.

The "rebalanced" hemostatic system in patients with liver disease, however, appears much more fragile compared to the hemostatic balance of healthy individuals. This precarious hemostatic balance contributes, in part, to the occurrence of both bleeding and thrombotic complications, which occur in a significant proportion of patients with liver diseases [6,16]. Furthermore, it has to be noted that the changes in the hemostatic system of patients with liver disease vary with the etiology of the disease. For instance, in patients with cholestatic liver disease hemostatic defects are milder as compared to that in patients with non-cholestatic liver disease [17]. In patients with acute liver failure, thrombocytopenia is less common, levels of coagulation factors are generally lower, and fibrinolysis is more inhibited compared to patients with cirrhosis [18]. Finally, studies have suggested a hypercoagulable state in patients with non-alcoholic fatty liver disease (NAFLD), which might contribute to their increased risk of thrombosis [19-22]. In Chapter 6 of this thesis, an extensive overview of the hemostatic system of patients with NAFLD will be given.

Thrombosis and anticoagulant drugs in patients with liver disease

Patients with chronic liver diseases are nowadays more commonly diagnosed with thrombotic complications. For instance, the development of portal vein thrombosis (PVT) is a common complication of cirrhosis [23]. In addition, numerous studies have shown a significant occurrence of venous thromboembolism (VTE, i.e. deep vein thrombosis (DVT) and pulmonary embolism (PE)) in patients with liver disease [24-28]. Finally, evidence suggests an increased prevalence of arterial thrombosis in patients with NAFLD (see Chapter 6).

Patients with liver disease may, thus, have multiple indications for antithrombotic therapy to prevent or treat thrombosis. A number of possible anticoagulant drugs are clinically available, however all these drugs have different advantages and disadvantages in patients with liver diseases. Heparins can be used for prevention or treatment of venous thrombosis and PVT. They act by binding to antithrombin to inhibit factor Xa and/or thrombin. Three classes of heparins are clinically available: unfractionated heparin, low molecular weight heparin (LWMH), and fondaparinux. LMWHs appear to be safe and effective in patients with cirrhosis and PVT or venous thrombosis [29-34]. However, heparin requires antithrombin to exert its anticoagulant effect and antithrombin levels are frequently decreased in patients with liver disease. Another disadvantage of heparins is their mode of administration (i.v. for unfractionated heparin, and s.c. for LMWH and fondaparinux), which may limit long-term use. Vitamin K antagonists are the most prescribed anticoagulants in the general medical population worldwide. Vitamin K antagonists act by decreasing vitamin K-dependent procoagulant factors II, VII, IX, X, and anticoagulant proteins C and S. A major advantage of vitamin K antagonist therapy is the oral mode of administration. However, a major drawback in patients with liver disease is the monitoring of the drug by INR levels which are already abnormal in cirrhotic patients. New-generation anticoagulant drugs, including direct factor Xa inhibitors (rivaroxaban, apixaban, and endoxaban) and direct factor IIa inhibitor (dabigatran), are garnering increasing interest due to their oral mode of administration and lack of need for monitoring. Reports of success in cirrhotic patients are emerging [35-38], however larger clinical trials on efficacy and safety of these drugs in cirrhotic patients are lacking.

Due to the profound alterations in the hemostatic system of patients with liver disease, anticoagulation in these patients is very challenging. While there are many antithrombotic agents available, little research has been done on the efficacy and safety of these agents in

patients with liver disease. As a result, the anticoagulant drug of choice and dosing for the various indications is still unknown. The prevention and treatment of thrombosis in patients with liver disease will be further discussed in Chapter 2 of this thesis.

Aim of this thesis

In this thesis, novel concepts of the functionality of (parts) of the hemostatic system in patients with liver disease will be described. Furthermore, we study the efficacy of current available anticoagulant drugs in plasma from patients with cirrhosis. Finally, the use of routine coagulation assays in the monitoring of different available anticoagulant drugs in plasma from patients with cirrhosis is examined. The major part of this thesis is focused on gaining a better understanding of the hemostatic system in patients with (fatty) liver disease and to contribute to better strategies to prevent or treat thrombosis in these patients in the future.

Chapter 2 describes the current literature on the hemostatic changes in patients with liver disease in detail. In this review we discuss the concept of a rebalanced hemostasis and its implications for clinical management of patients with liver disease. In **chapter 3,** plasma levels of protein S and TFPI in patients with cirrhosis are determined. Furthermore, we study the functionality of the TFPI/protein S anticoagulant system in these patients. **Chapter 4** describes the use of the novel Thrombodynamics analyzer to test plasma hemostatic capacity in patients with chronic liver disease.

Comparable to patients with liver disease, in patients following liver resection plasma levels of hemostatic proteins decrease, in part due to a reduced synthetic capacity of the liver remnant. Also conventional coagulation tests, such as the PT and APTT, suggest a hypocoagulable state in these patients. These tests are not reliable for the assessment of the overall hemostatic status in these patients, because they only asses isolated defects of procoagulants and are insensitive for anticoagulant factors. Thrombin generation testing is a more global hemostasis test and has been successfully used to reassess the hemostatic status of patients with liver disease. In **chapter 5**, we, therefore, study the perioperative coagulation status in patients undergoing hemi-hepatectomy using thrombin generation assays.

The next part of this thesis will focus on the hemostatic system in patients with NAFLD. **Chapter 6** gives an extensive overview of the current literature on the hemostatic changes in patients with NAFLD and its relation with increased risk of cardiovascular disease. Recent studies have suggested a role for a hypercoagulable state in the increased risk of thrombosis in patients with NAFLD. However, results of these studies are inconsistent, most studies only reported plasma levels of individual hemostatic proteins rather than functional tests of global hemostasis, and patients with cirrhosis were excluded from most of these studies. The hemostatic status across the spectrum of NAFLD stages thus remains unclear. In **chapter 7**, we therefore study all components of the hemostatic system (i.e., platelets, coagulation, and fibrinolysis) using both biomarkers and functional tests in patients with various histological severities of NAFLD.

In the last parts of this thesis, the focus will be on the evaluation of the different available anticoagulant drugs in patients with liver disease. In **chapter 8**, we evaluate the in vitro effect of different anticoagulant drugs in plasma from patients with cirrhosis. In the **appendix to chapter 8**, we examine the in vitro anticoagulant potency of Apixaban (direct factor Xa inhibitor), which we compare to the anticoagulant potency of Rivaroxaban (another direct

factor Xa inhibitor) by using thrombomodulin-modified thrombin generation testing. This is studied in response to two recent reports on the use of these new oral anticoagulant drugs in the treatment of PVT. Monitoring of anticoagulant drugs may be required in patients with liver disease, which however, appears to be difficult due to the hemostatic changes associated with the underlying liver disease. The aim of **chapter 9** is to determine whether different anticoagulant drugs can be reliably monitored using anti-Xa or anti-IIa tests in plasma from patients with cirrhosis. The **appendix to chapter 9** reports the issues with monitoring of unfractionated heparin in patients with cirrhosis.

In **chapter 10,** the results presented in the previous chapters are discussed in a broader perspective. In addition, the implications for clinical management of hemostatic abnormalities in patients with liver disease are discussed. Finally, recommendations for future research are given.

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CHAPTER
1

CHAPTER 2

Management of coagulation abnormalities in liver disease

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Abstract

Liver disease is characterized by changes in all phases of hemostasis. These hemostatic alterations were long considered to predispose patients with liver disease towards a bleeding tendency, as they are associated with prolonged conventional coagulation tests. However, these patients may also suffer from thrombotic complications, and we now know that the hemostatic system in patient with liver disease is, in fact, in a rebalanced state. In this review we discuss the concept of rebalanced hemostasis and its implications for clinical management of patients with liver disease. For instance, there is no evidence that the use of prophylactic blood product transfusion prior to invasive procedures reduces bleeding risk. Clinicians should also be aware of the possibility of thrombosis occurring in patients with a liver disease, and regular thrombosis prophylaxis should not be withheld in these patients.

Introduction

Chronic and acute liver diseases alter the hemostatic system tremendously [1]. Historically, patients with liver diseases were considered to be at high risk for bleeding complications due to the negative impact of the diseased liver on platelet synthesis and function, reduced production of coagulation factors and hyperfibrinolysis. However, the liver is also involved in the synthesis of various antihemostatic and antifibrinolytic proteins and therefore the hemostatic system is perceived to be rebalanced, as shown by recent laboratory studies and clinical observations [2]. This rebalance in the hemostatic system is not reflected by conventional coagulation tests, such as the prothrombin time (PT) and the activated partial thromboplastin time (APTT) [3]. Hence, these tests cannot be used to assess bleeding or thrombotic risk in patients with liver disease. Clinically, the rebalanced hemostatic system is reflected by the large proportion of patients with liver disease who can undergo major surgery without any requirement for blood product transfusion [4]. In fact, prophylactic use of blood products may even contribute to bleeding rather than prevent it. However, correcting the laboratory coagulopathy by transfusion of blood products prior to invasive procedures is still common practice in many centers [5,6].

The occurrence of both clinical thrombotic events and bleeding complications in patients with liver disease suggests that the balance of the hemostatic system is more fragile in these patients than in healthy individuals [7]. In fact, treatment and prevention for thrombotic complications is often necessary [8,9]. However, due to the profound hemostatic alterations and insufficient clinical experience, antithrombotic therapy is highly complicated in patients with liver disease and the best choice of drugs is still unknown.

This review summarizes the changes in the hemostatic system in patients with liver diseases. In addition, we review the limitations of laboratory tests in the investigation of bleeding or thrombotic risk in these patients. Finally, we discuss difficulties in management of both bleeding and thrombotic complications in liver disease patients.

The hemostatic system in patients with liver disease

The hemostatic system comprises platelet aggregation, coagulation and fibrinolysis also termed primary, secondary and tertiary hemostasis. Liver disease is associated with changes in all these phases of hemostasis (Table 1). Historically, these changes were interpreted as predisposing for a bleeding tendency due to the abnormal laboratory coagulation tests and the observation that spontaneous bleeding occurs frequently in this group of patients. However, several authors have pointed out shortcomings of this classical interpretation of the coagulopathy of liver disease [10–13] and in recent years the concept of 'rebalanced hemostasis' [2] has become widely accepted. In this concept, we suggested a new but more fragile balance within and between the procoagulant, anticoagulant and fibrinolytic systems.

Changes in primary hemostasis

In primary hemostasis, the formation of a platelet plug is initiated by vessel wall damage with the exposure of platelet adhesion proteins such as collagen. Concurrent tissue factor exposure activates the plasmatic coagulation cascade. Then, activated platelets capacitate the rapid production of a fibrin mesh by exposing activated clotting factors on their surface and producing a 'thrombin burst' through a positive feedback mechanism [14].

A reduced platelet count is common in patients with acute or chronic liver disease. The etiology of thrombocytopenia in patients with liver diseases is multifactorial. An important cause includes pooling of platelets and sequestration in the spleen due to congestive splenomegaly, which is related to portal hypertension [15]. Some authors have also suggested a role of antiplatelet antibodies [16] and decreased production of platelets because of lower levels of hepatic thrombopoietin [17]. Furthermore, platelet consumption, because of cirrhosis-related hypercoagulability resulting in systemic or intrahepatic platelet activation, has also been assumed to be an etiopathologic factor of thrombocytopenia in patients with chronic liver disease [18].

Besides thrombocytopenia, multiple mechanisms predisposing to functional platelet defects have also been described [11]. On the other hand, high plasma levels of von Willebrand factor (VWF) might compensate for defects in platelet number and function in patients with cirrhosis [19]. Indeed, it has been shown that VWF levels are elevated in proportion to the severity of liver disease [20]. The elevated levels of VWF may be a consequence of endothelial activation commonly observed in patients with liver disease. Endothelial activation is considered an important consequence of portal hypertension, and in fact, it was shown that VWF levels are associated with clinically significant portal hypertension [21]. Other possible mechanisms of elevated levels of VWF in cirrhosis are induction of synthesis of VWF in the cirrhotic liver itself or reduced liver-mediated clearance. Although the concept of platelet hypofunction in cirrhosis has long been widely accepted, more recent work suggests that platelet function might not be abnormal when studied under physiological test conditions. One study showed that although in-vitro platelet adhesion to subendothelial structures under conditions of flow is substantially reduced, this was fully attributable to the reduced platelet count and reduced hematocrit in these patients [22]. Another study even provided evidence for enhanced platelet function [23].

Changes in the coagulation cascade

In secondary hemostasis, complex reactions of the pro- and antihemostatic proteins in the coagulation cascade lead to the formation of a fibrin clot (Figure 1). The liver produces all plasma proteins involved in the generation of a fibrin mesh (except factor VIII). As a result, levels of coagulation factors V, VII, IX, X, XI and prothrombin are commonly reduced in both acute and chronic liver disease [24]. In contrast, factor VIII levels are often elevated [25], possibly due to upregulated synthesis from extrahepatic sites, such as the lung, spleen and kidney [26]. Other possible causes for the high levels of FVIII may be the elevated levels of VWF, the carrier protein of FVIII or reduced FVIII clearance. Fibrinogen levels are frequently reduced (except for patients with biliary cirrhosis, in which fibrinogen levels may be elevated), especially in patients with acute liver failure or advanced cirrhosis. In addition, fibrinogen is often functionally aberrant as a result of excessive sialic acid content leading to impaired fibrin polymerization [27].

The decreased production of procoagulant factors is mostly counterbalanced by decreased production of anticoagulant proteins, such as protein C, protein S, protein Z, protein Z-dependent protease inhibitor, antithrombin (AT), heparin cofactor II and a2-macroglobulin, which are all produced by the liver [1]. Tissue factor pathway inhibitor (TFPI) is synthesized by endothelial cells, and as a result of continuous activation of the endothelium in patients with liver disease, these patients are assumed to have increased levels of TFPI. However, studies

have shown either increased or normal levels of TFPI in patients with acute or chronic liver diseases. It has recently been established that protein S acts as a cofactor for TFPI in the downregulation of thrombin generation and, as a result, acquired and congenital protein S deficiencies are associated with a concomitant TFPI deficiency [28]. It may thus be that the increased TFPI release in patients with liver disease is masked by the decrease in protein S. Indeed, we recently reported that, despite a substantial decrease in protein S levels in patients with cirrhosis, TFPI levels are comparable between patients and healthy individuals. However, despite normal TFPI plasma levels, the TFPI/protein S anticoagulant system is functionally impaired in patients with cirrhosis [29, Chapter 3 of this thesis].

Studies using the thrombin generation test have examined the net balance of secondary hemostasis. As shown in Table 1, these studies have either shown a normo- or hypercoagulable state. These contrasting data may be attributed to differences in methodology, but probably also to differences in the disease severity of included patients. In fact, it has been hypothesized that progression of liver disease is correlated with a more hypercoagulable state, possibly due to the progressive decrease of protein C [30] and/or increase of FVIII levels [24,31] with increasing disease severity.

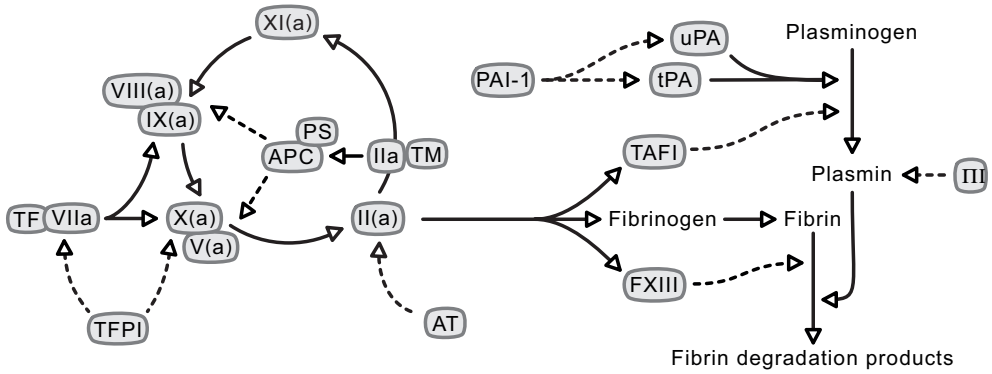

Figure 1. Schematic representation of the coagulation cascade and fibrinolytic system resulting in the generation and subsequent breakdown of a fibrin clot.
In this figure, activator processes are indicated by the uninterrupted lines, whereas regulatory or inhibitory steps are indicated by interrupted lines. After vessel wall damage, binding of coagulation factor VII to the exposed transmembrane protein TF initiates a series of enzymatic reactions in which proenzymes are activated into active forms. This cascade results in the generation of thrombin (IIa), which then cleaves fibrinogen into fibrin. Thrombin generation is downregulated by TFPI and AT, which inactivate factor VIIa and Xa, and thrombin, respectively. Furthermore, activated protein C, activated when thrombin binds to its endothelial receptor TM, inactivates cofactors Va and VIIIa. The fibrinolytic system can break down the fibrin clot, which is initiated by release of plasminogen activators tPA or uPA from endothelial cells, macrophages or renal epithelial cells. The plasminogen activators activate plasminogen to form plasmin, an enzyme that degrades fibrin into fibrin degradation products. Plasmin generation is regulated by PAI-1, a direct inhibitor of tPA and uPA, and by PI, which inactivates plasmin. Furthermore, activated FXIII and activated TAFI, both activated by thrombin generation in the coagulation cascade, render the fibrin clot more resistant to plasmin. AT: Antithrombin; PAI-1: Plasminogen activator inhibitor type 1; PI: Plasmin inhibitor; TAFI: Thrombin activatable fibrinolysis inhibitor; TF: Tissue factor; TFPI: Tissue factor pathway inhibitor; TM: Thrombomodulin; Tpa: Tissue-type plasminogen activator.

Changes in the fibrinolytic system

The liver synthesizes all proteins involved in the breakdown of a fibrin clot (fibrinolysis, see Figure 1), except for tissue-type plasminogen activator (tPA) and plasminogen activator inhibitor 1 (PAI-1). Indeed, levels of plasminogen, plasmin inhibitor, thrombin activatable fibrinolysis inhibitor and factor XIII are frequently reduced in both acute and chronic liver disease [32,33]. On the other hand, plasma levels of tPA are elevated as a result of enhanced release by activated endothelium cells and/or due to a reduction in the clearance of tPA by the diseased liver. Furthermore, PAI-1 levels are substantially increased in acute liver failure [34], but modestly increased in chronic liver disease

[35]. The net effect of these changes has often been described as hyperfibrinolytic, but its mechanistic role in bleeding is still debated [11]. Although contrasting results have been reported (Table 1), the balance of fibrinolysis is probably restored in patients with chronic liver disease by the parallel changes in the profibrinolytic and antifibrinolytic proteins [32]. However, in patients with acute liver failure the balance is probably shifted toward hypofibrinolysis due to the elevated levels of PAI-1 [34], and hyperfibrinolysis may occur during liver transplantation as a result of lack of clearance of tPA during the anhepatic phase.

Hemostatic phase	Prohemostatic drivers	Antihemostatic drivers	Studies showing either hypo-, normo-, or hypercoagulability
Primary hemostasis	Elevated levels of VWF [19] Low levels of ADAMTS13 [98]	Thrombocytopenia [99] Functional platelet defects [11]	Platelet defects shown in: [100-103] Normal platelet function shown in: [22,104] Platelet hyper function shown in: [23,105]
Secondary hemostasis	Low anticoagulant factors [1]: protein C, protein S, protein Z, protein Z-dependent protease inhibitor, antithrombin, heparin cofactor II, and α_2-macroglobulin High procoagulant factor VIII [25] Impaired TFPI-protein S anticoagulant system [29]	Low procoagulant factors [24]: fibrinogen, factors II, V, VII, IX, X, XI	Normocoagulability in patients with acute liver failure: [51] Normocoagulability in patients with cirrhosis suggested in: [3,49] Hypercoagulability in patients with cirrhosis suggested in: [3,9,24,31,50]
Tertiary hemostasis	Low levels of plasminogen [33] High levels of PAI-1 [34,35]	High levels of t-PA Low levels of TAFI, factor XIII, and alpha-2-antiplasmin [32]	Hypofibrinolysis in patients with acute liver failure: [34,51] Normofibrinolysis in patients with cirrhosis suggested in: [32] Hyperfibrinolysis in patients with cirrhosis suggested in: [35, 106-108]

Table 1. Summary of studies on the changes in all phases of the hemostasis in patients with liver disease. ADAMTS 13: a disintegrin and metalloprotease with thrombospondin type 1 motif 13; PAI: plasminogen activator inhibitor; TAFI: thrombin-activatable fibrinolysis inhibitor; t-PA: tissue plasminogen activator; VWF: von Willebrand Factor.

Rebalanced but fragile: bleeding & thrombosis risk

Thus, despite the profound hemostatic alterations, the hemostatic system appears to be rebalanced in patients with liver disease. However, this balance is far more precarious and potentially unstable compared with the hemostatic balance in healthy individuals, which explains the occurrence of both bleeding and thrombotic complications in these patients. In fact, there are a variety of disturbances that can predispose an individual liver disease patient to either bleeding or thrombosis. For instance, development of renal failure is common in advanced liver disease and this usually leads to a bleeding tendency as a result of acquired platelet dysfunction, abnormal platelet–vessel wall interaction and anemia [36]. Another important and often coexisting modulator of hemostasis is the appearance of bacterial infections. Endotoxins may inhibit platelet function by prostacyclin production and enhancement of nitric oxide and inhibit coagulation by stimulating generation of heparin-like substances [37]. Bacterial infection may thus increase the risk of initiation and failure to control bleeding. However, some investigators also suspect a potential direct effect of endotoxin in the activation of the clotting cascade [38], leading to disseminated intravascular coagulation. Indeed, during endotoxemia or sepsis, endotoxin induces tissue factor expression in macrophages or endothelial cells, possibly contributing to development of disseminated intravascular coagulation [39,40]. Prophylactic administration of antibiotic drugs to patients with chronic liver disease is known to reduce mortality and improve the hemostatic function [41], but the exact mechanism is unknown. It is therefore recommended to adequately diagnose and treat infections before invasive procedures.

Variceal bleeding is one of the most common bleeding events occurring in patients with advanced liver disease, occurring in 20–30% of patients with cirrhosis [42]. However, the occurrence of variceal bleeding in patients with cirrhosis is mostly unrelated to hemostasis and depends more on local vascular abnormalities and portal hypertension leading to increased vascular pressure [43]. Indeed, prevention and treatment of variceal bleeding is currently not aimed at improving the hemostasis by transfusion of blood products, as these products may cause increased portal vein pressure and thereby aggravate bleeding, but the use of nonselective b-adrenergic blockers and endoscopic band ligation is recommended [44].

Nowadays, there is an increasing recognition of the various thrombotic complications that may occur in patients with liver diseases. Indeed, portal vein thrombosis (PVT) is a common occurrence in patients with cirrhosis, occurring in up to 26% of cirrhotic patients with end-stage liver disease [45]. Furthermore, the occurrence of venous thrombosis is also not uncommon in patients with liver disease. In fact, some studies have even suggested a significantly higher relative risk of venous thrombosis in these patients [9]. The incidence of thrombosis in liver disease is possibly underreported because of nonspecific symptoms of deep vein thrombosis and pulmonary embolism and the lesser attention of clinicians to the possibility of thrombosis in these patients. One clinical survey reported that 40% of patients admitted to the hospital with decompensated liver disease suffered from bleeding events (about one-half nonvariceal) and 7% suffered from deep venous thrombosis [46]. Finally, patients with nonalcoholic fatty liver disease, an increasing cause of liver disease in western counties, are known to have a substantially increased prevalence of arterial thrombotic events [47].

Laboratory measurements in patients with liver disease

There is a frequent clinical need for predicting the risk of bleeding or thrombotic events during or after procedures. However, current clinical available tests are not accurate in predicting those risks in patients with liver diseases because they only evaluate narrow aspects of the hemostatic system. Table 2 summarizes the limitations of commonly available laboratory measurements in patients with liver disease.

The bleeding time, which assesses platelet function, is frequently prolonged in patients with liver disease. Furthermore, the classic platelet aggregation assays are also frequently disturbed in these patients. However, both tests correlate poorly with bleeding symptoms in patients with liver disease [11]. Blood platelet count has some clinical correlation with bleeding, but possibly only at low platelet levels (below 50 x 10E9/l) [48].

The PT is widely used as a general indicator of coagulation. Because coagulation tests, such as the PT and APTT, only measure procoagulant factors and are not sensitive to the reduction in anticoagulant factors, they cannot reliably predict the risk of bleeding in patients with profound hemostatic alterations such as in liver disease [11,13]. These coagulation tests thus cannot reflect the true hemostatic status of patients with liver diseases. Furthermore, the interlaboratory variability of the PT assay is substantial in these patients, making its clinical implication more difficult.

Recently, thrombin generation testing has been increasingly used to reassess the hemostatic capacity of patients with liver disease. This test measures the total amount of thrombin generated during in vitro coagulation, in contrast with the PT and APTT, in which the time to formation of a plasma clot is measured when only about 5% of the total thrombin has been generated. Especially in the presence of thrombomodulin, a transmembrane protein located on vascular endothelial cells acting as the main physiologic activator of protein C, the thrombin generation test is sensitive to all anticoagulant proteins in the plasma. Therefore, this test measures the true balance between the pro- and anticoagulant factors. In fact, thrombin generation testing in the presence of thrombomodulin has demonstrated normal or even superior thrombin generation in patients with chronic liver disease [3,31,49,50]. Furthermore, in patients with acute liver disease, the PT and APTT are substantially prolonged; however, this is not associated with lower levels of thrombin generation [51] or an increased risk of hemorrhage [52–54]. Some studies have also shown that thrombin generation testing is useful in identifying patients with an increased risk of thrombosis [55–57] or those with a bleeding tendency [58]. However, the thrombin generation test is not widely available, addition of thrombomodulin is not standardized yet and currently the test is too complicated for routine use in diagnostic laboratories. Therefore, studies are needed to further asses the clinical value of the thrombin generation assay in predicting hemostatic abnormalities in patients with liver disease and to standardize the test for routine use.

Laboratory measurements	Limitations
Bleeding Time	Correlates poorly with bleeding symptoms Limited availability Time consuming test Patient discomfort
Platelet count	Correlation with bleeding symptoms only at extremely low levels Does not indicate platelet function
Platelet function assays	Correlates poorly with bleeding symptoms Most assays are not calibrated for thrombocytopenia Most tests are not sensitive for VWF Not widely available
INR and PT	Measures only the procoagulant system Not predictive of bleeding risk Substantial interlaboratory variability in patients with cirrhosis
APTT	Measures only the procoagulant system Usually not reflective of hepatic dysfunction Usually (nearly) normal in liver disease
Thrombin generation test	Not widely available Too complicated for routine use Addition of thrombomodulin is not standardized yet
Thromboelastography	Not validated in predicting bleeding or thrombosis in nonsurgical patients Most parameters not standardized Need for fresh whole blood samples Experience required to interpret tracings
Anti-Xa assay in the monitoring of heparins	Underestimates masses of AT-dependent anticoagulant drugs

Table 2. Limitations of commonly available laboratory measurements in patients with liver disease
APTT: activated partial thromboplastin time; AT: antithrombin; INR: international normalized ratio, PT: prothrombin time; VWF: von Willebrand Factor.

Another possible potent technique for measurement of hemostasis in liver disease is thromboelastography (TEG) [59,60]. By using whole blood, this test measures speed and strength of clot formation continuously and can thus theoretically analyze all components of the hemostatic system [61]. Currently there are two commercially available devices, TEG and rotational thrombelastometry, and both have been routinely used for guiding transfusion of platelet concentrates, factor repletion and fibrinolytic therapy during liver transplantation [60]. Although most studies imply that thromboelasthography provides an accurate assessment of bleeding risk in patients with liver disease, to date no studies have directly tested this hypothesis. Therefore, studies to assess the clinical value of the TEG to guide hemostatic management in patients with liver disease are urgently needed.

Prevention & treatment of bleeding complications

As mentioned before, patients with liver disease are not as prone to hypocoagulation-associated bleeding as clinicians formerly thought, and routine coagulation tests cannot be reliably used to assess a bleeding tendency in these patients. Therefore, management of bleeding complications should be aimed at encountering clinical relevant problems instead of correcting abnormal routine laboratory values. This policy is evidenced by the experience

in liver transplantation, in which currently many patients can undergo transplantation without or with minimal transfusion of blood products [4].

Minimal transfusion of blood products prior to invasive procedures

Currently it is in many centers no longer common practice to use blood transfusion products prior to or during liver transplantation except in the occurrence of active bleeding. Indeed, there are no studies showing a beneficial effect of administration of fresh frozen plasma (FFP) or platelet concentrates on prevention of bleeding, specifically for patients with cirrhosis. There are, however, published data on the lack of efficacy of FFP administration in the general population [62,63]. In addition, a clinical trial in which platelet count was increased prior to invasive procedures by a thrombopoietin receptor agonist (Eltrombopag) was prematurely terminated due to thrombotic complications [64], suggesting that correction of thrombocytopenia in patients with liver disease does more harm than good. This may be due to the fact that thrombocytopenia is balanced by the highly elevated levels of VWF in patients with liver disease and that elevated levels of VWF in the context of normalized platelet counts result in a prothrombogenic state.

Despite the lack of benefits, prophylactic use of blood products prior to smaller invasive procedures, such as biopsies, thoracentesis and smaller surgical procedures, is still common practice. Indeed, FFP is frequently used to correct a prolonged PT in patients with liver disease, while recent transfusion guidelines specifically state that it is highly unlikely that these patients benefit from FFP [65]. Furthermore, in one randomized controlled trial in cirrhotic patients undergoing dental extractions usage of intranasal desmopressin appeared as effective, more convenient, better tolerated and less expensive than transfusion of FFP in the prevention of bleeding [66]. This indicates that there is little clinical benefit from prophylactic FFP transfusion before low-risk invasive procedures in patients with liver disease. Moreover, the response to FFP administration is unpredictable in these patients and frequently does not lead to a full normalization of the PT or international normalized ratio (INR) [67]. Prophylactic administration of FFP may even lead to volume overload and exacerbation of portal hypertension, and thereby paradoxically increasing the risk of bleeding [11]. Other side effects of FFP may be the risk of infection and the risk of transfusion-related acute lung injury. Since the benefits of FFP are unclear and given the side effects that may occur, we strongly advise against routinely correcting a prolonged PT or INR with transfusion of FFP prior to procedures in patients with liver disease. Instead, only those patients with significant bleeding should be treated. However, exceptions may include very high-risk procedures in which bleeding is unlikely to be detected in time to intervene before irreversible damage occurs (e.g., intracranial pressure monitor placement in patients with acute liver failure). Under these circumstances, it seems reasonable to (partially) correct prolonged coagulation times with FFP in spite of limited clinical data to support this practice. Because highly elevated levels of VWF appear to balance thrombocytopenia in patients with liver disease, and given the potential side effects, prophylactic platelet transfusion should also not be administered routinely. However, it has been suggested that correcting a platelet count below 50,000 or 60,000/ml may be advisable for high-risk procedures [14]. For example, the Society of Interventional Radiology guidelines advises to correct a platelet count <50,000/ml [68]. However, there is little clinical evidence to suggest that the bleeding risk indeed increased with platelets counts below a certain threshold. A single observational study demonstrated an increased bleeding risk following invasive procedures in patients with a platelet count below 75,000/ml [69]. Additional studies

to investigate whether such a threshold for correction of thrombocytopenia exists, and whether administration of platelet concentrates prior to invasive procedures has a beneficial risk/benefit ratio at platelet counts below such a threshold are required.

In contrast to the discussed transfusion products, administration of low-volume prohemostatics, such as prothrombin complex concentrates, that contain both procoagulant and anticoagulant proteins, may be useful in the prevention of bleeding during liver transplantation. The safety and efficacy of this preoperative administration of prothrombin complex concentrate in patients undergoing liver transplantation is currently under investigation [70].

Treatment strategies for bleeding complications

In summary, the occurrence of hemorrhage in patients with liver disease is frequently unrelated to hemostasis and depends more on portal hypertension, endothelial dysfunction, bacterial infection or renal failure. These risk factors should therefore also be addressed when preventing (re)bleeding. Moreover, excessive transfusion of red blood cells or large volumes of plasma should be avoided due to the increase in portal vein pressure and resultant increased (re)bleeding risk [14]. Indeed, in a recent randomized controlled trial in patients with acute upper gastrointestinal bleeding (a frequent complication in chronic liver disease) survival was improved and rebleeding risk reduced by the use of a restrictive red blood cells transfusion policy [71].

Instead of administration of blood products, treatment of coagulation abnormalities in liver disease patients may be addressed by the following drugs: Desmopressin (DDAVP, 1-deamino-8-D-arginine vasopressin), antifibrinolytics such as aprotinin and tranexamic acid and recombinant tissue factor VIIa (rFVIIa). DDAVP has been shown to correct the bleeding time [72]; however, several studies have shown that administration to patients with an acute variceal bleeding, undergoing hepatectomy or liver transplantation, has no effect on blood loss [73,74]. Therefore, the hemostatic effect of DDAVP in patients with liver disease is questionable. During liver transplantation, the usage of antifibrinolytics seems justified as it resulted in substantial reduction of blood loss in randomized studies [75,76]. In addition, in a recent Cochrane review [77], it was confirmed that antifibrinolytics may potentially reduce blood loss and transfusion requirements. However, one of the most frequently used antifibrinolytic drug, aprotinin, has been retracted from the market due to reported side effects in cardiac surgery [78]. However, alternatives such as tranexamic acid and epsilon aminocaproic acid are still available and equally effective [76]. Currently, antifibrinolytics are assessed for their potential in the prevention of gastrointestinal bleeding in the HALT-IT trial [79]. Finally, the use of rFVIIa during liver transplantation resulted in reduced blood loss in one pilot study [80], which was, however, not confirmed by larger randomized trials [81,82] and also not shown during liver resection. An effect of rFVIIa on variceal bleeding has recently been reported, but its use was associated with an elevated risk of thrombosis [83]. Thus, the evidence of the efficacy of rFVIIa in reducing bleeding complications is limited, and there is considerable evidence of its thrombogenic potential. Therefore, recently published guidelines from the European Society of Anaesthesiology on the management of severe perioperative bleeding recommended against the prophylactic use of rFVIIa in patients with liver disease and suggested that it should be used only as a rescue therapy for uncontrollable bleeding [84].

Prevention & treatment of thrombotic complications

As mentioned before, thrombotic complications do occur frequently in patients with liver disease and these patients may even be in a hypercoagulable state. Therefore, we strongly advise not to withhold thrombosis prophylaxis in patients with liver disease, even in the presence of abnormal routine tests of hemostasis, when risk factors for thrombotic complications are present. Examples of such risk factors for thrombosis prophylaxis include hospitalization and immobilization, undergoing invasive procedures, and the presence of (hepatocellular) cancer [85]. However, for general antithrombotic prophylaxis, especially in patients both at risk for thrombosis and bleeding, careful clinical decision making on an individual basis is needed. Finally, as a result of limited clinical experience, the choice and dosage of anticoagulant drugs for the various indications is still unknown.

Different anticoagulant drugs & their pros & cons in patients with liver disease

Vitamin K antagonists have been used for decades for the long-term prevention and treatment of thromboembolic events, and they are the most prescribed anticoagulants in the general medical population worldwide. A major drawback of vitamin K antagonist therapy in patients with liver disease is the monitoring of the drug by the INR because INR levels are already abnormal in these patients. Therefore, target ranges of the INR for patients with liver disease are unclear and an optimal anticoagulant efficacy will be difficult to achieve. Indeed, studies investigating vitamin K antagonists in patients with chronic liver disease have shown an unacceptably high level of bleeding complications [86,87].

The use of low-molecular-weight heparin in selected patients with cirrhosis has shown to be both safe and effective in the prevention and treatment of PVT [45,88]. Anticoagulant therapy may even be beneficial in these patients by preventing progression of cirrhosis [45]. In addition, the prevention of venous thrombosis with heparins appears safe and effective [89]. However, also heparins have drawbacks, especially long-term use of these drugs may be limited by the mode of administration as well as the concern for heparin-induced thrombocytopenia. Furthermore, we [90, Chapter 8 of this thesis] and Senzolo et al. [91] have recently shown that heparin and low-molecular-weight heparin have a more profound anticoagulant effect in plasma from patients with cirrhosis compared with healthy individuals. In addition, monitoring of heparins is complicated because the anti-Xa assay underestimates drug levels of LMWH in patients with cirrhosis [92,93]. This is most probably related to the reduced AT levels in patients with liver disease. Indeed, we have recently shown that the anti-Xa assay not only underestimates the LMWH mass, but the test also underestimates the masses of other AT-dependent anticoagulant drugs in plasma from patients with cirrhosis [94, Chapter 9 of this thesis]. However, when excess AT is added to the anti-Xa assay, the assay can be used in the monitoring of heparins in patients with cirrhosis [94], but this modification of the test is not readily available in many routine diagnostic laboratories.

New-generation antithrombotic drugs

Newer anticoagulant drugs, such as the direct factor Xa inhibitor rivaroxaban and the thrombin inhibitor dabigatran, have several theoretical advantages over the currently used anticoagulant drugs. The advantages include an oral mode of administration, rapid onset of action, fewer drug to drug interactions, lack of need for monitoring and no need for titration or dose adjustments [95]. However, the major advantage of no need for laboratory monitoring is at the same time also a disadvantage as it may increase the risk for noncompliance. Previously,

one of the major concerns for these drugs was the absence of an established reversal agent. However, this may be addressed in the future, as specific reversal agents for direct Xa and IIa inhibitors are in clinical development.

Both drugs may be applicable in the prevention and treatment of thrombotic complications in patients with liver disease. They may be advantageous especially in long-term anticoagulant strategies given the oral mode of administration. However, clinical data on the use of these drugs in patients with chronic liver disease are still lacking, as these patients have been excluded from clinical trials. According to the package insert, rivaroxaban is even contraindicated in patients with Child B and Child C cirrhosis due to a perceived bleeding risk. Since these new drugs are cleared by the liver and kidneys, the pharmacokinetics may potentially be altered in patients with liver disease. Nevertheless, in a recent case report, 6 months of therapy with rivaroxaban resulted in complete resolution of acute PVT in a patient with Child A cirrhosis, without any adverse effect [96]. Furthermore, in another study, five patients with cirrhosis and PVT safely received oral factor Xa inhibitor anticoagulants (rivaroxaban or apixaban) and in two patients repermeation of the portal vein occurred after 6 months of therapy [97]. We recently studied the potency of old and new anticoagulant drugs in plasma from patients with cirrhosis [90, Chapter 8 of this thesis] and observed a substantially increased anticoagulant response to dabigatran. In contrast, rivaroxaban resulted in a reduced response in plasma from patients with cirrhosis. Thus, the new oral anticoagulants may work differently in liver disease patients compared with patients with intact liver function, which is a potential caveat. Drug-specific dose adjustments, taking both the pharmacokinetics and the altered anticoagulant potency of the drug into account, may be required for patients with liver disease. Laboratory tests meeting these specific needs need to be developed.

Thus, antithrombotic treatment is frequently required in patients with liver disease and thrombosis prophylaxis should not be withheld from these patients. Clinical studies on efficacy and safety of the available anticoagulant drugs in patients with liver disease are urgently needed in order to better recommend the appropriate choice and dosage of anticoagulant drug for the prevention and treatment of the various thrombotic complications.

Conclusion

The concept of a rebalanced but more precarious hemostatic system in patients with liver disease, with the risk of both bleeding and thrombotic complications, is increasingly accepted. Routine laboratory tests, such as the platelet count, PT and APTT, are poor in predicting the bleeding risk in these patients, and more sophisticated tests of hemostasis, such as thrombin generation testing, are not available for routine use in diagnostic laboratories yet. Therefore, it is currently impossible for clinicians to identify individual patients with an increased risk for bleeding, except when risk factors, such as portal hypertension, endothelial dysfunction, bacterial infection or renal failure, are present. Future research should focus on developing a method that can reliably predict the bleeding risk of an individual liver disease patient.

In this paper, we have summarized the available evidence that the use of prophylactic transfusion products prior to invasive procedures, to correct prolonged routine coagulation tests, does not reduce bleeding risk. Instead, the use of blood products may paradoxically cause bleeding as a result of volume overload and has several other severe side effects.

These new insights should be established in common practice and only those patients with an active bleed should be treated. Furthermore, clinicians should be aware of the risk factors for hemorrhage and these should also be addressed.

It has also been highlighted that regular thrombosis prophylaxis should not be withheld in patients with liver disease, especially in the presence of risk factors, such as hospitalization and immobilization, undergoing invasive procedures, and the presence of (hepatocellular) cancer. However, we also acknowledge that future clinical research focusing on the efficacy and safety of anticoagulant therapies in these patients is necessary as specific guidelines for dosing and monitoring of these drugs for the prevention or treatment of thrombosis are lacking.

Expert commentary

Medical research on the hemostasis in liver disease has recently begun to expand, and laboratory testing and clinical observations have shifted the assumption of a hypocoagulation-based bleeding tendency in patients with liver disease toward the concept of a rebalanced hemostasis in these patients. However, these patients may suffer from both clinical relevant bleeding and thrombotic complications. Current laboratory tests fail to accurately predict bleeding and thrombosis risk in patients with liver diseases, and we therefore discourage using these tests in the prevention of procedural-related bleeding. Thrombin generation assays may be promising in assessing hemostatic imbalance in patients with liver diseases, as it is a good marker of global hemostasis. However, future studies are needed to further asses the clinical value of the test in predicting hemostatic abnormalities and to standardize it for routine use.

Current knowledge on the occurrence of bleeding complications in patients with liver disease has shown that its etiopathophysiology is frequently unrelated to a coagulopathy and depends more on risk factors, such as portal hypertension, renal failure or infections. This suggests that prophylactic transfusion of blood products prior to invasive procedures is not helpful in the prevention of bleeding, and indeed research has shown no benefits of these products. Its use may even contribute to bleeding rather than prevent it. We therefore strongly advise against prophylactic transfusion of blood products prior to invasive procedures in patients with liver disease. Instead, only those patients with significant bleeding should be treated, and the risk factors for bleeding should be addressed in the prevention of (re)bleeding.

Finally, since clinical observations have provided evidence for the occurrence of thrombotic complications in patients with liver diseases, we believe that these patients should not be withheld from thrombosis prophylaxis.

Five-year view

The occurrence of thrombotic complications and the necessity of antithrombotic therapy in patients with liver diseases is increasingly recognized. In the future, a further increase in the use of anticoagulant therapy in these patients may be expected due to the increased incidence of thrombotic complications over time related to increasing rates of fatty liver disease and generally longer survival times of patients with chronic liver disease.

Moreover, although our knowledge on bleeding in patients with cirrhosis has tremendously expanded, there still are patients with severe hemostasis-related bleeding, either spontaneously or procedure-related. First of all, it would be useful if we could predict which patients are at risk for thrombosis or bleeding. Currently, two tests may be promising in the prediction of bleeding or thrombosis risk in these patients, thrombin generation assays and TEG, as they both measure global hemostasis. However, future research is necessary to further asses the clinical value of these tests in predicting thrombosis and bleeding risk and their use in routine laboratories.

Furthermore, as the incidence of thrombotic complications may increase in patients with liver disease, both thrombosis prophylaxis and treatment should be used more frequently in these patients. Currently, specific guidelines on the choice and dose of anticoagulant drugs in patients with liver disease are lacking, and the available drugs have various advantages and disadvantages. Therefore, future clinical studies focusing on the efficacy and safety of anticoagulant therapies in these patients are urgently needed.

By far the most exciting advance in the field is the accumulating data showing that antithrombotic treatment may slow down progression of liver disease. If confirmed, this finding may cause a revolution in the management of patients with cirrhosis. If antithrombotic treatment truly prevents progression of disease and substantially delays decompensation, the need for liver transplant, or death, this will profoundly impact the clinical management of patients with (early) cirrhosis. However, future clinical studies on the benefits and safety of such treatment strategies in different patient groups are required to further apply this in clinical practice.

Finally, in the field of bleeding management in patients with liver disease prophylactic use of transfusion products prior to invasive procedures is not recommended because there is no evidence that this will reduce the bleeding risk. However, in the future it may be useful to investigate whether administration of low-volume prohemostatics can prevent bleeding during invasive procedures.

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CHAPTER
2

CHAPTER 3

Decreased TFPI-dependent anticoagulant capacity in patients with cirrhosis who have decreased protein S but normal TFPI plasma levels

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Abstract

Protein S acts as a cofactor for tissue factor pathway inhibitor (TFPI) in down regulation of thrombin generation, and acquired and congenital protein S deficiencies are associated with a concomitant TFPI deficiency. In contrast, in patients with liver diseases, decreased protein S, but normal or increased levels of TFPI have been reported. We compared TFPI and protein S plasma levels between 26 patients with cirrhosis and 20 healthy controls and found that TFPI levels were comparable between patients (111 ± 38%) and controls (108 ± 27%), despite reduced protein S levels (74 ± 23% in patients vs. 98 ± 10% in controls). Subsequently, we quantified the activity of the TFPI-protein S system by measuring thrombin generation in the absence and presence of neutralizing antibodies to protein S or TFPI. Ratios of peak thrombin generation in the absence and presence of these antibodies were calculated. Both the protein S and the TFPI ratios were increased in patients with cirrhosis compared to controls. Protein S ratios were 0.62 [0.08-0.93] in patients vs. 0.32 [0.20-0.54] in controls; TFPI ratios were 0.50 [0.05-0.90] in patients vs. 0.18 [0.11-0.49] in controls. Thus, although the acquired protein S deficiency in patients with cirrhosis is not associated with decreased TFPI levels, the TFPI/protein S anticoagulant system is functionally impaired.

Introduction

Protein S is a vitamin K-dependent plasma protein that is synthesized in the liver and by endothelial cells. It is well known that protein S acts as a cofactor for activated protein C (APC) in the proteolytic inactivation of coagulation factors Va and VIIIa [1]. It has been shown that protein S also effectively inhibits thrombin generation in plasma in the absence of APC [2,3]. This APC-independent anticoagulant property of protein S was especially observed at low TF concentrations. These observations led to the identification of a new role of protein S as a cofactor for tissue factor pathway inhibitor (TFPI) in the inhibition of factor Xa (FXa) [4]. Based on these findings, it has been proposed that protein S deficiency not only increases the risk of thrombosis by impairing the protein C system, but also by reducing the ability of TFPI to down-regulate the coagulation pathway. In fact, it has been shown that TFPI plasma levels are reduced in both congenital and acquired protein S deficiency [5], which is most probably due to the existence of a complex between protein S and TFPI in plasma. Deficiency of either protein is associated with an increased risk of venous thrombosis [6,7]

Castoldi et al. [5] assessed the functional consequences of combined (partial) protein S and TFPI deficiency by comparing thrombin generation in plasma from heterozygous type I protein S-deficient individuals with age- and sex-matched controls. At a low TF concentration, thrombin generation in plasma from patients with a protein S deficiency was 3-fold higher than in plasma from healthy individuals. Furthermore, simultaneous normalization of both protein S and TFPI levels completely corrected the elevated thrombin generation, while normalization of the protein S level alone hardly affected thrombin generation and normalization of the TFPI level alone reduced the peak height of thrombin generation by half.

In addition, Maurissen et al. [8] quantified the activity of the TFPI-protein S system in plasma by measuring thrombin generation in the absence and presence of neutralizing antibodies to protein S or TFPI. These studies showed that protein S and TFPI ratios, determined as the ratio of thrombin peaks in the absence and presence of neutralizing antibodies, were elevated in protein S-deficient individuals, indicating an impairment of the TFPI-protein S system. Furthermore, both ratios correlated well with full-length TFPI levels, which were significantly lower in protein S-deficient patients compared to family members with normal levels of protein S. This decrease in TFPI levels in conjunction with protein S deficiency was proposed to exacerbate the hypercoagulable phenotype of protein S deficiency [5].

We recently reported that plasma levels of protein S are markedly reduced, with median levels of only 16% of normal, in patients with acute liver failure [9]. Also, protein S levels were reported to be decreased in plasma from patients with cirrhosis [10,11]. Whether this decrease can be fully attributed to decreased synthesis, or whether protein S consumption also contributes is at present unclear. TFPI is not produced by the liver, but the microvascular endothelium is thought to be the principal source of TFPI [12]. Therefore, it has previously been proposed that patients with liver disease have normal plasma levels of TFPI. However, the literature is scarce and also conflicting. Increased TFPI levels have been described in patients with hepatic inflammatory diseases [13,14]. Also in those patients with acute liver failure, in whom we reported substantially decreased protein S levels, plasma levels of TFPI were substantially increased, by more than 2-fold, as compared to healthy controls [15]. In contrast, another study reported normal levels of the inhibitor in patients with advanced chronic hepatocellular disease [16]. In yet another study, patients with chronic hepatocellular disease appeared to have normal or elevated levels of TFPI, but patients with fatal hepatic

dysfunction had either low, normal, or high levels of TFPI [17]. Finally, one study has shown that the TFPI concentration decreases in advanced liver disease [18].

Although the literature is not consistent, several studies have shown normal or even increased levels of TFPI in patients with liver disease, despite a reduced plasma level of protein S. Thus, liver disease may be an exception to the rule that protein S deficiency is accompanied by TFPI deficiency. Here we studied protein S and TFPI levels in a well characterized cohort of patients with cirrhosis. In addition, we studied the functionality of the TFPI/protein S system in these patients by comparing thrombin generation profiles generated in plasma of cirrhotic patients to profiles generated in plasma from healthy individuals in the presence and absence of inhibitory antibodies to protein S or TFPI.

Patients and methods

Patients

Twenty-six adult patients with a previous clinical diagnosis of liver cirrhosis, who were under routine control for their disease by the Department of Hepatology of the University Medical Center Groningen (UMCG) or who were admitted to the Hepatology ward of the UMCG, were included in the study. The patients were classified according to the Child-Pugh classification [19]. Ten patients with Child's A cirrhosis, ten patients with Child's B cirrhosis, and six patients with Child's C cirrhosis were studied. Exclusion criteria were documented history of congenital coagulation disorders, presence of active infection (<2 weeks), presence of acute liver failure, use of anticoagulant drugs in the past 10 days, pregnancy, human immunodeficiency virus (HIV) positivity, and recent (<7 days) transfusion with blood products.

The control group consisted of twenty adult healthy volunteers working at our institution. Exclusion criteria for the control group were documented history of congenital coagulation disorders, documented history of hepatic disease, recent viral infection (<2 weeks), use of anticoagulant drugs in the past 10 days, pregnancy, use of oral contraceptives, and HIV positivity.

This study was approved by the local medical ethical committee and informed consent was obtained from each subject before inclusion in the study.

Plasma samples

Blood samples from each patient and control were drawn by clean venepuncture and collected into vacuum tubes containing 0.129 mol/l trisodium citrate as an anticoagulant, at a blood to anticoagulant ratio of 9:1, and was stored at room temperature for a maximum of 1 hour. Platelet-poor plasma (PPP) was prepared by double centrifugation at 2000 and 10 000 g, respectively for 10 min at 18°C. Plasma was aliquoted, snap-frozen and stored at -80°C until use.

Thrombin generation-based assays for measurement of the activity of the TFPI-protein S system

Thrombin generation was determined using calibrated automated thrombography. Methods were based on the thrombin generation-based assays to measure the activity of the TFPI-protein S system described by Maurissen et al. [8]. Plasma (68 µl) was incubated for 15 min at 37°C with 4 µl of corn trypsin inhibitor (CTI, 33 µg/ml final concentration; Hematologic Technologies, Essex Junction, VT, USA) and 8 µl of either HEPES-NaCl buffer (25 mmol/l

HEPES, pH 7.4, 175 mmol/l NaCl), polyclonal antibodies to protein S (to a final concentration of 2.80 µmol/l; DakoCytomation, Glostrup, Denmark), or monoclonal antibodies to TFPI (to a final concentration of 0.66 µmol/l, MW1848; Sanquin, Amsterdam, The Netherlands). Coagulation was initiated with 20 µl PPP 5 pmol/l reagent (Thrombinoscope B.V., Maastricht, The Netherlands) diluted with 0.9% NaCl, to obtain a final concentration of 2.0 pmol/l TF and 1.6 µmol/l phospholipids in the reaction mixture. After addition of 20 µl of $CaCl_2$ and fluorogenic substrate (Thrombinoscope B.V.), substrate conversion by thrombin was followed in a Fluoroskan Ascent reader (Thermo Labsystems, Helsinki, Finland) with 390 nm excitation and 460 nm emission filter sets. Peak heights of thrombin generation were calculated using software obtained from Thrombinoscope B.V. [20]. The TFPI cofactor activity of protein S was expressed as the ratio of thrombin peaks determined in the absence and presence of anti-protein S antibodies (protein S ratio). Furthermore, the activity of the TFPI-protein S system was expressed as the ratio of thrombin peaks determined in the absence and presence of anti-TFPI antibodies (TFPI ratio).

Determination of factor levels in plasma

Total protein S antigen was assayed by enzyme-linked immunosorbent assay (ELISA) using antibodies from DAKO (Glostrup, Denmark). Free protein S was measured by precipitating the C4b-binding protein-bound fraction with polyethylene glycol 8000 and measuring the concentration of free protein S in the supernatant. Plasma TFPI levels were measured by ELISA as previously described [8]. Levels of protein S and TFPI were expressed as percentages of pooled normal plasma, which was obtained by combining plasma samples from >200 healthy volunteers.

Statistical analysis

Data are expressed as means (with standard deviations [SDs]), medians (with ranges), or numbers (with percentages) as appropriate. Means of two groups were compared by Student's t-test or Mann–Whitney U-test as appropriate. Multiple groups were compared using one-way analysis of variance (ANOVA; with the Bonferroni post-test) or Kruskal–Wallis H-test (with Dunn's post-test) as appropriate. Spearman's correlation coefficient was used to assess correlation between continuous variables and Pearson chi-square or Fisher's exact test with Pearson Correlation Coefficient for dichotomous variables. P-values of 0.05 or less were considered statistically significant. GRAPHPAD PRISM (San Diego, CA, USA) and IBM SPSS STATISTICS 20 (New York, NY, USA) were used for analyses.

Results

Patient characteristics

The main characteristics of the study population are reported in Table 1. Twenty-six patients with cirrhosis (14 males and 12 females; mean age 55 ± 11 years) were included and categorized according to the severity of liver disease as expressed by the Child Pugh score [19]. The most common aetiologies of cirrhosis were alcoholic disease and non-alcoholic steatohepatitis (NASH). Twenty healthy subjects (10 males and 10 females; mean age 35 ± 11 years) were included as controls. None of the patients or controls used oral contraceptives or hormone replacement therapy.

Plasma protein S and TFPI levels

Plasma TFPI levels were comparable between patients and controls (108 ± 27% in patients vs. 111 ± 38% in controls, P = 0.815; Table 2). In contrast, protein S levels were substantially decreased in patients (total Protein S: 74 ± 23% in patients vs. 98 ± 10% in controls, P < 0.001; free protein S: 83 ± 23% in patients vs. 103 ± 12% in controls, P = 0.0006), and the decrease in protein S levels was proportional to the severity of the disease (Table 2).

	Cirrhotic patients			P-value
Characteristic	Child A n=10	Child B n=10	Child C n=6	
Age (yrs)	55 [20-72]	54 [41-61]	58 [53-68]	.497
Sex (male)	5 (50)	5 (50)	4 (67)	.781
BMI	26.0 [5.3]	28.9 [6.0]	30.5 [6.9]	.320
Smoking	4 (40)	2 (20)	2 (33.3)	.668
Alcohol (U per week)	0 [0-10]	0 [0-7]	0 [0-130]	.408
Aetiology of liver disease:				
Alcoholic	3 (30)	2 (20)	6 (100)	.005
HCV	0 (0)	1 (10)	0 (0)	1.000
NASH	2 (20)	2 (20)	0 (0)	.639
Hemochromatosis	0 (0)	2 (20)	0 (0)	.323
PBC	1 (10)	0 (0)	0 (0)	1.000
PSC	0 (0)	1 (10)	0 (0)	1.000
Auto-immune	1 (10)	1 (10)	0 (0)	1.000
Wilson's disease	1 (10)	0 (0)	0 (0)	1.000
Alcoholic + NASH	0 (0)	1 (10)	0 (0)	1.000
Alcoholic + HCV	1 (10)	0 (0)	0 (0)	1.000
Hemochromatosis + NASH	1 (10)	0 (0)	0 (0)	1.000
Co-morbidity:				
Cardiovascular	4 (40)	2 (20)	0 (0)	.258
DM	4 (40)	1 (10)	0 (0)	.227
Plasma levels:				
Serum bilirubin (µmol/L)	10 [4-26]	40 [18-121]	93 [63-255]	.0001
Serum albumin (g/L)	42.7 [3.9]	33.1 [4.8]	27.2 [2.6]	<.0001
Serum creatinin (µmol/L)	62.4 [19.2]	72.8 [32.3]	86.7 [18.5]	.193
Haemoglobin (mmol/L)	8.7 [0.6]	7.2 [0.9]	6.3 [0.8]	<.0001
Leukocytes (10^9/L)	6.9 [2.7]	4.9 [2.1]	6.5 [3.7]	.238
Platelets (10^9/L)	134 [49-321]	71 [28-471]	73 [48-114]	.084
INR	1.1 [0.1]	1.3 [0.2]	1.6 [0.2]	<.0001

Table 1. Demographic and clinical characteristics of the study population
Data are expressed as number (%), mean [SD], or median [range].
HCV: Hepatitis C virus, NASH: Non-alcoholic steatohepatitis, PBC: Primary biliary cirrhosis, PSC: Primary sclerosing cholangitis, DM: Diabetes Mellitus, INR: International Normalized Ratio.

Thrombin generation

Plasma from cirrhotic patients and controls were incubated with CTI in the absence or presence of neutralizing antibodies to protein S or TFPI for 15 min at 37°C. Thrombin generation was then initiated with a low TF concentration (2.0 pmol/l). The median peak heights in the absence of antibodies were 25.7 [9.6–66.5] nmol/l (median [range]) in plasma from controls and 61.4 [3.2–132.7] nmol/l in plasma from patients (P = 0.01). Peak heights of thrombin generation without antibodies were significantly higher in Child class B (81.1 [3.2–132.7] nmol/l) and Child class C (81.0 [48.8–116.5] nmol/l) cirrhotic patients compared

to healthy controls (P = 0.04 and P = 0.007 respectively). Addition of antibodies to protein S increased the thrombin peaks to 78.1 [41.2–147.4] nmol/l in controls and to 97.7 [34.8–188.6] nmol/l in patients (P = 0.203; Table 2; Fig 1). Furthermore, in the presence of protein S antibodies, average peak heights were no longer significantly higher in the Child class B and C cirrhotic patients compared to controls (Table 2), suggesting that decreased protein S levels in cirrhotic patients explain the difference in thrombin generation between cirrhotic Child class B and C patients and healthy controls. Complete inhibition of the TFPI-protein S system was achieved through addition of inhibitory antibodies against TFPI, resulting in further increases of peak heights to 128.2 [90.4–202.2] nmol/l in controls and 116.2 [59.4–197.7] nmol/l in patients (Table 2; Fig 1).

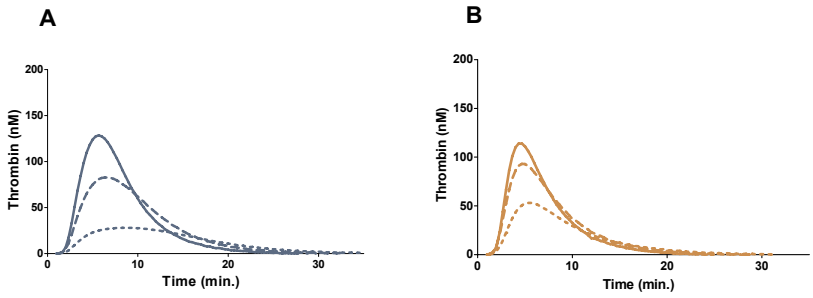

Figure 1. Effects of anti-protein S and anti-tissue factor pathway inhibitor (TFPI) antibodies on thrombin generation at a low tissue factor concentration (2.0 pmol/l). Average of (**A**) 20 separate thrombin generation curves in plasma of healthy controls and (**B**) 26 separate thrombin generation curves in plasma of patients with cirrhosis, without addition of antibodies (dotted line), with anti-protein S antibodies (dashed line) or with anti-TFPI antibodies (solid line).

The anticoagulant activities of protein S and TFPI were expressed as the ratio of peak heights obtained in the absence and presence of the respective inhibitory antibodies. The median protein S ratios were 0.32 and 0.62 in controls and patients respectively. In other words, protein S, via its TFPI cofactor activity, reduced thrombin generation by approximately 68% in controls and by 38% in patients. The median TFPI ratios were 0.18 and 0.55, respectively, indicating that the TFPI-protein S system as a whole reduced thrombin generation by approximately 82% in controls and by 45% in patients. Both protein S and TFPI ratios were significantly increased in patients compared to controls, P = 0.03 and P = 0.009, respectively (Fig 2). In particular in Child class B and C patients, both ratios were markedly increased compared to controls (Table 2).

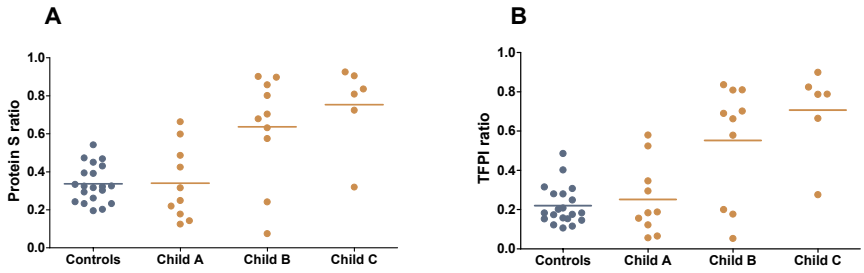

Figure 2. Functional tissue factor pathway inhibitor (TFPI)–protein S assays in controls and in patients with Child A, B, or C cirrhosis. (**A**) Protein S ratio in plasma from controls and patients. (**B**) TFPI ratio in plasma from controls and patients.

Interestingly, peak thrombin generation in the absence of inhibitory antibodies (basal peak thrombin generation) was strongly correlated to protein S ratios, both in patients and controls ($r = 0.88$; $P < 0.0001$ and $r = 0.82$, $P < 0.0001$ respectively). This positive correlation was also observed between basal peak thrombin generation in plasma and TFPI ratios ($r = 0.92$; $P < 0.0001$ and $r = 0.87$, $P < 0.0001$, respectively; Fig 3).

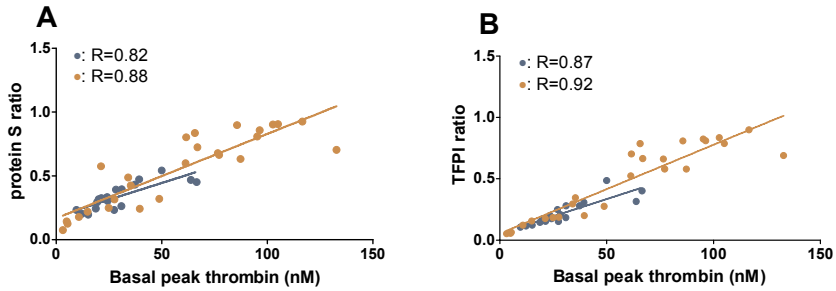

Figure 3. Correlation between basal peak thrombin generation (nmol/l) and (A) protein S ratio and (B) TFPI ratio, in plasma from 20 healthy individuals (●) and 26 patients with cirrhosis (●).

Furthermore, plasma protein S levels strongly correlated with the protein S ratio in patients ($r = -0.66$, $P = 0.0002$; Fig 4). Plasma protein S levels were also correlated with the TFPI ratio in both patients ($r = -0.76$, $P < 0.0001$) and controls ($r = -0.51$, $P = 0.02$; Fig 4). Free plasma protein S levels also correlated with both the protein S ratio ($r = -0.41$, $P = 0.04$) and the TFPI ratio ($r = -0.48$, $P = 0.01$) in patients. In addition, plasma TFPI levels strongly correlated with the TFPI ratios in both patients ($r = -0.67$, $P < 0.0001$) and controls ($r = -0.75$, $P = 0.0001$; Fig 5). No correlation between plasma TFPI levels and protein S ratios was observed in both patients and controls.

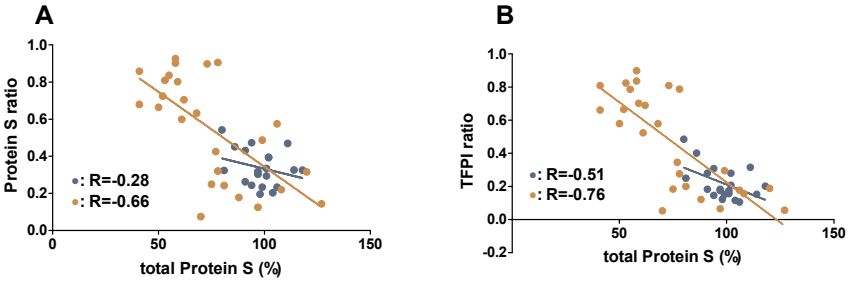

Figure 4. Correlations between total protein S levels and (A) protein S ratio and (B) TFPI ratio, in plasma from 20 healthy individuals (●) and 26 patients with cirrhosis (●).

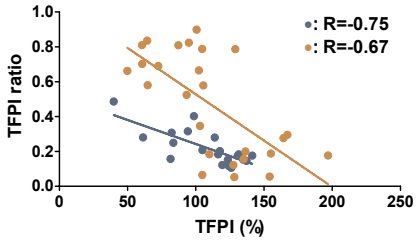

Figure 5. Correlations between tissue factor pathway inhibitor (TFPI) levels and TFPI ratio in plasma from 20 healthy individuals (●) and 26 patients with cirrhosis (●).

	Controls (n=20)	Patients Child A (n=10)	Child B (n=10)	Child C (n=6)
Peak height (nmol/l)	25.7 [9.6-66.5]	26.2 [5.0-77.1]	81.1 [3.2-132.7]*	81.0 [48.8-116.5]**
Peak height + α-protein S (nmol/l)	78.1 [41.2-147.4]	76.5 [34.8-116.2]	112.5 [36.9-188.6]	116.9 [78.6-152.5]
Peak height + α-TFPI (nmol/l)	128.2 [90.4-202.2]	108.7 [82.9-146.5]	119.6 [59.4-197.7]	122.6 [83.4-177.0]
Protein S ratio	0.32 [0.20-0.54]	0.28 [0.13-0.66]	0.69 [0.08-0.90]	0.82 [0.32-0.93]*
TFPI ratio	0.18 [0.11-0.49]	0.19 [0.06-0.58]	0.68 [0.05-0.84]	0.79 [0.28-0.90]**
TFPI (%)	108.1 [27.1]	121.5 [32.1]	96.2 [46.2]	116.0 [26.5]
Protein S total (%)	98.4 [9.9]	90.2 [24.9]	65.9 [19.1]**	62.2 [12.3]**
Protein S free (%)	103.0 [12.3]	94 [25.5]	72.6 [20.0]**	82.8 [20.4]

Table 2. Tissue factor pathway inhibitor (TFPI)–protein S parameters in cirrhotic patients and controls. Data are expressed as mean [SD] or median [range]. *P < 0.05; **P < 0.01.

Discussion

Protein S and TFPI act together in down-regulating thrombin formation [4] and it has been shown that both hereditary and acquired protein S deficiency states are accompanied by a partial deficiency of full-length TFPI [5]. However, we show that in patients with cirrhosis the acquired protein S deficiency is not accompanied by a decrease in TFPI plasma levels. Furthermore, patients with cirrhosis showed a reduced activity of the TFPI-protein S system when compared to healthy controls.

Despite the substantial decrease in protein S plasma levels in cirrhotic patients, TFPI levels are comparable between patients and controls. In addition, we have previously shown increased TFPI levels despite substantially decreased protein S levels in patients with acute liver failure [15]. This indicates that the decrease in TFPI levels in conjunction with acquired protein S deficiency as described previously [5] does not occur in patients with liver diseases.
A possible explanation for normal TFPI levels in patients with liver diseases despite decreased plasma levels of protein S may be that TFPI release in these patients is substantially increased compared to that in healthy individuals, but that this increased TFPI release is masked, in part, by the protein S deficiency. In both chronic and acute liver diseases, continuous activation of the endothelium is common, resulting in increased plasma levels of endothelial-derived proteins, such as von Willebrand factor [21,22]. The combination of continuous endothelial cell activation and protein S deficiency may thus result in normal to slightly elevated TFPI levels. Endothelial activation was probably absent in the acquired protein S deficiencies studied by Castoldi et al (use of oral contraceptives or vitamin K antagonists) [5], explaining the divergent results between our study and the published data.

Protein S and TFPI levels are significantly lower in females than in males [8,23]. In this study there were slightly more males in the patient group (14 males and 12 females) than in the control group (10 males and 10 females). However, this could not explain the differences in protein S levels between patients (with lower protein S levels) and controls observed here. Furthermore, the mean age in the patient group was substantially higher than in the control group. However, levels of TFPI are hardly influenced by age [7] and only in females does total protein S levels increase with increasing age [23] which is unlikely to explain the decreased protein S levels in the (older) patients compared to the (younger) controls observed here.

The decreased TFPI-dependent anticoagulant capacity in patients with cirrhosis resulted in an increased thrombin generation as compared to controls. The difference in peak thrombin generation between patients and controls largely disappeared upon addition of anti-protein S antibodies, indicating that the low protein S levels were responsible for the impairment of the TFPI–protein S system in the patients with cirrhosis.

We have previously proposed that the haemostatic system in patients with liver disease is in a 'rebalanced' status due to a concomitant decrease in pro- and anticoagulants [24]. Furthermore, we proposed the rebalanced haemostatic status of patients with liver disease to be unstable, resulting in a propensity to switch to both a hypo- and hypercoagulable status. The experiments performed in this study suggest that the haemostatic status of a patient with liver disease also depends on the strength of the initiating trigger. At the suboptimal concentrations of TF and lipids used in this study, thrombin generation profiles in healthy individuals are clearly marginal (Fig 1A), and plasma from patients with cirrhosis clearly

generate more thrombin compared to that in healthy volunteers (Fig 1B). This hypercoagulable phenotype was much less pronounced when thrombin generation in the same patients was tested using a high TF trigger (data not shown).
The observed increased peak thrombin generation in this study is in line with the clinical observation of thrombotic complications in patients with cirrhosis. Several studies have shown an increased risk of venous thromboembolism (VTE) in patients with cirrhosis [25-27]. Portal vein thrombosis forms another frequently occurring venous thrombosis in patients with cirrhosis [28]. These thrombotic complications cause increasing morbidity and mortality. The increase in factor VIII in combination with decreased protein C is proposed to be responsible for the hypercoagulable status in patients with chronic liver disease [29]. However, in the current study we have shown that the impaired activity of the TFPI-protein S system in patients with cirrhosis may also contribute to this hypercoagulable state, especially at a low TF concentration. Thus, the increased risk of thrombotic complications, such as venous thrombosis and portal vein thrombosis, which are probably not associated with massive exposure of TF, may be explained by the decreased TFPI-dependent anticoagulant capacity in patients with cirrhosis.

In conclusion, the acquired protein S deficiency in patients with cirrhosis is not associated with a decrease in TFPI plasma levels. Despite normal TFPI plasma levels, the TFPI/protein S anticoagulant system is functionally impaired in patients with cirrhosis, which may contribute to thrombotic complications.

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CHAPTER 4

Preserved clot formation detected by the Thrombodynamics analyzer in patients with cirrhosis

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Abstract

Introduction: Patients with cirrhosis have substantial alterations in their hemostatic system, which are paradoxically associated with the risk of both bleeding and thrombotic complications. However, it still remains difficult to predict those risks, because results from conventional coagulation tests, such as the prothrombin time (PT) and activated partial thromboplastin time (APTT), do not reflect the complex hemostatic changes in these patients. More sophisticated global hemostasis tests, such as thrombin generation assays, are not standardized for routine use yet. Here we examined the spatial clot growth in plasma from patients with cirrhosis using the novel Thrombodynamics assay, which uses a fundamentally new approach to test plasma hemostatic capacity.

Materials and Methods: Thrombodynamics assays were performed in plasma from thirty-one patients with cirrhosis and twenty-five healthy controls. Results were compared to results with thrombin generation testing and PT/APTT test results.

Results: Rates of clot growth, clot size, and clot density from the Thrombodynamics assay were comparable between patients and controls. Thrombin generation in the presence of thrombomodulin was increased in the patients, despite prolonged PT and APTT test results. There was little correlation between parameters derived from the Thrombodynamics assay and the PT, APTT, or thrombin generation data.

Conclusions: The Thrombodynamics assay showed preserved clot formation in plasma from patients with cirrhosis, which is in line with the results of the thrombin generation assay in this study and previously reported by others.

Introduction

Conventional coagulations tests, such as the prothrombin time (PT) and activated partial thromboplastin time (APTT), are frequently prolonged in patients with cirrhosis suggesting a hypocoagulable state [1]. Traditionally, these findings have been considered as pointing towards a bleeding tendency in patients with a chronic liver disease. However, these coagulation tests are only sensitive for selected procoagulant factors and do not take the reduction in anticoagulant factors, which also occurs in these patients, into account. In fact, the hemostatic system in patients with a chronic liver disease is nowadays considered to be rebalanced [2]. However, this balance is more unstable compared to that in healthy individuals and there is thus a frequent clinical need for predicting the risk of bleeding or thrombosis in patients with a liver disease [2,3].

In contrast to the conventional coagulation tests, the thrombin generation test in the presence of thrombomodulin, the main activator of protein C, is sensitive to all anticoagulant systems in plasma and thus measures the true balance between the pro- and anticoagulant proteins. This test has shown normal to even increased thrombin generation in patients with a chronic liver disease [4–8]. However, the thrombin generation assay is not widely available yet. The test is currently too complicated for routine use in diagnostics laboratories, and the addition of thrombomodulin is not yet standardized.

Recently a new plasma-based global hemostasis assay, Thrombodynamics, has been developed that allows a continuous monitoring of clot growth in non-stirred plasma initiated by a thin layer of immobilized tissue factor (TF) [9,10]. This assay was designed to better mimic the in vivo conditions of clot formation by taking into account both the biochemical reactions of the coagulation cascade and the spatial aspects of clot formation. Indeed, while in other coagulation tests (e.g. PT and thrombin generation) clotting is activated by TF that is homogenously distributed over the plasma sample, in this test only a thin layer of plasma is exposed to TF and clot formation starts on this surface and propagates into the bulk of plasma. This spatial clot growth assay previously showed defective clot formation (with lower rate of clot growth and thinner clots) in both patients with hemophilia A and B [11]. In addition, another study showed that spatial clot growth in plasma can be used to predict an increase in D-dimer levels in sepsis patients [9]. Furthermore, the test was useful for detecting procoagulant changes caused by an aptamer antagonist of tissue factor pathway inhibitor [10], recombinant factor VIIa [12], or platelet microparticles [13].
Here we aimed to examine the spatial clot growth in plasma from patients with a chronic liver disease using the novel Thrombodynamics assay and compared results with thrombin generation testing and PT/APTT test results.

Materials and Methods

Patients

Thirty-one adult patients with cirrhosis, who were seen on an outpatient basis or were admitted to the department of Hepatology of the University Medical Center Groningen, were included in the study. Patients were classified according to the Child-Pugh classification [14]. Eleven patients were classified as Child A, ten patients as Child B, and ten patients as Child C cirrhosis. Exclusion criteria were documented history of congenital coagulation disorders, presence of active infection (<2 weeks), presence of acute liver failure, use of anticoagulant

drugs in the past 10 days, pregnancy, human immunodeficiency virus (HIV) positivity, and recent (<7 days) transfusion with blood products.

Twenty-five healthy volunteers working at our institute were included as controls. Exclusion criteria for the control group were a documented history of congenital coagulation disorders, documented history of hepatic disease, recent viral infection (<2 weeks), use of anticoagulant drugs in the past 10 days, pregnancy, and HIV positivity.

The study protocol was approved by the medical ethical committee of the University Medical Center Groningen, Groningen, The Netherlands and written informed consent was obtained from each subject before inclusion in the study.

Plasma Samples

Blood samples from each patient and control were drawn by venipuncture and collected into vacuum tubes containing 3.8% trisodium citrate as an anticoagulant, at a blood to anticoagulant ratio of 9:1. Platelet poor plasma was prepared by double centrifugation at 2000 g and 10.000 g respectively for 10 min. Plasma was snap-frozen in liquid nitrogen and stored at -80 °C until use.

Thrombodynamics Assay

The general concept of the Thrombodynamics test was previously described by others [9,10,15,16]. Briefly, in a thin layer of plasma coagulation is activated when it is brought in contact with tissue factor (TF) immobilized on a plastic surface. The clot formation starts on the activator and propagates into the bulk of plasma in which no TF is present. Light scattering by fibrin allows observation of spatial clot formation in real time by using time lapse imaging [17].

In this study, the Thrombodynamics assay was performed using an experimental device provided by HemaCore LLC (Moscow, Russia). Reagents (Thrombodynamics kit, Hemacore LLC, Mosow, Russia) and protocols from the manufacturer were used. According to these instructions, plasma was pre-treated with Corn Trypsin Inhibitor for 10minutes at 37 degrees Celcius prior to initiation of the assay. The following parameters were analyzed: lag time, initial and stationary rates of cloth growth, clot density, and clot size at 30minutes. The lag time is defined as the time between clotting initiation and actual appearance of the fibrin clot. The initial rate of clot growth (Vi) is the slope of the curve on a clot vs. time graph during the first 2-6 minutes of cloth growth. Stationary rate of clot growth (Vst) is measured as a slope of the curve on a clot size vs. time graph within the interval 15–25 minutes after clot growth begins.

Coagulation Tests

Thrombin generation testing was performed using platelet-poor plasma (PPP) with the fluorimetric method described by Hemker, Calibrated Automated Thrombography® (CAT) [18]. Reagents and protocols were purchased from Thrombinoscope BV, Maastricht, The Netherlands. Coagulation was activated using commercially available reagents containing recombinant TF (final concentration 5 pM), phospholipids (final concentration 4 mM), in absence or presence of soluble thrombomodulin. To calibrate the thrombin generation curves, Thrombin Calibrator (Thrombinoscope BV) was added, and a fluorogenic substrate

with CaCl2 (FluCa-kit, Thrombinoscope BV, Maastricht, The Netherlands) was used to allow a continuous registration of thrombin generation. Fluorescence was read in time by a fluorometer, Fluoroskan Ascent® (ThermoFisher Scientific, Helsinki, Finland).

The PT, APTT, and fibrinogen levels were assessed on an automated coagulation analyzer (ACL 500 TOP) with reagents (Recombiplastin 2G for PT, SynthaSil for APTT, and QFA thrombin (Hemosil) for fibrinogen) and protocols from the manufacturer (Instrumentation Laboratory, Breda, the Netherlands).

Statistical Analysis

Data are expressed as means (with standard deviations (SDs)), medians (with interquartile ranges), or numbers (with percentages) as appropriate. Means of two groups were compared by Student's t-test or distributions in the two groups by Mann-Whitney U test as appropriate. Multiple groups were compared using one-way ANOVA (with the Bonferroni post test) or Kruskal-Wallis H test (with Dunn's post test) as appropriate. Spearman's correlation coefficient was used to assess correlation between continuous variables. P values of 0.05 or less were considered statistically significant. GraphPad Prism (San Diego, USA) and IBM SPSS Statistics 20 (New York, USA)) were used for analyses.

Results

Patient Characteristics

The main characteristics of the study population are presented in Table 1. Thirty-one patients with cirrhosis (20 males and 11 females) were included, and they were categorized according to the severity of liver disease as expressed by the Child Pugh classes (11 Child A, 10 Child B and 10 Child C patients). Twenty-five healthy subjects (10 males and 15 females) with a mean age of 33.9 ± 11.1 (mean ± SD) were included as controls. The most common etiology of liver disease was alcoholic, especially in the Child class C patients. Three healthy subjects and none of the patients used oral contraceptives (P=0.08).None of the healthy subjects and three patients used antiplatelet agents or nonsteroidal anti-inflammatory drugs (two used carbasalate calcium and one used naproxen) (P= 0.25).

Thrombodynamics Assay

The results of the Thrombodynamics test in the plasma from patients and controls are presented in Table 2 and Figure 1. The lag time was slightly, but significantly prolonged in the patients compared to the controls (P = 0.025). Vi was significantly increased in the Child class C patients (63.7 μm/min (59.4-68.6) (median with range)) compared to controls (57.0 μm/min (50.0-63.4); P < 0.01). However, the Vst was comparable between patients and controls. Clot size was also comparable between patients and controls. Finally, clot density was decreased in the Child class C patients (8093 arbitrary units (a.u.) (4712-10923)) compared to controls (11228 a.u. (6181-20868); P < 0.01).

Correlations Between Thrombodynamics Results and Other Coagulation Tests

There was no significant correlation between the Thrombodynamics data and the PT, APTT, or thrombin generation data in the controls. The PT was significantly prolonged in the patients (12.8 s (9.8-23.8)) compared to the controls (10.8 s (9.9-12.1); P < 0.0001), especially in the Child class B and C patients (Table 2). There was a significant negative correlation between

the PT and the clot density of the Thrombodynamics test in the patients (r = -0.72; P < 0.001). Furthermore, the Vi of the Thrombodynamics test showed a positive correlation with the PT (r = 0.53; P = 0.002). The APTT was also significantly prolonged in the patients (38.7 s (33.3-48.7)) compared to the controls (33.4 s (27.6-39.1), P < 0.0001). There was a significant negative correlation between the APTT and the clot density of the Thrombodynamics test in the patients (r= -0.51; P = 0.004).

The endogenous thrombin potential (ETP) was decreased in the patients compared to the controls (Table 2). However, in the presence of thrombomodulin, the ETP was increased in the patients (616.4 nM*min (144.6-1046.7)) compared to the controls (355.4 nM*min (168.3-1251.4); P = 0.003), especially in the Child class C patients (Table 2). Peak thrombin generation was also increased in the patients compared to the controls in the presence of thrombomodulin (Table 2). In the patients, both ETP and peak thrombin generation showed a significant negative correlation with clot density (r= -0.61; P=0.0002 for ETP and r=-0.46; P=0.01 for peak) and a positive correlation with Vi of the Thrombodynamics test (r= 0.48; P = 0.006 for ETP and r = 0.43; P = 0.02 for peak).

Characteristics	Cirrhotic patients Child A n=11	Child B n=10	Child C n=10	P-value
MELD score	7 [6-10]	11 [7-18]	17 [13-25]	<.0001
Age (years)	61.0 [11.4]	49.0 [12.5]	52.2 [7.5]	.042
Sex (male)	5 (45.5)	8 (80)	7 (70)	.261
BMI	25.8 [18.5-35.0]	27.9 [23.6-41.4]	26.3 [22.0-37.1]	.146
Smoking	4 (36.4)	2 (20)	4 (40)	.705
Alcohol (U per week)	0 [0-1]	0 [0-7]	0 [0-490]	.638
History of variceal bleeding (number of patients)	3 (27.3)	2 (20)	1 (10)	.849
History of portal vein thrombosis (number of patients)	0 (0)	0 (0)	2 (20)	.194
Etiology of liver disease				
Alcoholic	4 (36.4)	2 (20)	9 (90)	.005
HCV	1 (9.1)	1 (10)	0 (0)	1.000
NASH	2 (18.2)	2 (20)	0 (0)	.511
Hemochromatosis	0 (0)	1 (10)	0 (0)	0.645
PSC	1 (9.1)	2 (20)	0 (0)	.511
Auto-immune	2 (18.2)	0 (0)	0 (0)	.312
Alcoholic + NASH	0 (0)	1 (10)	0 (0)	0.645
Alcoholic + HCV	1 (9.1)	0 (0)	0 (0)	1.000
Unknown	0 (0)	1 (10)	1 (10)	.527
Plasma levels				
Serum bilirubin (µmol/L)	13 [4-26]	29 [18-61]	82 [36-121]	<.0001
Serum albumin (g/L)	42 [28-58]	33 [29-63]	26 [24-29]	<.0001
Serum creatinin (µmol/L)	67 [25]	77 [30]	69 [28]	.655
Hemoglobin (mmol/L)	8.4 [1.1]	7.3 [0.9]	6.4 [0.9]	.0005
Leukocytes (10^9/L)	4.6 [3.4-10.4]	5.1 [2.1-8.7]	6.0 [3.4-19.1]	.601
Platelets (10^9/L)	128 [77-274]	90 [28-471]	79 [44-165]	.118
Fibrinogen (mg/ml)	2.7 [2.1-3.9]	2.6 [1.0-5.3]	1.7 [1.3-2.5]	.015

Table 1. Clinical and demographic characteristics of the study population.
Data are expressed as number (%), mean [SD], or median [range]. NSAIDs: Nonsteroidal anti-inflammatory drugs, HCV: Hepatitis C virus, NASH: Non-alcoholic steatohepatitis, PSC: Primary sclerosing cholangitis.

In addition, the lag time of the thrombin generation assay showed a significant positive correlation with the clot density of the Thrombodynamics assay (r = 0.47; P = 0.007), and the velocity index of the thrombin generation correlated with the clot density (r = -0.45; P = 0.01) as well as the Vi of the Thrombodynamics test (r= 0.45; P = 0.01). Finally, fibrinogen levels strongly correlated with the clot density of the Thrombodynamics test in the patients (r= 0.97; P < 0.0001).

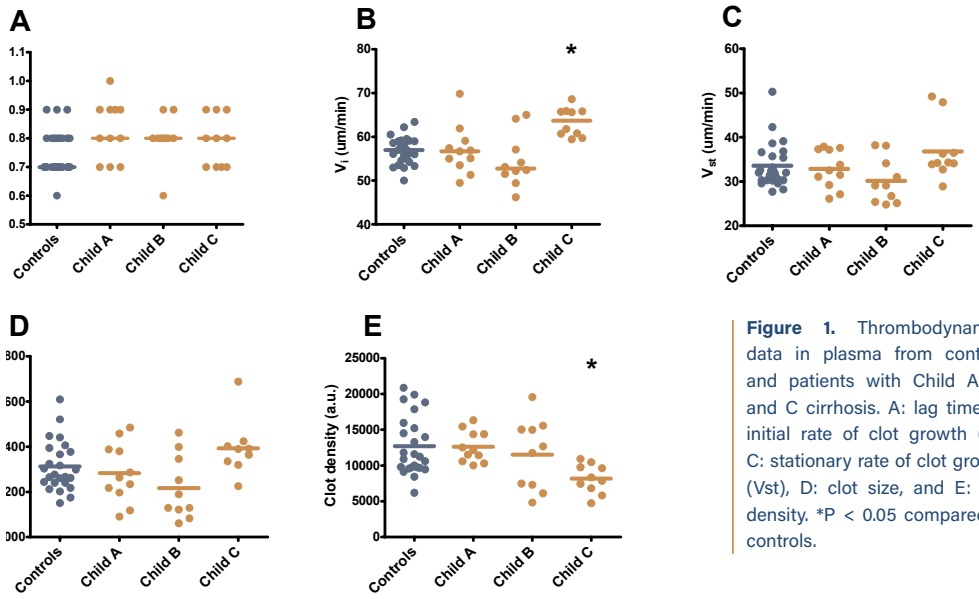

Figure 1. Thrombodynamics data in plasma from controls and patients with Child A, B, and C cirrhosis. A: lag time, B: initial rate of clot growth (Vi), C: stationary rate of clot growth (Vst), D: clot size, and E: clot density. *P < 0.05 compared to controls.

Discussion

This study shows preserved clot formation in plasma from patients with cirrhosis using the novel Thrombodynamics assay. These results are in line with the results of previous studies using the thrombin generation in the presence of thrombomodulin, which demonstrated normal to increased thrombin generation in patients with a chronic liver disease [4,6,7]. Also in this study we observed increased thrombin generation in patients with cirrhosis when thrombomodulin was added to the plasma.

Although overall the Thrombodynamics assay showed comparable test results between patients and controls, in patients with Child C cirrhosis both hyper- and hypocoagulable features were present, with an increase in initial rate of clot growth and decrease in clot density, respectively. The decrease in clot density might be partially explained by a decrease in fibrinogen in patients with severe cirrhosis [19,20]. Patients with, especially severe, cirrhosis may thus have a precarious rebalanced hemostasis, explaining the risk of both bleeding and thrombotic complications [3,21].

Despite the fact that both the Thrombodynamics assay and the thrombomodulin-modified thrombin generation assay showed similar results in patients with cirrhosis, correlation between test results was poor. Especially in the controls, we observed no significant correlation

between any of the Thrombodynamics data and the PT, APTT, or thrombin generation data. This may be explained by the fact that this novel Thrombodynamics assay uses a fundamentally different principle to assess the coagulation cascade than both the thrombin generation assay and the conventional coagulation tests. Whereas in these tests clotting is activated by homogeneously dissolved TF, in the Thrombodynamics test clotting is activated by a surface with immobilized TF, which better resembles in vivo clot formation. The correlations observed between Thrombodynamics test results and PT, APTT, or thrombin generation test results in patients were confusing and conflicting. For example, the negative correlation between the PT and clot density is not in line with the negative correlation between the ETP and clot density. Also the positive correlation between the PT and the initial rate of clot growth is difficult to explain.

The preserved clot formation in plasma from patients with cirrhosis observed with the novel Thrombodynamics assay in this study together with similar conclusions drawn from thrombomodulin-modified thrombin generation tests supports the notion that hemostasis in cirrhosis is rebalanced and even has hypercoagulable features [2,19,21]. The clinical consequences of this include a restrictive use of prophylactic prohemostatic agents in patients undergoing invasive procedures [3,22]. Furthermore, routine thrombosis prophylaxis should

Parameters	Controls	All patients	Child A	Child B	Child C
PT	10.8 [9.9-12.1]	12.8 [9.8-23.8]***	11.4 [9.8-13.1]	12.6 [10.3-22.3]*	17.4 [15.0-23.8]***
APTT	33.4 [27.6-39.1]	38.7 [33.3-48.7]***	38.7 [35.7-48.7]***	39.8 [33.3-47.0]**	37.5 [33.8-46.1]**
Thromodynamics					
Lag time (min)	0.7 [0.6-0.9]	0.8 [0.6-1.0]*	0.8 [0.7-1.0]	0.8 [0.6-0.9]	0.8 [0.7-0.9]
V_i (umol/min)	57.0 [50.0-63.4]	57.4 [46.2-69.8]	56.7 [49.5-69.8]	52.8 [46.2-65.0]	63.7 [59.4-68.6]**
V_{st} (umol/min)	33.6 [5.0]	33.3 [5.6]	32.9 [4.3]	30.2 [5.1]	36.8 [6.5]
Clot size (umol)	1313 [110]	1294 [147]	1284 [131]	1217 [141]	1393 [125]
Clot density (a.u.)	11228 [6181-20868]	10568 [4712-19552]	12156 [10006-16331	12210 [4812-19552]	8093 [4712-10923]**
Thrombin generation					
ETP	908.5 [684.1-1813.4]	771.7 [419.0-1262.3]*	692.0 [419.0-906.1]**	889.5 [641.1-1262.3]	822.6 [585.6-1030.6]
ETP TM	355.4 [168.3-1251.4]	616.4 [144.6-1046.7]**	370.9 [181.8-687.5]	726.0 [144.6-980.7]	745.3 [556.1-1046.7]**
Peak	166.6 [83.7-278.2]	155.7 [55.7-241.1]	139.7 [55.7-188.1]	183.0 [98.0-241.1]	155.9 [115.2-186.5]
Peak TM	94.1 [45.8-221.3]	145.3 [34.5-225.1]*	97.6 [46.7-162.0]	170.6 [34.5-225.1]*	150.3 [113.1-184.7]*
Velocity index	67.9 [16.7-165.7]	76.6 [8.8-121.4]	55.7 [8.8-89.8]	83.4 [27.8-121.4]	78.9 [54.9-98.2]
Velocity index TM	49.1 [22.9-92.1]	74.6 [14.1-119.5]*	48.8 [21.6-86.4]	85.2 [14.1-119.5]	75.2 [51.2-105.3]*
Lag time	1.7 [1.3-2.7]	1.4 [1.1-3.7]	1.8 [1.4-3.7]	1.5 [1.3-2.0]	1.3 [1.1-1.6]***
Lag time TM	1.4 [1.0-2.0]	1.4 [1.1-2.0]	1.7 [1.3-1.7]	1.4 [1.2-2.0]	1.3 [1.1-1.7]

Table 2. PT, APTT, Thrombodynamics, and thrombin generation data in patients with cirrhosis and healthy subjects. Data are expressed as mean [SD] or median [range]. *P<0.05; **P<0.01; ***P<0.001 versus controls. PT: prothrombin time, APTT: activated partial thromboplastin time, Vi: initial rate of clot growth, Vst: stationary rate of clot growth, a.u.: arbitrary units, ETP: endogenous thrombin potential, TM: thrombomodulin.

not be withheld in these patients, even though routine coagulation tests are abnormal and prolonged [3,23].

Since patients with cirrhosis can suffer from both thrombotic and bleeding complications, there is a frequent clinical need for predicting the risk of bleeding or thrombotic events before, during, or after procedures in these patients. Currently, two tests may be useful in assessing overall hemostasis, thrombin generation assays and thromboelastography. However, the thrombin generation test is not widely available, the addition of thrombomodulin is not standardized yet, and currently the test is too complicated for routine use in diagnostic laboratories [3,24]. Thromboelastography also has some major drawbacks, including its unique set of pre-analytic and analytic variables that impact test reliability and reproducibility. In addition, thromboelastography does not allow quantification of the contribution of individual components of the hemostatic system to abnormalities in the thromboelastographic tracing. Finally, there are various methods to perform the test and these results poorly correlate [25–27].

The Thrombodynamics assay is a global coagulation assay with a fundamentally new test principle. Given the stability of the assay, provided a strict blood processing protocol is followed, this test may have merit in the clinical setting [17]. It may hold promise in the prediction of bleeding or thrombotic risk in patients with a chronic liver disease, which awaits clinical validation studies.

In conclusion, we observed preserved clot formation in plasma from patients with cirrhosis using the novel Thrombodynamics assay, which is in line with the results of the thrombin generation assay in this study and previously reported by others [4,6,7]. The Thrombodynamics assay may be promising in assessing the hemostatic imbalance in patients with liver diseases, as it uses a fundamentally different principle to measure the coagulation than the other coagulation tests, which may better resemble the in vivo clot formation. However, future studies are needed to assess the clinical value of the test in predicting hemostatic abnormalities and to further standardize it for routine use.

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CHAPTER 5

Hypercoagulability following major partial liver resection - detected by thrombomodulin -modified thrombin generation testing

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Abstract

Background: Conventional coagulation tests are frequently prolonged after liver surgery, suggesting a post-operative hypocoagulability. However, these tests are unreliable for assessment of the haemostatic status in these patients. In contrast, thrombin generation testing measures the true balance between pro- and anti-coagulant factors.

Aim: To study the perioperative coagulation status in patients undergoing hemi-hepatectomy using thrombin generation assays.

Methods: We examined thrombin generation profiles in serial plasma samples taken from seventeen patients undergoing right hemi-hepatectomy. Results were compared to ten patients undergoing pancreatic resection and twenty-four healthy volunteers. In addition, we measured conventional coagulation tests and plasma levels of several haemostatic proteins.

Results: Following liver resection, the endogenous thrombin potential (ETP) slightly decreased until post-operative day 7. However, in the presence of thrombomodulin, the ETP increased [from 542 nM*min (417–694) at baseline to 845 nM*min (789–1050) on post-operative day 3] to values higher than that in healthy subjects (558 nM*min (390–680); $P < 0.001$), which contrasts with substantially prolonged PT levels. Normal to decreased thrombin generation was observed following pancreatic resection. Thrombin generation was only slightly affected by thrombomodulin after hemi-hepatectomy, while thrombin generation in healthy subjects decreased profoundly upon addition of thrombomodulin. This hypercoagulability following liver resection may be explained by decreased levels of protein C, S, and antithrombin and by elevated levels of factor VIII.

Conclusions: Thrombin generation in the presence of thrombomodulin revealed hypercoagulability in patients following liver resection. These results support the recently advocated restrictive use of plasma during liver resection and the exploration of more extensive use of post-operative thrombosis prophylaxis.

Introduction

Major abdominal surgery is associated with a post-operative hypercoagulable state and this hypercoagulability has been recognised as a factor that contributes to the occurrence of thromboembolic complications, such as deep vein thrombosis and pulmonary embolism [1]. The underlying mechanisms include increased activation of platelets, reduced concentrations of anti-coagulants, and impaired fibrinolysis [2], as well as more general risk factors for thrombosis, such as immobilisation, tissue damage and the presence of cancer.

Conventional coagulation tests, such as the activated partial thromboplastin time (APTT), and the prothrombin time (PT) and related international normalised ratio (INR), are frequently elevated after liver surgery [3–6], suggesting a post-operative hypocoagulability. These tests are, however, not reliable for assessment of the overall haemostatic status in these patients, because they only evaluate narrow aspects of haemostasis. Specifically these tests are only sensitive for circulating levels of pro-coagulant factors, and do not test functionality of the natural anti-coagulant systems. Indeed, venous thromboembolism also occurs after liver surgery and, in fact, the risk increases with the extent of hepatectomy [7, 8]. The thrombotic risk after liver resection may result from a hypercoagulable state induced by extensive tissue injury or reduction in anti-coagulant factors determined by increased consumption, blood loss or haemodilution [9–13]. Despite this, elevation of routine coagulation tests (PT, APTT) following liver resection frequently leads to transfusion of fresh frozen plasma (FFP) [14–16]. However, several serious side effects of blood product transfusion may occur, including the risk of infection and the risk of transfusion-related acute lung injury [17]. In addition, elevation of routine coagulation tests often leads clinicians to delay thrombosis prophylaxis, based on the assumption that the patient is 'auto-anticoagulated'. This potentially increases the risk of deep vein thrombosis and pulmonary embolism in these patients [7, 18]. Using thromboelastography (TEG), which analyses all components of the haemostatic system, it was shown that after living donor liver transplantation the majority of donors were hypercoagulable in spite of elevated routine coagulation tests [10]. Also patients undergoing partial liver resection for malignant disease demonstrated a brief hypercoagulable state, followed by normal clot formation as shown byTEG [5, 19], and yet another study using TEG reported normocoagulability after liver resection [6].

Thromboelastography is routinely used for guiding transfusion of blood products during massive bleeding and liver transplantation by many centres [20, 21]. However, it is still uncertain if thromboelastography-guided transfusion strategies improve outcome in patients with massive bleeding [22], or decrease blood loss during liver transplantation [23]. Furthermore, although thromboelastography assesses the haemostatic status in a whole blood environment, the technique has three major drawbacks. (1) There are multiple ways to perform a thromboelastographic analysis. Specifically, tests with non-anticoagulated blood as well as with citrated blood are widely used, and the results from these various methods poorly correlate [24]. When using citrated blood, various triggers are added to initiate coagulation (tissue factor/kaolin).

Furthermore, two devices (TEG and ROTEM) are used clinically, and results from these methods are also not always in accordance [25,26]. (2) Quantification of the contribution of individual components of the haemostatic system to abnormalities in the thromboelastographic tracing is not possible, although it has been demonstrated that alterations in some components of the traces are dominated by platelets, coagulation or fibrinogen levels [25, 26]. (3) Thromboelastography suffers from a unique set of pre-analytic and analytic variables that impact test reliability and reproducibility [27].

The thrombin generation assay, which measures the total amount of thrombin generated during in vitro coagulation, has been successfully used to reassess the haemostatic status of patients with liver disease [28–31]. This global test, which takes plasma concentrations of both pro- and anti-coagulants into account, offers a valid alternative to the conventional coagulation tests which only test functionality of some of the pro-coagulant factors. Thrombin generation testing has demonstrated normal or even superior thrombin generation in patients with cirrhosis [28–31], despite a prolonged PT or APTT. It has been well established that plasma levels of haemostatic proteins decrease following a partial liver resection, which has been attributed in part to a reduced synthetic capacity of the liver remnant [6, 9, 10]. However, the net results of these changes in plasmatic coagulation have not been established. We therefore studied the perioperative coagulation status in patients undergoing right hemi-hepatectomy using both conventional coagulation tests and thrombin generation assays. Furthermore, we compared results to those of patients undergoing a pancreatic resection which is a surgical procedure of a similar extent, but without a decrease in post-operative synthetic capacity of the liver.

Materials and Methods

Patients

Seventeen adult patients, who underwent a right (n = 15) or extended right (n = 2) hemi-hepatectomy were included in the study. The control group consisted of ten patients who underwent a pylorus preserving pancreaticoduodenectomy (PPPD). Twenty-four adult healthy volunteers were included to establish reference values for the various tests performed. All patients were included in the University Medical Center Groningen, the Netherlands. Exclusion criteria were age younger than 18 years, pre-existing coagulation disorders, pre-operative anti-coagulation, and use of non-steroidal anti-inflammatory drugs or aspirin 1 week before surgery. Routine surgical and anaesthetic procedures were adopted.
The study protocol was approved by the local medical ethical committee and informed consent was obtained from each subject before inclusion in the study.

Plasma samples

Plasma samples for analyses were, for both groups, drawn at the following time points: after induction of anaesthesia (baseline), at the end of surgery, and on the post-operative days 1, 3, 5, 7 and 30. Following surgery, all patients received standard thromboprophylaxis with (once-daily) low molecular weight heparin (LMWH) and at each post-operative day blood was drawn just prior to the administration of the LMWH.

Blood samples from each subject were drawn by venepuncture and collected into vacuum tubes containing 3.8% trisodium citrate as an anti-coagulant, at a blood to anti-coagulant

ratio of 9:1. Platelet-poor plasma was prepared by double centrifugation at 2000 g and 10 000 g, respectively, for 10 min. at 18 °C. Plasma was aliquoted, snap-frozen and stored at -80 °C until use.

Thrombin generation

Thrombin generation testing was performed using platelet-poor plasma (PPP) with the fluorimetric method described by Hemker, Calibrated Automated Thrombography (CAT) [32]. Coagulation was activated by using a commercial trigger composed of recombinant tissue factor (TF, final concentration 5 pM) and phospholipids (final concentration 4 μM), in the presence or absence of soluble thrombomodulin (TM). These reagents were purchased from Thrombinoscope BV, Maastricht, The Netherlands. To calibrate the thrombin generation curves, thrombin Calibrator (Thrombinoscope BV) was added. A fluorogenic substrate with CaCl2 (FluCa-kit, Thrombinoscope Thrombinoscope BV, Maastricht, the Netherlands) was dispensed in each well to permit a continuous registration of thrombin generation. Fluorescence was read in time by the fluorometer Fluoroskan Ascent (ThermoFisher Scientific, Helsinki, Finland). All procedures were followed according to the protocol suggested by Thrombinoscope B.V. Thrombin generation variables analysed were endogenous thrombin potential (ETP), peak thrombin generation, lag-time (time needed for thrombin concentration to reach 1/6th of the peak concentration) and velocity index (slope between the end of lag-time and peak thrombin generation). Furthermore, a normalised thrombomodulin sensitivity ratio (TM-SR) was determined by dividing the ETP in the presence of TM divided by the ETP in the absence of TM of an individual, by the ETP in the presence of TM divided by the ETP in the absence of TM of pooled normal plasma. A TM-SR >1 reflects a decreased anti-coagulant response to TM in comparison to pooled normal plasma.

Conventional coagulation tests

The PT was assessed on an automated coagulation analyser (ACL 500 TOP) with reagents (Recombiplastin 2G) and protocols from the manufacturer (Instrumentation Laboratory, Breda, the Netherlands). Levels of factor (F) VIII and II, antithrombin (AT), and protein C were measured on an automated coagulation analyser (ACL 500 TOP) with reagents and protocols from the manufacturer (Recombiplastin 2G for FII, Hemosil (R) SynthASil for FVIII, Liquid Antithrombin reagent for AT, and IL reagent for protein C) (Instrumentation Laboratory).

Statistical analyses

Values are expressed as means (with S.D.), medians (with interquartile ranges), or numbers (with percentages) as appropriate. Differences between values of pre-operative coagulation tests and thrombin generation tests, and follow-up values were evaluated by mixed linear models. To calculate differences between continuous data among independent groups, the t-test for independent samples or the Mann–Whitney U-test, as appropriate, was used. Differences between patient values and levels measured in healthy controls were compared using one-way ANOVA (with the Bonferroni post-test) or Kruskal–Wallis H test (with Dunn's post-test) as appropriate. P-values of 0.05 or less were considered statistically significant. GraphPad Prism (San Diego, CA, USA) and IBM SPSS Statistics 20 (New York, NY, USA) were used for analyses.

Results

Patient characteristics

Seventeen patients who underwent a right (n = 15) or extended right (n = 2) hemi-hepatectomy, ten patients who underwent a PPPD, and twenty-four controls were included in the study. The main characteristics of the study population are reported in Table 1. The most common indication for liver resection was liver metastases from colorectal cancer and the most common indication for PPPD was pancreatic cancer. The median estimated blood loss was 700 mL in the patients who underwent a hemi-hepatectomy and 400 mL in the patients who underwent a PPPD. None of the patients suffered from venous thrombosis within 30 days after surgery.

Conventional coagulation tests

Baseline PT was 10.8 s (9.9–11.2) [median (interquartile range)] in the controls, 11.5 s (10.9–12.0) in the patients undergoing hepatectomy ($P < 0.05$ compared to controls), and 11.6 s (10.6–12.2) in the patients undergoing PPPD ($P > 0.05$ compared to controls). The PT progressively increased after both liver and pancreatic surgery (Figure 1), with a peak PT of 18.3 s (16.8–21.3) in the patients undergoing hepatectomy ($P < 0.001$ compared to baseline PT) and 14.4 s (13.5–15.1) in the patients undergoing PPPD ($P = 0.002$ compared to baseline PT) on post-operative day 1. After post-operative day 1 the PT decreased to baseline levels on post-operative day 3 in the patients undergoing PPPD and on post-operative day 7 in the patients undergoing hepatectomy.

Characteristics	Hemi-hepatectomy (n=17)	PPPD (n=10)	Controls (n=24)	P-value
Sex (male)	6 (35)	7 (70)	13 (54)	0.201
Age (years)	62 [9.8]	67 [7.9]	27 [4.5]	<0.001
Surgical indications				
Colon cancer metastasis	9 (53)	1 (10)		
Hepatocellular carcinoma (HCC)	2 (12)	0		
Pancreatic cancer	0	7 (70)		
Cholangiocarcinoma	2 (12)	1 (10)		
Neuroendocrine tumor	1 (5.9)	1 (10)		
Leiomyosarcoma	1 (5.9)	0		
Adrenocortical Carcinoma metastasis	1 (5.9)	0		
Benign lesion	1 (5.9)	0		
Length of surgery (min)	616 (480-650)	631 (551-739)		0.414
Estimated blood loss (ml)	700 (200-1000)	400 (300-1125)		0.980
Amount of fluids administered (ml)	4441 [1580]	5225 [1239]		0.192
RBC transfusion (units)	0 (0-0)	0 (0-0)		0.902
FFP transfusion (units)	0	0		1.000
Length of hospital stay (days)	14 (9-21)	15 (11-21)		0.863
Hemoglobin before surgery (mmol/L)	8.6 [1.2]	8.2 [1.1]		0.407
Hemoglobin after surgery (mmol/L)	6.7 [1.3]	6.8 [1.2]		0.841

Table 1. Patient characteristics.
PPPD: pylorus preserving pancreaticoduodenectomy
Data are expressed as number (%), mean [SD], or median (interquartile range).
* To convert values for hemoglobin to g/dL, multiply by 1.650.

The APTT was 34.2 s (31.1–38.5) in the controls, 37.9 s (32.3–43.5) in the patients undergoing hepatectomy ($P > 0.05$ compared to controls), and 30.2 s (25.6– 36.3) in the patients undergoing PPPD ($P > 0.05$ compared to controls) at baseline. The APTT slightly decreased

following hepatectomy [reaching 31.8 s (28.8– 34.6) on post-operative day 3; P = 0.009], but increased following PPPD [reaching 39.6 s (36.4–43.2) on post-operative day 1; P < 0.001; Figure 1].

At baseline, AT levels were slightly lower in patients undergoing hepatectomy [85.0% (78.0–92.0), P < 0.001)] and patients undergoing PPPD [83.0% (71.3–85.8), P < 0.001] compared to the controls [109.0% (99.3– 117.0)]. Levels of AT substantially and significantly decreased at the end of surgery after both the hepatectomy [52.0% (40.0–63.5), P < 0.001 compared to baseline value] and PPPD [61.0% (35.3–68.5), P < 0.001 compared to baseline value]. AT levels further decreased in the patients undergoing hepatectomy until post-operative day 3, after which levels slowly increased towards baseline levels which were reached on post-operative day 30. In the patients undergoing PPPD, levels of AT progressively increased after post-operative day 1, reaching levels exceeding baseline levels on post-operative day 30 (P = 0.002; Figure 1).

Baseline levels of protein C were comparable between patients undergoing hepatectomy, patients undergoing PPPD, and controls [96.0% (82.0–111.0), 94.5% (80.0– 118.8) and 104.0% (93.0–112.3), respectively]. Protein C levels decreased until post-operative day 1 in both the patients undergoing hepatectomy [44.0% (31.0–51.5), P < 0.001 compared to baseline value] and the patients undergoing PPPD [53.0% (45.0–75.5), P < 0.001 compared to baseline

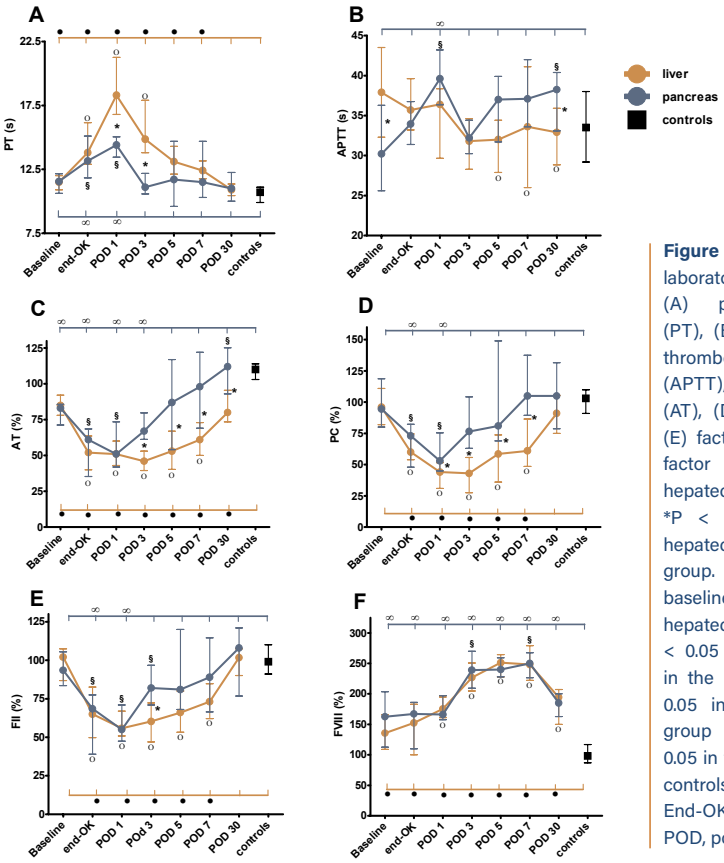

Figure 1. Changes in laboratory measurements (A) prothrombin time (PT), (B) activated partial thromboplastin time (APTT), (C) anti-thrombin (AT), (D) Protein C (PC), (E) factor II (FII), and (F) factor VIII (FVIII) after hepatectomy and PPPD.
*P < 0.05 between the hepatectomy and PPPD group. °P < 0.05 vs. baseline levels in the hepatectomy group. §P < 0.05 vs. baseline levels in the PPPD group. ●P < 0.05 in the hepatectomy group vs. controls. ∞P< 0.05 in the PPPD group vs. controls.
End-OK, end of surgery; POD, post-operative day.

value]. Protein C levels decreased significantly more after hepatectomy compared to PPPD (P = 0.03), and the time to reach baseline levels was substantially longer after hepatectomy (levels returned to baseline on post-operative day 30 after hepatectomy, compared to post-operative day 3 after PPPD) (Fig. 1).

Levels of FII at baseline were comparable between the groups, with 99.0% (91.0–110.0) of FII in the controls, 102.0% (86.8–107.4) in the patients undergoing hepatectomy, and 93.5% (83.5–105.5) in the patients undergoing pancreatic surgery. FII levels decreased following surgery to 55.8% (50.9–67.0) (P < 0.001 compared to baseline value) in patients undergoing hepatectomy and 55.0% (47.5–71.0) (P < 0.001 compared to baseline value) in patients undergoing PPPD on post-operative day 1. Baseline levels of FII were reached again on post-operative day 4 after PPPD, but not until post-operative day 30 after hepatectomy (Figure 1). FVIII levels were, at baseline, significantly higher in both the patients undergoing hepatectomy [135.6% (109.0–165.8), P < 0.05] and the patients undergoing PPPD [162.5% (112.5–203.5), P < 0.01] compared to the controls [92.5% (85.5–114.8)]. Levels of FVIII increased in both groups following surgery, with the highest levels of FVIII on post-operative day 5 after hepatectomy [251.1% (227.8–264.4), P < 0.001 compared to baseline value], and on post-operative day 7 after PPPD [250.0% (234.0–261.0), P = 0.03 compared to baseline value]. After post-operative day 7 the levels of FVIII substantially decreased in both groups although levels remained elevated compared to the healthy controls (Fig. 1).

Thrombin generation

At the start of surgery, the ETP measured in the absence of TM, was comparable between the patients undergoing hepatectomy, the patients undergoing PPPD and healthy volunteers (Table 2). In the patients undergoing hepatectomy, the ETP decreased until post-operative day 7 (P < 0.001 compared to baseline value), and recovered until baseline values on day 30. In the patients undergoing PPPD, the decrease in ETP was more pronounced as compared to the decrease in the patients undergoing hepatectomy (P = 0.01 compared to baseline value, and P = 0.045 compared to hepatectomy on post-operative day 3). The ETP in the patients undergoing PPPD also recovered to baseline values on post-operative day 30 (Figure 2).

Despite the decrease in thrombin generation in the absence of TM, in the presence of TM thrombin generation increased following hepatectomy (Table 2; Figure 2). The ETP increased from 542 nM*min (417–694) at baseline (P > 0.05 compared to controls) to 845 nM*min (789–1050) on post-operative day 3 (P < 0.001 compared to baseline; P < 0.001 compared to controls). In comparison, total thrombin generation only slightly increased in the patients following PPPD (Figure 2). When these data were recalculated to a normalised thrombomodulin sensitivity ratio (TM-SR), it became evident that after hepatectomy thrombomodulin was less effective at regulating thrombin generation in these patients compared to controls (Figure 2). The TM-SR increased from 1.2 ± 0.4 (mean ± S.D.) at baseline to 2.1 ± 0.2 on post-operative day 3 in the patients undergoing hepatectomy (P < 0.001 compared to baseline; P < 0.001 compared to controls). In comparison, the TM-SR only slightly increased in the patients following PPPD.

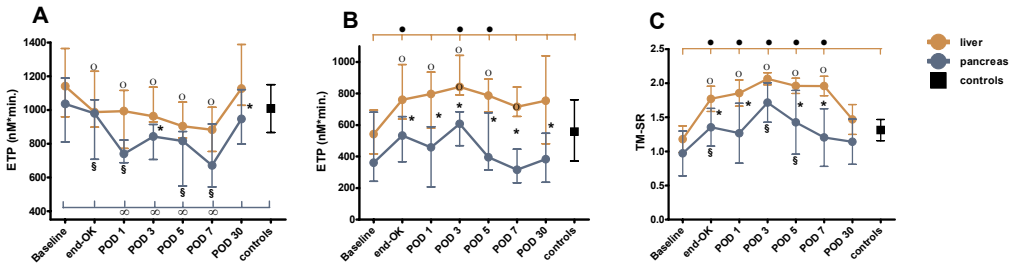

Figure 2. Changes in the thrombin generation assay [(A) endogenous thrombin potential (ETP) without thrombomodulin (TM), (B) endogenous thrombin potential (ETP) with TM, (C) thrombomodulin sensitivity ratio (TMSR)] after hepatectomy and PPPD.

*P < 0.05 between the hepatectomy and PPPD group. °P < 0.05 vs. baseline levels in the hepatectomy group. §P < 0.05 vs. baseline levels in the PPPD group. ●P < 0.05 in the hepatectomy group vs. controls. ∞P < 0.05 in the PPPD group vs. controls. End-OK, end of surgery; POD, post-operative day.

	TM- ETP	Peak	Lag-time	Velocity index	TM+ ETP	Peak	Lag-time	Velocity index
Controls	1009 [856-1154]	233 [191-245]	1.9 [1.7-2.0]	94 [58-105]	558 [390-680]	142 [99-176]	1.7 [1.7-2.0]	67 [49-87]
Patients following hepatectomy								
Baseline	1141 [960-1364]	201 [183-201]	1.8 [1.7-2.2]	68 [51-95]	542 [417-694]	128 [97-156]	1.7 [1.7-2.0]	57 [41-78]
End-OK	987 [915-1223]+	200 [170-220]	1.7 [1.3-1.8]	85 [75-97]+	760 [593-878]*+	161 [124-164]+	1.6 [1.3-1.7]	77 [61-87]+
POD1	993 [781-1179]+	194 [146-212]	2.0 [1.7-2.0]	85 [71-98]	820 [628-956]+	167 [135-185]	2.0 [1.7-2.0]	79 [64-93]
POD3	981 [931-1150]+	203 [186-227]	2.0 [1.7-2.0]	99 [88-106]+	845 [789-1050]*+	189 [177-203]*+	2.0 [1.7-2.0]	94 [88-102]*+
POD5	907 [823-1073]+	203 [180-218]	1.7 [1.7-2.0]	95 [88-103]+	787 [721-896]*+	181 [174-208]+	1.7 [1.7-2.0]	93 [79-104]*+
POD7	883 [745-994]+	199 [176-215]	1.7 [1.7-2.0]	89 [82-106]+	715 [654-847]	186 [162-198]+	1.7 [1.7-2.0]	93 [74-103]*+
POD30	1173 [1080-1173]	243 [194-293]	1.8 [1.7-2.0]	103 [58-126]+	818 [501-1101]	179 [116-248]+	1.7 [1.7-2.0]	77 [50-115]+
Patients following PPPD								
Baseline	1036 [810-1189]	165 [118-205]*	1.8 [1.7-2.3]	49 [33-87]*	361 [243-680]	78 [55-168]*	1.7 [1.7-1.9]	36 [25-80]*
End-OK	980 [686-1112]+	152 [116-193]*	1.8 [1.5-2.2]	64 [36-80]*	559 [243-823]	110 [63-127]*	1.7 [1.7-1.9]	53 [32-62]
POD1	772 [656-874]*+	128 [96-162]*+	2.3 [1.6-3.3]	47 [26-72]*	388 [178-691]	91 [35-140]*	2.0 [1.6-2.7]	42 [11-63]*
POD3	868 [658-962]*+	194 [146-212]*	1.7 [1.7-2.1]	79 [65-99]+	627 [469-754]	154 [120-187]+	1.8 [1.7-2.1]	70 [59-94]+
POD5	825 [524-882]*+	182 [122-207]*	2.0 [1.9-2.2]	67 [49-104]	399 [347-701]	119 [98-185]	2.0 [1.7-2.3]	59 [47-83]
POD7	733 [549-966]*+	162 [127-212]*	2.2 [1.9-2.5]	69 [45-84]*	317 [259-601]	89 [74-165]	2.0 [1.9-2.5]	49 [32-83]
POD30	979 [901-1121]	185 [149-206]*	2.2 [1.9-2.3]	70 [43-78]*	476 [331-602]	112 [74-136]*	2.0 [1.7-2.1]	48 [32-66]*

Table 2. Parameters derived from the thrombin generation test in patients following hepatectomy, patients following PPPD, and healthy controls.

Data are expressed as mean [SD] or median [range]. *P<0.05; **P<0.01; ***P<0.001 versus controls.

PT: prothrombin time, APTT: activated partial thromboplastin time, Vi: initial rate of clot growth, Vst: stationary rate of clot growth, a.u.: arbitrary units, ETP: endogenous thrombin potential, TM: thrombomodulin.

Table 2 shows the other parameters derived from the thrombin generation test. In the absence of TM, also peak thrombin generation levels remained comparable to baseline values following hepatectomy, and slightly decreased in the patients following PPPD on post-operative day 1. Following surgery, the lag-time remained comparable to baseline values for both patient groups. The velocity index increased until post-operative day 30 following hepatectomy, but only slightly increased on post-operative day 3 and declined again thereafter following PPPD. In the presence of TM, peak thrombin generation also gradually increased following hepatectomy, compared to only an increase on post-operative day 3 following PPPD. Following surgery, the lag-time remained the same in both patient groups. The velocity index, however, increased in both groups following surgery, with a maximum on post-operative day 3.

Discussion

In the present study, we observed a hypercoagulable status in samples taken from patients following a right hemi-hepatectomy, despite substantially prolonged conventional coagulation tests. Our results using thrombomodulin-modified thrombin generation testing are in line with several recent studies that found a normal to hypercoagulable state following liver surgery [5,6,10,19]. In contrast to previous work, our study provides a mechanistic explanation for the hypercoagulable state, i.e. a profound and sustained post-operative deficiency of the natural anti-coagulants AT and protein C.

Samples taken after liver resection were profoundly resistant to the anti-coagulant action of thrombomodulin, the physiological activator of the natural anti-coagulant protein C. While thrombin generation substantially decreased in healthy volunteers when thrombomodulin was added to the plasma, thrombin generation only slightly decreased by the addition of thrombomodulin in plasma from patients following hemi-hepatectomy. Consequently, thrombin generation in the presence of thrombomodulin was higher than that of healthy volunteers. The thrombomodulin resistance following liver surgery is in part explained by the decreased levels of protein C and elevated levels of FVIII [28]. Combined with the low levels of AT, the net effect is a normal or even supranormal thrombin generation when tested in the presence of thrombomodulin after hemi-hepatectomy, despite decreased levels of pro-coagulant proteins.

The decrease in coagulation factors following hemi-hepatectomy is in part explained by the decreased synthetic capacity of the liver remnant. However, we also observed a transient decrease in FII, AT, and protein C following pancreatic surgery. Therefore, it is likely that consumption of coagulation proteins as a result of surgical damage may also play a role. Finally, haemodilution may also partly explain the decrease in coagulation factor levels, and the decrease in haemoglobin following surgery combined with the limited blood loss indeed suggests our patients to be slightly haemodiluted.

Despite similar effects of both liver and pancreas surgery on levels of coagulation factors, we observed a difference in thrombin generation following hemi-hepatectomy and PPPD. Although thrombin generation tested in the presence of thrombomodulin was increased following liver surgery, thrombin generation under these experimental conditions was normal in patients following pancreatic surgery. Furthermore, when thrombin generation was tested in the absence of thrombomodulin, we observed a decrease in thrombin generation in both groups, but this decrease was much more pronounced following pancreatic surgery. De

Pietri et al. [6] have also shown signs of hypocoagulability in patients undergoing pancreatic surgery using TEG. As the decrease in protein C and AT were more extensive following hemi-hepatectomy compared to the decrease in patients following PPPD, this may in part explain the difference in thrombin generation between both groups.
As reported before [5,6,19,28], we also observed a substantial prolongation of conventional coagulation tests in patients following liver surgery. However, these tests cannot reflect the true haemostatic status of patients undergoing liver surgery, as they are only sensitive for levels of pro-coagulant factors and do not take the reduction in anti-coagulant factors, which also occur following liver surgery, into account. The thrombomodulin-modified thrombin generation test is, however, sensitive to all anti-coagulant proteins in the plasma. Therefore, this test measures the true balance between the pro- and anti-coagulant factors. Thrombin generation testing in the presence of thrombomodulin has demonstrated normal or even superior thrombin generation in patients with chronic liver disease, despite prolonged conventional coagulation tests [28,29,31]. These findings have challenged the long held dogma of chronic liver disease being associated with a bleeding tendency due to changes in the plasmatic coagulation system [33]. Our present findings show comparable findings in patients that underwent liver resections, which may have important clinical consequences. First, we strongly advise not to correct routine coagulation tests by using blood product transfusion during or after hepatectomy. In fact, several serious side effects of blood product transfusion may occur, including the risk of infection and the risk of transfusion-related acute lung injury [17]. Furthermore, routine thrombosis prophylaxis should not be withheld in patients undergoing liver resection, as this may result in higher rates of venous thromboembolism in post-operative liver disease patients. More extensive prophylaxis may even be required as VTE rates are still substantial in those patients receiving optimal routine thromboprophylaxis [34].

In conclusion, although conventional coagulation tests point to hypocoagulability in patients undergoing liver resection, thrombin generation in the presence of thrombomodulin revealed hypercoagulability following liver resection. This hypercoagulability was associated to a profound thrombomodulin resistance which was likely attributable to decreased levels of protein C and elevated levels of FVIII. Therefore, clinicians should be aware of the limitations of the use of conventional coagulation tests to guide haemostatic management during liver surgery. Furthermore, the results of our study support the exploration of more extensive use of anti-coagulant medication in the post-operative period, as was previously suggested by others [7,18,34].

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CHAPTER 6

Vascular disease in patients with nonalcoholic fatty liver disease

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Abstract

Nonalcoholic fatty liver disease (NAFLD) is increasingly being diagnosed and is considered to be the most frequent chronic liver disorder in Western countries. It represents a histopathological spectrum ranging from simple hepatic steatosis to steatohepatitis and finally cirrhosis. NAFLD is considered as the hepatic manifestation of the metabolic syndrome and is associated with increased mortality. Increasing evidence now suggests that NAFLD is also associated with higher cardiovascular disease (CVD) morbidity and mortality independent of conventional cardio metabolic risk factors (such as obesity, insulin resistance, and diabetes mellitus). The exact mechanisms linking NAFLD to increased CVD risk are still incompletely understood and likely reflect multiple coexisting pathways. Recent evidence suggests a contributive effect of an altered hemostasis in patients with NAFLD. For example, patients with NAFLD have higher levels of pro-thrombotic factors (e.g., von Willebrand factor, fibrinogen, factor VII activity, and plasminogen activator inhibitor-1), which correlate with underlying histological severity of the disease. The current review focuses on these hemostatic abnormalities in NAFLD and the link with increased CVD risk.

Introduction

Nonalcoholic fatty liver disease (NAFLD) is the most common cause of abnormally elevated aminotransferases [1,2] with an estimated prevalence ranging from 6.3 to 33% with a median of 20% in the general population [2]. Its prevalence increases from 70 to 90% among those with diabetes or obesity [1,2]. NAFLD is characterized by fat accumulation in the liver in the absence of excessive alcohol consumption (less than 20 g/day in women and less than 40 g/day in men) and exclusion of other secondary causes of hepatic steatosis [3–6]. NAFLD represents a histopathological spectrum ranging from simple hepatic steatosis to steatohepatitis (NASH), characterized by necroinflammatory changes which increase the risk for progression to advanced fibrosis and cirrhosis [6–8]. NAFLD is considered the hepatic manifestation of the metabolic syndrome and is associated with increased mortality, particularly cardiovascular disease (CVD)-related mortality [7–9]. The metabolic syndrome is a cluster of metabolic abnormalities that predicts the risk of developing diabetes and CVD better than any of its individual components [10]. The metabolic syndrome predicts the development of NAFLD, and the risk of having NAFLD increases significantly with the addition of each of the component of the metabolic syndrome [11,12].

Cardiovascular Disease and Nonalcoholic Fatty Liver Disease

Patients with NAFLD have multiple traditional and nontraditional risk factors for CVD, and CVD-related mortality is the leading cause of death in those with NAFLD [7,8,13] Although NAFLD is commonly seen with other features of the metabolic syndrome, the increased CVD risk in NAFLD is independent of obesity, diabetes, and other features of the metabolic syndrome [14,15]. Accumulating evidence implicates NAFLD as a risk factor not only for CVD events but also for subclinical and premature CVD [16] Presence of NAFLD is associated with increased carotid artery intimal medial thickness (IMT), a validated marker of subclinical atherosclerosis [14]. These findings were corroborated in a larger meta-analysis that clearly showed that the presence of NAFLD was associated with carotid artery IMT and prevalence of carotid artery atherosclerotic disease [17]. The IMT was the greatest in those with steatohepatitis, then in those with simple hepatic steatosis, and the lowest in healthy individuals [14]. In addition, the association between NAFLD and the increased risk of coronary artery disease (as measured by coronary artery calcium score or CAC) is independent of conventional risk factors, metabolic syndrome features, insulin resistance, and even preexisting CVD [18].

The risk of clinically evident CVD is also much higher in those with NAFLD and is independent of classical CVD risk factors, features of metabolic syndrome, and insulin resistance [15,19]. In addition, the presence of NAFLD was associated with lower remodeling lesions or lipid core plaques of coronary arteries, thereby implicating NAFLD as a risk factor for vulnerable coronary artery plaques [20]. In a community-based cohort those with NAFLD had a much higher 10-year FraminghamRisk Score (FRS) than matched controls [21]. In fact, in a recent meta-analysis, presence of NAFLD was associated with increased overall mortality (odds ratio [OR]: 1.57; confidence interval [CI]: 1.18–2.10) deriving from liver related and cardiovascular-related mortality [22]. The exact mechanisms linking NAFLD to increased CVD risk are incompletely understood and likely reflect multiple coexisting pathways, one of which is altered hemostasis in those with NAFLD. The current review focuses on these hemostatic abnormalities in NAFLD.

Hemostasis in Nonalcoholic Fatty Liver Disease

A link between NAFLD and CVD is their close association with pro-inflammatory markers, such as high sensitivity C-reactive protein (hs-CRP), interleukin-6 (IL-6), tumor necrosis factor-a (TNF-a), and other acute phase reactants [23–26]. In addition to these pro-inflammatory markers, NASH is also considered to be associated with a pro-thrombotic state and the studies investigating this association are summarized in Table 1.

Nonalcoholic Fatty Liver Disease and Platelets

It is well known that platelet activation is increased in the metabolic syndrome and obesity [46–51], likely reflecting the altered biophysical state of the platelet membrane component in these patients. Platelets of these individuals have an enhanced expression of the platelet surface of adhesive receptors, such as P-selectin, αIIbβ3, and the glycoprotein (GP)Ib/IX/V complex. The increased expression of the latter two receptors most probably causes more frequent episodes of platelet activation and degranulation [52]. Patients with metabolic syndrome also have concurrent endothelial dysfunction which may trigger platelet activation. Finally, hypertriglyceridemia, a key clinical feature of NASH, is a known trigger for platelet activation and may contribute to the underlying thrombotic risk [53,54]. Studies have also shown an increase in mean platelet volume, a reliable indicator of platelet activation, in patients with NAFLD [27–29]. In fact, in a cohort of 100 consecutive patients undergoing liver biopsy for NAFLD, the mean platelet volume increased in a stepwise fashion from those with no steatosis to those with simple steatosis to those with NASH [30]. Furthermore, in a group of 44 NASH patients, an increase in platelet count was observed [55]. However, the increase in platelet count is followed by a steady decrease in platelet count when the disease progresses to fibrosis/cirrhosis [56]. Finally, von Willebrand factor (VWF), a mediator of platelet adhesion and aggregation, was found to be significantly elevated in patients with NAFLD and this may be a consequence of endothelial injury [31,34,43].

Nonalcoholic Fatty Liver Disease and Hemostatic Abnormalities

In addition to the increased platelet activation, patients with NAFLD also have higher levels of pro-thrombotic factors (e.g., VWF, fibrinogen, factor VII activity, and plasminogen activator inhibitor-1 [PAI-1]) and this directly correlates with underlying histology [57–59] For example, Kotronen et al observed that the plasma levels of factors VIII, IX, XI, and XII were increased in 54 patients with NAFLD compared with 44 controls [32]. The associations between NAFLD and these coagulation factors were independent of age, gender, and body mass index, suggesting that the presence of hepatic steatosis independently contributes to the thrombosis risk. More recently, Verrijken et al found significantly higher levels of PAI-1 in patients with NASH than controls and this association correlated with underlying histological parameters [31]. However, in patients with NAFLD plasma levels of fibrinogen, factor VIII, VWF, and antithrombin did not correlate with liver histology, but did correlate with features of the metabolic syndrome. Elevated levels of PAI-1 might, therefore, partly explain the increased cardiovascular risk seen in patients with NAFLD. Indeed, increased levels of PAI-1, with resultant reduction in fibrinolysis, have been extensively reported as a risk factor for thrombosis and cardiovascular events [60], although some studies suggest PAI-1 merely to be a reflection of general cardiovascular risk factors [61]. The liver is the site of synthesis for a majority of pro-coagulant proteins. Several studies have now documented an increased intrahepatic expression of these proteins in patients with NAFLD [35,36], suggesting that this increase may be because of increased hepatic synthesis [58,59]. These findings are further

corroborated by previous studies that have shown dysregulation of several candidate genes responsible for accelerated atherogenesis in the liver of those with NASH [62–64]. Despite the initial findings between NAFLD and hemostatic abnormalities, the data should be interpreted with caution as most of these studies are limited by their relatively small sample size and cross-sectional design. In addition, patients with cirrhosis were excluded from most of these studies, so it is not clear how progression to cirrhosis impacts these parameters. Nevertheless, some evidence does suggest that patients with NASH-related compensated cirrhosis have an increased risk of atherothrombotic events [65]. In a prospective study, it was demonstrated that, while patients with compensated cirrhosis because of NASH have a lower overall mortality rate than patients with hepatitis C virus-associated cirrhosis, cardiovascular mortality is increased in patients with NASH related cirrhosis than those with hepatitis C virus-associated cirrhosis [66].

To date, only one recent study has examined the coagulation status in patients with NASH-related cirrhosis [33]. The authors observed a pro-coagulant imbalance characterized by increased levels of factor VIII and a reduction in protein C in patients with NAFLD and these differences were more pronounced in those with NASH and NASH-cirrhosis than those with simple steatosis. However, they found no differences in alcoholic/viral-related cirrhosis, in which a pro-coagulant imbalance was also observed, as has been previously noted [67–69]. This observation, therefore, does not explain the increased risk of cardiovascular disease in NASH cirrhosis compared with alcoholic/viral cirrhosis. Furthermore, the authors only show the endogenous thrombin potential (ETP) ratio, which represents the resistance of the plasma to the anticoagulant action of thrombomodulin, and do not depict the original thrombin-generation data. More specifically, they do not compare original thrombin-generation data, for example, ETP in the presence of thrombomodulin between patients with NAFLD and controls. If the original ETP values are indeed significantly increased in patients with NAFLD compared with controls, this implies a hypercoagulable status in plasma of patients with NAFLD. Thus, to confirm these findings, further studies are needed to investigate the hemostatic abnormalities in patients with advanced NAFLD.

Primary hemostasis: Platelet dysfunction	**Secondary hemostasis:** Hypercoagulability	**Tertiary hemostasis:** Hypofibrinolysis
Increased platelet activation [27-30] Elevated levels of VWF [31,34,43]	High procoagulant factors VIII, IX, XI, and XII [31-33] High plasma levels of factor VII clotting activity [37] Elevated levels of fibrinogen [24,31,34,44] Low levels of antithrombin III [31] Levels of protein C either increased [44,45] or decreased [33]	Elevated levels of PAI-1 [15,24,31,32,34-42] Low levels of TAFI antigen [38] Low levels of t-PA [37]

Table 1. Summary of studies on the changes in all phases of the hemostasis in patients with nonalcoholic fatty liver disease.
VWF: von Willebrand Factor, PAI: Plasminogen activator inhibitor; TAFI: Thrombin-activatable fibrinolysis inhibitor, t-PA: Tissue plasminogen activator

Risk of Venous Thrombosis in Nonalcoholic Fatty Liver Disease

Recent epidemiological studies have challenged the notion that patients with a chronic liver disease are auto-anticoagulated, and, hence, are protected against venous thrombosis (including deep vein thrombosis [DVT] and pulmonary embolism) [70–74]. Rather, there is evolving evidence to suggest a higher propensity for developing venous thrombosis in patients with a liver disease [75–77].

While there are multiple case-control studies that have shown an increase in circulating levels of various pro-thrombotic proteins in plasma of patients with NAFLD (see previous section and Table 1), there is currently little published research that assessed the association between NAFLD/NASH and the risk of venous thrombosis. Although there is lack of data in NAFLD patients, there are numerous studies that implicate obesity, a common feature of NAFLD, as a risk factor for the development of venous thromboembolism (VTE) [78–80]. In the limited data available, an independent association between NAFLD and idiopathic VTE was noted and the prevalence of NAFLD increased nearly threefold in those with VTE [81]. A possible cause of the increased risk of venous thrombosis in NAFLD patients might be the elevated levels of PAI-1 resulting in hypofibrinolysis in these patients. However, there is currently little known about the incidence of venous thrombosis in NASH-related cirrhosis. In addition, a recent retrospective study reported an increased prevalence of portal vein thrombosis (PVT) in patients with NASH related cirrhosis, and the presence of NASH was an independent risk factor for the diagnosis of PVT [82]. Because of the limited number of studies, these data should be interpreted with caution and well-designed prospective studies are needed to further explore the relationship between NAFLD/NASH fibrosis stage and the risk of venous thrombosis.

Role of Hypercoagulability in Liver Fibrogenesis

The progression from chronic liver injury to advanced hepatic fibrosis or cirrhosis is a complex process, mediated through the interaction between genetic and environmental factors. Recent evidence suggests a role for the activity of the coagulation cascade in hepatic fibrogenesis [75,83]. For example, epidemiological evidence has shown an association between congenital coagulation abnormalities (e.g., increased expression of factor VIII, protein C deficiency, and factor V Leiden mutation) and accelerated progression to cirrhosis in patients with a chronic hepatitis C infection [84–87]. Furthermore, Assy et al observed a correlation between the presence of thrombotic risk factors and the extent of hepatic fibrosis in patients with NASH [45] Based on recent evidence, there may be two hypotheses that could explain the involvement of the coagulation cascade in the rate of liver fibrogenesis: tissue ischemia because of intrahepatic thrombi (also referred to as parenchymal extinction) and the activation of stellate cells by coagulation proteases [83].

Accumulating animal data suggest that antithrombotic treatment may retard the progression of fibrosis by inhibiting the coagulation or the platelet function [88–92]. Fujita et al have shown that antiplatelet therapy reduces hepatic steatosis, inflammation, and hepatic fibrosis in rats [93]. Also, anticoagulant therapy decreased fatty liver disease in an experimental animal model [94]. If antithrombotic treatment truly prevents progression of disease and substantially delays decompensation, the need for liver transplantation, or death, this will profoundly impact the clinical management of patients with a chronic liver disease. Nevertheless, several recent experimental animal studies suggest that platelets and fibrin

may, on the contrary, have beneficial effects on liver injury [95–98]. Future clinical studies on the benefits and safety of antithrombotic treatment in different patient groups are, therefore, required before this may be considered in routine practice. NAFLD might be a reasonable disease state to further investigate these treatment strategies in human trials, because of the suggested hypercoagulable state and the relative slow course of the disease. A major limitation to evaluating coagulation defects clinically is the lack of readily available laboratory tests to measure hypercoagulability in patients with a chronic liver disease.

Conclusion

NAFLD, the most common cause of liver disease globally, is associated with higher CVD morbidity and mortality. Multiple processes probably contribute to this increased risk of CVD, including abnormal glucose, fatty acid, and lipoprotein metabolism, chronic inflammation, increased oxidative stress, deranged adipokine function, endothelial dysfunction, atherosclerosis, and cardiac lipotoxicity. Several studies have also suggested a role for the presence of a hypercoagulable state in the increased risk of CVD in patients with NAFLD.

The present evidence strongly highlights the importance of evaluating the risk of CVD and related pro-inflammatory/pro-thrombotic state in patients with NAFLD. Clinicians should recognize the increased risk of CVD among patients with NAFLD, which warrants evaluation and treatment as much as the liver disease itself.

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CHAPTER 7

Preserved hemostatic status in patients with non-alcoholic fatty liver disease

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Abstract

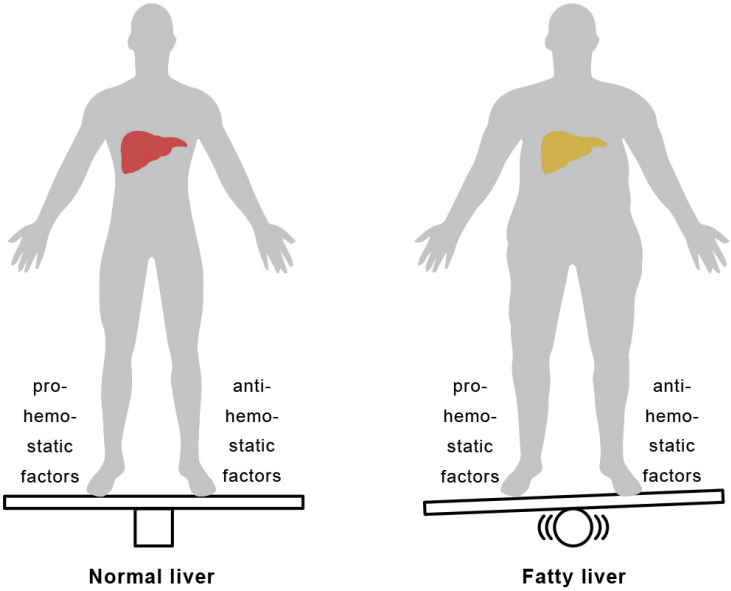
Background & Aims: Non-alcoholic fatty liver disease (NAFLD) is associated with an increased risk of thrombosis. However, it remains unclear if hypercoagulability contributes to this risk. We, therefore, determined an in-depth hemostatic profile in a cohort of well-defined patients with NAFLD.

Methods: We drew blood samples from 68 patients with biopsy-proven NAFLD (simple steatosis n = 24, NASH n = 22, and NASH cirrhosis n = 22), 30 lean controls, 30 overweight controls (body mass index (BMI) >25 kg/m2), and 15 patients with alcoholic (ASH) cirrhosis, and performed in-depth hemostatic profiling.

Results: Basal and agonist-induced platelet activation, plasma levels of markers of platelet activation, and plasma levels of the platelet adhesion regulators von Willebrand factor and ADAMTS13 were comparable between patients with non-cirrhotic NAFLD and controls. Agonist-induced platelet activation was decreased in patients with cirrhosis. Thrombomodulin-modified thrombin generation was comparable between all patients and controls, although patients with cirrhosis had a reduced anticoagulant response to thrombomodulin. Thromboelastography test results were comparable between controls and non-cirrhotic NAFLD patients, but revealed moderate hypocoagulability in cirrhosis. Plasma fibrinolytic potential was decreased in overweight controls and non-cirrhotic NAFLD, but accelerated fibrinolysis was observed in ASH cirrhosis. Clot permeability was decreased in overweight controls and patients with NAFLD.

Conclusions: The overall hemostatic profile is comparable between patients with non-cirrhotic NAFLD and controls. Additionally, pro-thrombotic features (hypofibrinolysis and a prothrombotic structure of fibrin clot) in patients with NAFLD are likely driven by obesity. Our study suggests a limited role for hyperactive hemostasis in the increased thrombotic risk in NAFLD.

Graphical abstract



Introduction

Non-alcoholic fatty liver disease (NAFLD) is associated with an increased risk of cardiovascular disease (CVD). Increasing evidence suggests that the higher incidence of cardiovascular disease (CVD) morbidity and mortality in patients with NAFLD is independent of conventional cardiometabolic risk factors (such as obesity, insulin resistance, and diabetes mellitus) [1–3]. However, the exact mechanisms linking NAFLD to increased risk of CVD are incompletely understood and likely reflect multiple coexisting pathways [3]. Furthermore, rates of venous thromboembolism (VTE) and portal vein thrombosis (PVT) appear also increased in patients with NAFLD [4,5]. Recent studies have suggested a role for a hypercoagulable state in the increased risk of thrombosis in patients with NAFLD. Increased plasma levels of various pro-thrombotic factors (e.g., fibrinogen, factor VIII, and plasminogen activator inhibitor 1 (PAI-1)) have been described in patients with NAFLD [6–9]. Furthermore, studies have shown hypercoagulable features in patients with NAFLD detected with either thromboelastography (TEG) [10] or thrombin generation testing [11]. Platelet hyperactivity has also been implicated as a contributor of the increased risk of cardiovascular disease in patients with the metabolic syndrome [12–14], but its role in NAFLD remains unclear [3].

Nevertheless, results on the hemostatic status in NAFLD are inconsistent [3]. Furthermore, most studies have reported plasma levels of individual hemostatic proteins rather than functional tests of hemostasis. In addition, no study has profiled all components of the hemostatic system simultaneously, and patients with cirrhosis were excluded from most of these studies. The hemostatic status across the spectrum of NAFLD stages thus remains unclear. Whether the hemostatic status might explain the increased risk of thrombosis in these patients also remains to be firmly established. We, therefore, determined an in-depth hemostatic profile by performing functional hemostatic tests of platelets, coagulation, fibrinolysis, and fibrin clot structure in a cohort of well-defined patients with NAFLD. Furthermore, we compared the hemostatic status of patients with NASH-related cirrhosis to that of patients with alcoholic-(ASH) related cirrhosis. This is the first study that comprehensively investigated all components of the hemostatic system (i.e., platelets, coagulation, and fibrinolysis) using both biomarkers and functional tests in patients with various histological severities of NAFLD.

Patients and methods

Patients

All subjects (healthy controls, patients with various severity of NAFLD, and patients with ASH cirrhosis) were enrolled through the NASH Clinic at the Virginia Commonwealth University (Richmond, VA). The study protocol was IRB approved and written informed consent was obtained from each subject before inclusion in the study. NAFLD was defined by the evidence of hepatic steatosis on liver biopsy and the absence of causes for secondary hepatic fat accumulation (such as significant alcohol consumption) [15]. The liver biopsy was graded according to the NASH Clinical Research Network (NASH-CRN) scoring system, and the NAFLD activity score (NAS) was based on the unweighted sum of steatosis, lobular inflammation, and hepatocellular ballooning scores [16]. The NAFLD cohort was further subdivided into patients with simple hepatic steatosis (defined as the presence of hepatic steatosis with no evidence of hepatocellular injury (ballooning), n = 24), patients with non-alcoholic steatohepatitis (NASH; defined as the presence of hepatic steatosis and inflammation with hepatocyte injury with or without fibrosis, n = 22), and patients with NASH-related cirrhosis (defined as the presence of

cirrhosis with current or previous histological evidence of steatosis or steatohepatitis, n = 22) [15]. Two control groups of lean (BMI <25 kg/m2; n = 30) and overweight (BMI >25 kg/m2; n = 30) subjects with no evidence of chronic liver disease were included to establish reference values for the various tests performed. The absence of liver disease was established by normal liver enzymes and a normal liver sonogram. Furthermore, fifteen patients with alcoholic (ASH) cirrhosis were included as a control group for the patients with NASH-related cirrhosis. Exclusion criteria were documented history of congenital coagulation disorders, presence of active infection (<2 weeks), use of anticoagulant or anti-platelet drugs, pregnancy, human immunodeficiency virus positivity, and recent (<7 days) transfusion with blood products.

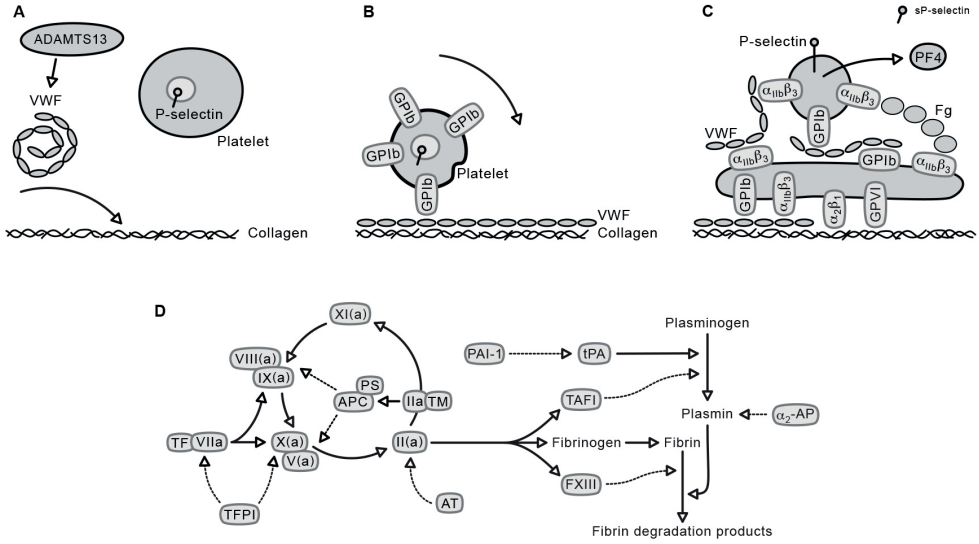


Figure 1. Schematic representation of the hemostatic system. Upon vessel wall injury, subendothelial collagen is exposed (A). Plasma VWF, which is a globular protein in circulation, unwinds over the collagen surface and becomes adhesive to platelets. The multimeric size of VWF is regulated by the protease ADAMTS13. Platelets lose velocity by rolling over collagen-bound VWF (B), and eventually stably adhere by multiple receptors (C). Platelets become activated by multiple triggers including collagen and thrombin, which results in a conformational change in αIIbβ3. This conformational change allows platelet-platelet interactions mediated by VWF and fibrinogen. During platelet activation, proteins within platelet α- granules (among which PF4) are excreted. In addition, the α-granule transmembrane protein P-selectin is translocated to the outer membrane during α-granule secretion. Part of this P-selectin is shed from the platelet surface and soluble P-selectin can be found in plasma. Simultaneously, vessel wall injury exposes tissue factor (TF), which initiates coagulation (D, left section). Thrombin (coagulation factor IIa) is the terminal enzyme of the coagulation system, and is responsible for cleavage of fibrinogen to fibrin. Thrombin generation is the net result of the work of pro- and anticoagulant systems. The anticoagulant systems are indicated by the interrupted line. The fibrin clot is degraded by the fibrinolytic system, which is activated by release of tPA from endothelial cells, resulting in conversion of plasminogen to plasmin (D, right section). Multiple regulatory steps in fibrinolysis are depicted with interrupted lines. Importantly, thrombin not only cleaves fibrinogen to fibrin, but also activates two major inhibitors of fibrinolysis (FXIII and thrombin activatable fibrinolysis inhibitor; TAFI).

Blood samples

Blood was drawn by venapuncture in 3.8% citrate tubes. A sample was processed directly for flow cytometry and thromboelastography. The remainder of the blood was processed to platelet-poor plasma (PPP) by double centrifugation at 2000g and 10.000g respectively for 10 min. Plasma was snap-frozen and stored at -80 C until use.

Figure 1 shows an overview of hemostasis and all tests performed are indicated in this schematic.

Platelet activation status

The platelet activation status was assessed using flow cytometry in whole blood. Details of sample processing have been described previously [17]. Platelets were kept in a resting state or activated by either adenosine diphosphate (ADP, 15 µM, Sigma-Aldrich, St. Louis, USA) or thrombin receptor activating peptide (TRAP6, 15 µM, Bachem, Bubendorf, Switserland). Samples were analyzed within six hours after processing using a BD LSRFortessa™ X-20 cell analyzer (BD Biosciences, Franklin Lakes, NJ, USA). Samples were gated on the basis of their forward and sideward scatter properties. The percentage of platelets expressing P-selectin and the geometric mean fluorescence intensity (MFI) of the platelet population were recorded. The percentage of platelets positive for P-selectin after activation was corrected for the percentage of platelets positive for P-selectin in the non-activated sample of each patient and control. The increase in the percentage of P-selectin positive platelets obtained in this manner represents the extent to which platelets in a given sample can be activated by a given activator (i.e., the platelet activatability).

Thromboelastography

Thromboelastography (TEG) was performed using the Thrombelastograph Hemostasis Analyzer 5000 (Haemonetics Corp., Haemoscope Division, Niles, IL, USA) as described previously [18]. Briefly, 1 ml of citrated whole blood (3.2% citrate) was subjected to kaolin-activated TEG within two hours of the blood draw. Kinetic changes in clot formation and clot dissolution were measured for 30 minutes after reaching maximal clot firmness. The following parameters were recorded: reaction time (R-time: the time taken for a clot to begin forming), kinetics time (K-time: the time from initial clot formation required to reach a specific clot firmness, α-Angle (the kinetics of clot formation), Maximum amplitude (MA: the maximal clot strength), and lysis at 30 minutes (Lysis-30 the percentage of clot lysis 30 minutes after MA is established).

Thrombin generation, PT, APTT

Thrombin generation testing was performed in platelet-poor plasma (PPP) with the fluorimetric method described by Hemker, Calibrated Automated Thrombography® (CAT) [19]. Reagents and protocols were purchased from Thrombinoscope BV, Maastricht, the Netherlands. Details of thrombin generation testing have been described previously [20]. The following parameters were recorded: endogenous thrombin potential (ETP; which represents the total enzymatic work performed by thrombin during the time that it was active), peak, velocity index (slope between the end of lag time and peak thrombin), and lag time (time needed for thrombin concentration to reach 1/6th of the peak concentration). In addition, a normalized thrombomodulin sensitivity ratio (TM-SR) was determined by dividing the ETP in the presence

of TM divided by the ETP in the absence of TM of an individual, by the ETP in the presence of TM divided by the ETP in the absence of TM of pooled normal plasma. A TM-SR > 1 reflects a decreased anticoagulant response to TM in comparison to pooled normal plasma.

The prothrombin time (PT) and activated partial thrombin time (APTT) were assessed on an automated coagulation analyzer (ACL 300 TOP) with reagents (Recombiplastin 2G for PT and SynthaSil for APTT) and protocols from the manufacturer (Instrumentation Laboratory, Breda, the Netherlands).

Clot lysis

Lysis of a tissue factor–induced clot by exogenous tPA was studied by monitoring changes in turbidity during clot formation and subsequent lysis as described previously [21]. Clot lysis time was determined as the time from the midpoint of the clear to maximum turbid transition, which characterizes clot formation, to the midpoint of the maximum turbid to clear transition, which represents clot lysis.

Fibrin structure

The average pore size of the fibrin clot (expressed as the Darcy constant Ks) was determined in permeation studies as described previously [22,23].

Fibrin density was assessed by laser-scanning confocal microscopy of clots supplemented with fluorescently labeled fibrinogen. Plasma clots were generated as described previously [24]. Clots were visualized with a Leica TCS SP8 confocal laser scanning microscope using a 63X/1.40 NA oil objective (Leica Microsystems, Eindhoven, The Netherlands). Alexa Fluor-488 fibrinogen was excited at 488 nm with an argon laser. Optical sections were taken at three randomly chosen areas throughout the clot to visualize the fibrin network. Fiber density was determined by counting the number of fibers crossing 100 µm lines placed in the image using the Image J plug-in grid (Fiji, National Institute of Health, Bethesda, Maryland, USA).

Fibrinogen was isolated from plasma samples as described previously [25]. Carbonylation of purified fibrinogen samples was quantified using a commercially available ELISA kit (Enzo Life Sciences, Farmingdale, NY) following the manufacturer's instructions.

Plasma markers of primary hemostasis

Soluble P-selectin (sP-selectin) and platelet factor 4 (PF4) levels were determined using a commercially available sP-selectin or PF4 enzyme-linked immunosorbent assay (ELISA) (R&D Systems, Abingdon, UK). Levels of von Willebrand Factor antigen (VWF) were assessed with an in-house ELISA using commercially available polyclonal antibodies against VWF (DAKO, Glostrup, Denmark). Plasma a disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13 (ADAMTS13) activity was measured using the FRETS-VWF73 assay (Peptanova, Sandhausen, Germany). Levels of VWF and ADAMTS13 in pooled normal plasma were set at 100%, and values obtained in test plasmas were expressed as a percentage of pooled normal plasma.

Plasma markers of coagulation

Levels of fibrinogen, factor (F) VII, FVIII, antithrombin, protein C, and D-dimer were assessed on an automated coagulation analyzer (ACL 300 TOP) with reagents (QFA thrombin (Hemosil) for fibrinogen, Factor VII deficient plasma and Recombiplastin for FVII, Factor VIII deficient plasma and Hemosil (R) SynthASil for FVIII, Liquid Antithrombin reagent for antithrombin, IL reagent for protein C, and Ddimer 500 HS for D-dimer) and protocols from the manufacturer (Instrumentation Laboratory, Breda, the Netherlands).

Plasma markers of fibrinolysis

PAI-1 levels were determined with an ELISA kit from R&D systems (Abingdon, UK). Levels of tissue plasminogen activator (tPA) were measured using an ELISA kit from Sekisui (Lexington, USA).

Statistical analysis

Data are expressed as means (with standard deviations (SDs)), medians (with ranges), or numbers (with percentages) as appropriate. Means of two groups were compared by Student's t-test or distributions in the two groups by Mann-Whitney U test as appropriate. Multiple groups were compared using one-way ANOVA (with the Bonferroni posttest) or Kruskal-Wallis H test (with Dunn's posttest) as appropriate. Spearman's correlation coefficient was used to assess correlation between continuous variables. P values of 0.05 or less were considered statistically significant. GraphPad Prism (San Diego, USA) and IBM SPSS Statistics 20 (New York, USA) were used for analyses.

Results

Patient characteristics

Patient characteristics are reported in Table 1. Sixty-eighth patients with biopsy-proven NAFLD (simple steatosis n = 24, NASH n = 22, and NASH cirrhosis n = 22), thirty lean controls (BMI 25 kg/m2), and fifteen patients with alcoholic (ASH) cirrhosis were included. None of the patients were diagnosed with another form of liver disease (e.g., hepatitis B, hepatitis C, autoimmune hepatitis, hereditary haemochromatosis etc.). The NAS score increased from patients with simple steatosis to patients with NASH and patients with NASH-related cirrhosis.

Data from all hemostasis tests performed are summarized in Table 2; parts of these data are also graphically represented herein.

Parameters	Lean controls n=30	Overweight controls n=30	Steatosis n=24	NASH n=22	NASH cirrhosis n=22	ASH cirrhosis n=15	P-value
Age (years)	43 [13]	42 [11]	50 [15]	53 [12]$^{\#}$	61 [10]$^{***\#\#\#}$	54 [8]$^{\#}$	<0.0001
Gender (male)	10 (33)	5 (17)	10 (42)	8 (36)	9 (41)	12 (80)*	0.003
BMI (kg/m^2)	22.6 [1.4]	30.4 [3.8]***	33.6 [6.5]***	34.6 [9.2]***	35.2 [5.4]***	27.2 [5.7]	<0.0001
Alcohol (units/week)	1 [0-8]	1 [0-42]	0 [0-7]	0 [0-3]	0 [0-3]	0 [0-130]	0.032
Race							
Caucasian	18 (60)	23 (77)	19 (79)	22 (100)*	21 (95.5)	10 (67)	<0.001
African American	4 (13)	6 (20)	4 (17)	0	1 (4.5)	5 (33)	
Asian	8 (27)	1 (3)	1 (4)	0	0	0	
OAC use	4 (13)	3 (10)	0	0	0	0	0.076
Diabetes mellitus	0	0	6 (25)	12 (54.5)$^{*\#}$	15 (68.2)$^{*\#}$	1 (6.7)	<0.001
Insulin dependent	0	0	1 (4.2)	4 (18.2)	3 (13.6)	0	0.008
Hemoglobin (g/dL)			14.0 [1.4]	14.5 [1.6]	13.3 [1.2]	12.2 [2.2]	0.0003
Leukocytes (10^9/L)			6.5 [3.2-12.2]	7.5 [3.4-11.2]	5.3 [3.1-15.4]	5.5 [3.9-9.2]	0.060
Platelets (10^9/L)			236 [53]	243 [64]	131 [69]	134 [64]	<0.0001
AST (U/L)	17 [7-66]	18 [12-35]	32$^{**\#\#\#}$ [20-68]	44$^{***\#\#\#}$ [15-184]	52$^{***\#\#\#}$ [18-128]	36$^{***\#\#\#}$ [20-571]	<0.0001
ALT (U/L)	12 [5-65]	15 [4-31]	40$^{***\#\#\#}$ [16-119]	56$^{***\#\#\#}$ [17-217]	35$^{***\#\#\#}$ [8-169]	28$^{**\#}$ [13-230]	<0.0001
Alk phos (U/L)			77 [43-141]	81 [38-138]	90 [29-437]	108 [81-545]	0.010
Bilirubin (mg/dL)	0.3 [0.2-1.0]	0.2 [0.2-1.1]	0.6$^{**\#\#\#}$ [0.4-1.2]	0.6$^{**\#\#\#}$ [0.2-2.6]	0.9$^{***\#\#\#}$ [0.3-4.0]	1.1$^{***\#\#\#}$ [0.2-5.1]	<0.0001
Cholesterol							
Total (mg/dL)	155 [31]	164 [17]	180 [46]	159 [37]	163 [39]	134 [31]	0.004
LDL (mg/dL)	81 [23]	85 [19]	110 [36]$^{**\#\#}$	88 [35]	89 [29]	73 [19]	0.0006
HDL (mg/dL)	48 [15]	42 [14]	53 [14]	49 [15]	50 [21]	37 [11]	0.018
Triglycerides (mg/dL)	91 [64]	136 [95]	121 [47]*	154 [70]	129 [54]	72 [38]$^{\#}$	0.001
NAS score			3 [1-4]	5 [4-6]	5 [4-8]		<0.0001
MELD score					9.1 [3.0]	11.3 [3.4]	0.053

Table 1. Patient characteristics

NASH: Non-alcoholic steatohepatitis, ASH cirrhosis: alcoholic cirrhosis, OAC: oral contraceptive, AST: aspartate transaminase, ALT: alanine transaminase, Alk phos: alkaline phosphatase, LDL: low-density lipoprotein, HDL: high-density lipoprotein, NAS: NAFLD activity score, MELD: model for end-stage liver disease. *P<0.05, **P<0.01, ***P<0.001 compared to lean controls. #P<0.05, #P<0.01, #P<0.001 compared to overweight controls. Data are expressed as number (%), mean [SD], or median [range].

Platelet activation status

The basal platelet activation status and the agonist-induced platelet activatability in patients and controls are reported in Figure 2. There was no statistically significant difference in the number of P-selectin positive platelets at baseline between patients with NAFLD, patients with ASH cirrhosis, and lean controls, although few individual patients appeared to have a slightly increased basal platelet activation status. Also the MFI of the P-selectin signal was comparable between all patients and controls. When platelets were activated in vitro using either TRAP or ADP, the percentage of platelets expressing P-selectin were decreased in patients with cirrhosis compared to controls when corrected for baseline values, although the difference did not reach statistical significance. Furthermore, the MFI after activation with TRAP or ADP was also decreased in patients with cirrhosis, although the difference did not reach statistical

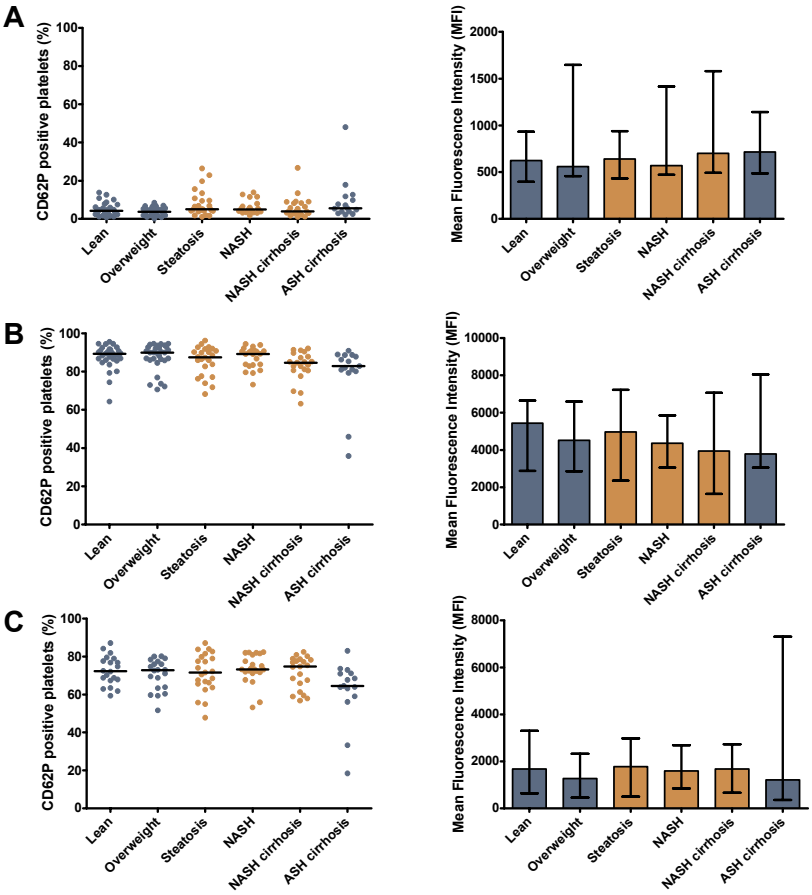


Figure 2. Basal and agonist-induced platelet activation.

The basal platelet activation status (A) and the agonist-induced platelet activatability using TRAP (B) and ADP (C) as assessed by flow cytometry for P-selectin in lean controls, overweight controls, patients with simple steatosis, patients with non-alcoholic steatohepatitis (NASH), patients with NASH cirrhosis, and patients with alcoholic (ASH) cirrhosis. Shown are the percentage of P-selectin positive platelets (left panels) and the mean fluorescent intensity (MFI) of the same flow cytometry experiments (right panels). Agonist-induced platelet activatability values were corrected for baseline values. Horizontal lines represent medians. Bars indicate medians with the error bars representing ranges.

significance compared to controls. Neither basal platelet activation status nor platelet activatability in patients with NAFLD correlated with BMI, lipid levels (high density lipoprotein (HDL)-C, low density lipoprotein (LDL)-C, total cholesterol, and triglycerides (TGs)), NAS score or individual histological parameters (steatosis, inflammation, and ballooning), aspartate aminotransferase (AST), alanine aminotransferase (ALT), bilirubin, or c-reactive protein (CRP).

Thromboelastography

TEG test results were comparable between lean and overweight controls, patients with simple steatosis, and patients with NASH. However, TEG revealed moderate hypocoagulability in both NASH and ASH cirrhosis as demonstrated by a prolonged K-time (NASH cirrhosis vs. overweight controls p <0.01) and a decreased a-angle (NASH cirrhosis vs. overweight controls p <0.01) and MA (ASH cirrhosis vs. overweight controls p <0.05).

Thrombin generation, PT, APTT

Compared to lean controls, there was a trend towards increased thrombin generation in overweight controls but this did not reach statistical significance. In the absence of TM, thrombin generation was decreased in patients with cirrhosis, as demonstrated by a decrease in ETP and peak. However, in the presence of TM, thrombin generation was comparable between all patient groups and controls (Fig. 3). When these data were recalculated to a normalized thrombomodulin sensitivity ratio (TM-SR), it became evident that TM was less effective at regulating thrombin generation in patients with cirrhosis compared to controls (Fig. 3). There was a strong negative correlation between TM-SR and levels of protein C (r = -0.60; p <0.0001) and levels of antithrombin (r = -0.57; p <0.0001). In addition, the TM-SR correlated with the ratio of factor VIII to protein C (r = 0.50; p <0.0001). However, TM-SR values did not correlate with BMI, lipid levels, or NAS score.

None of the thrombin generation parameters within the patients with NAFLD correlated with BMI, lipid levels (HDL-C, LDL-C, total cholesterol, and TGs), NAS score or individual histological parameters (steatosis, inflammation, and ballooning), AST, ALT, bilirubin, or CRP.

Both PT and APTT were comparable between patients with non-cirrhotic NAFLD and controls. However, in patients with cirrhosis the PT (p <0.001 compared to lean controls) and APTT (p <0.01 compared to lean controls) were prolonged.

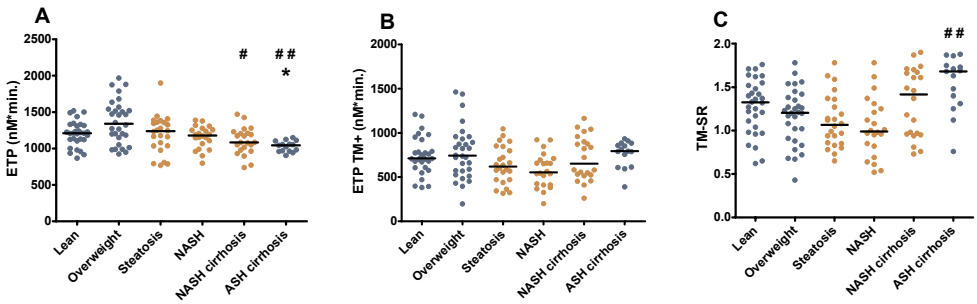


Figure 3. Results from the thrombin generation test.
Endogenous thrombin potential (ETP) (A), ETP in the presence of thrombomodulin (TM+) (B), and the thrombomodulin sensitivity ratios (TM-SR) (C) in lean controls, overweight controls, patients with simple steatosis, patients with non-alcoholic steatohepatitis (NASH), patients with NASH cirrhosis, and patients with alcoholic (ASH) cirrhosis.
* P <0.05, ** P <0.01 compared to lean controls. # P <0.01, ## P <0.01 compared to overweight controls.

Fibrinolysis

The clot lysis time was higher in overweight controls and in patients with non-cirrhotic NAFLD compared to lean controls, although differences did not reach statistical significance. The clot lysis time was comparable between patients with NASH-related cirrhosis and lean controls, but was decreased in patients with ASH-related cirrhosis (Fig. 4).

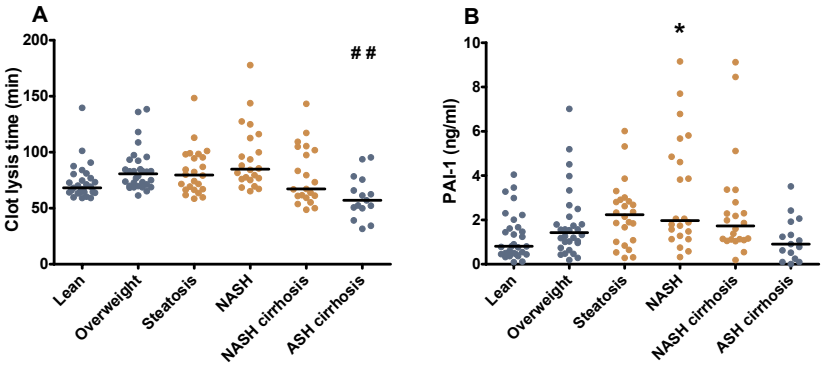


Figure 4. Fibrinolytic potential.

Clot lysis time (A) and levels of plasminogen activator inhibitor-1 (PAI-1) (B) in lean controls, overweight controls, patients with simple steatosis, patients with non-alcoholic steatohepatitis (NASH), patients with NASH cirrhosis, and patients with alcoholic (ASH) cirrhosis. Horizontal lines represent medians.

* P <0.05, ** P <0.01 compared to lean controls. # P <0.01, ## P <0.01 compared to overweight controls.

Fibrin structure

Clot permeability was decreased in overweight controls and patients with NAFLD, indicating more dense clots compared to lean controls, although differences did not reach statistical significance (Fig. 5). Clot permeability was similar in patients with ASH-related cirrhosis compared to lean controls, whereas clots generated in NASH-related cirrhosis had substantially reduced permeability.

Confocal microscopy revealed a stepwise increase in fiber density from lean controls to overweight controls and patients with simple steatosis to patients with NASH and NASH cirrhosis (Fig. 5). Fiber density was also increased in patients with ASH-related cirrhosis compared to lean controls ($p < 0.001$).

The protein carbonyl content of fibrinogen purified from patients with simple steatosis, NASH, and NASH cirrhosis was increased compared to lean controls, although differences did not reach statistical significance. However, the carbonyl content of fibrinogen purified from patients with ASH-related cirrhosis was significantly elevated ($p < 0.01$ compared to lean controls).

We observed a negative correlation between fibrinogen levels and clot permeability ($r = -0.66$, $p < 0.0001$). Furthermore, fiber density correlated with the BMI ($r = 0.35$, $p < 0.0001$). However, within patients with NAFLD, values of clot permeability or fiber density did not correlate with lipid levels, NAS score, AST, ALT, bilirubin, or CRP.

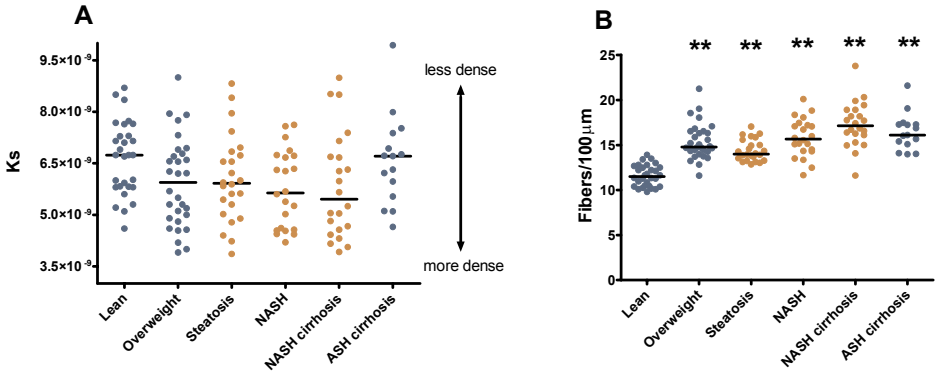


Figure 5. Fibrin structure.
The permeability coefficient (Ks, calculated following Darcy's Law) (A) and fibrin fiber density assessed by laser-scanning confocal microscopy (expressed as the number of fibers per 100 µm) (B) in plasma clots of lean controls, overweight controls, patients with simple steatosis, patients with non-alcoholic steatohepatitis (NASH), patients with NASH cirrhosis, and patients with alcoholic (ASH) cirrhosis. Horizontal lines represent medians. * P <0.05, ** P <0.01 compared to lean controls.

Plasma levels of proteins involved in primary hemostasis

Levels of sP-selectin were slightly, but non-significantly increased in patients with NASH, NASH cirrhosis, and ASH cirrhosis compared to lean and overweight controls. In contrast, levels of PF4 were comparable between all patient groups and controls. Plasma levels of VWF were comparable between controls and patients with non-cirrhotic NAFLD. However, VWF levels were increased in patients with cirrhosis compared to patients with non-cirrhotic NAFLD and controls (p <0.001). In contrast, ADAMTS13 activity was comparable between all cohorts.

Plasma levels of proteins involved in coagulation

Although fibrinogen levels were higher in overweight controls and patients with NAFLD compared to lean controls, this did not reach statistical significance. Patients with cirrhosis and lean controls had comparable fibrinogen levels. In NAFLD patients, levels of fibrinogen modestly correlated with BMI (r = 0.21; p = 0.02). Furthermore, fibrinogen levels increased with increasing severity of steatosis on liver biopsy (2.24 mg/ml [2.08–2.65] (median [range]) in steatosis <5%, 3.38 mg/ml [2.21–5.91] in steatosis 5–33%, 3.09 mg/ml [1.49–5.07] in steatosis 34–66%, and 3.80 mg/ml [3.44–4.43] in steatosis >66%; p <0.007). However, fibrinogen levels did not correlate with other individual histological parameters (inflammation or ballooning) or with lipid levels. Plasma levels of FVII were slightly, but non-significantly increased in patients with non-cirrhotic NAFLD compared to lean controls. In contrast, in patients with cirrhosis levels were decreased (NASH cirrhosis p <0.05; ASH cirrhosis p <0.001 compared to lean controls). Levels of FVIII were increased in patients with NASH-related cirrhosis compared to lean controls (p <0.05). In contrast, levels were similar between patients with ASH-related cirrhosis and controls. Both protein C and antithrombin levels were decreased in patients with NASH- and ASH-related cirrhosis (p <0.001 for both protein C and antithrombin compared to lean controls). Levels of D-dimer were non-significantly increased in patients with non-cirrhotic NAFLD, but levels were significantly increased in patients with cirrhosis compared to lean controls (p <0.001).

Parameters	Lean controls n=30	Overweight controls n=30	Steatosis n=24	NASH n=22	NASH cirrhosis n=22	ASH cirrhosis n=15	P-value
Flow cytometry							
Basal platelet activation (%)	4.3 [0.7-13.8]	3.8 [0.2-8.5]	5.0 [1.1-26.4]	4.9 [1.8-13.9]	3.9 [1.1-26.7]	5.6 [2.2-48.0]	0.063
TRAP-induced platelet activatability	89.3 [64.3-95.6]	89.9 [70.6-94.6]	87.5 [68.2-96.2]	89.3 [73.2-94.4]	84.6 [63.2-92.1]	82.9 [35.8-90.9]	0.012
ADP-induced platelet activatability	71.1 [59.3-87.1]	71.3 [51.6-84.2]	71.6 [47.8-87.1]	73.3 [53.1-82.3]	74.7 [56.8-82.4]	64.5 [18.4-83.0]	0.118
MFI	624 [398-933]	560 [457-1646]	641 [431-939]	570 [473-1416]	701 [492-1579]	715 [486-1142]	0.015
MFI TRAP activatability	5432 [2880-6645]	4513 [2861-6591]	4962 [2354-7216]	4359 [3057-5859]	3947 [1648-7071]	3787 [3050-8043]	0.025
MFI ADP activatability	1677 [637-3300]	1268 [457-2326]	1776 [497-2978]	1592 [846-2686]	1678 [669-2731]	1214 [364-7304]	0.061
Plasma markers of primary hemostasis							
sP-selectin (ng/ml)	28.5 [10.8-64.0]	30.6 [13.8-60.3]	30.2 [13.8-71.8]	36.5* [21.9-70.8]	36.2 [19.5-64.6]	35.6 [21.3-62.9]	0.007
PF 4 (ng/ml)	204 [77-716]	211 [110-600]	288 [122-889]	271 [117-1465]	229 [54-717]	275 [78-1938]	0.188
VWF (%)	106 [33-251]	111 [49-228]	115 [53-217]	127 [57-212]	240$^{***\#\#\#}$ [138-474]	205$^{***\#\#\#}$ [141-575]	<0.0001
ADAMTS13 (%)	103 [53-141]	93 [39-130]	110 [69-143]	115$^{\#}$ [51-210]	106 [47-202]	91 [23-177]	0.033
Thromboelastography							
R-time (min)	5.3 [2.8-7.7]	4.8 [3.8-7.2]	4.3 [1.7-6.0]	4.8 [3.7-5.9]	5.4 [2.2-7.8]	4.8 [3.2-7.2]	0.290
K-time (min)	1.4 [0.9-2.4]	1.2 [0.8-2.1]	1.1 [0.9-9.3]	1.3 [0.9-2.2]	1.6$^{\#\#}$ [1.1-3.5]	1.7 [1.0-2.5]	0.0002
α-Angle (degrees)	69.3 [57.7-76.3]	73.1 [62.1-77.5]	72.8 [61.2-76.5]	71.2 [59.4-77.0]	66.9$^{\#\#}$ [59.0-73.9]	68.3 [56.5-77.4]	0.0008
MA (mm)	59.7 [46.7-74.1]	67.0 [44.0-76.6]	68.9 [30.8-74.7]	67.5 [48.8-73.8]	60.0 [43.7-71.3]	56.7$^{\#}$ [49.4-68.9]	0.001
LY30 (%)	0.60 [0-10.0]	0.20 [0-7.90]	0.30 [0-20.9]	0 [0-5.60]	0 [0-2.40]	0.05 [0-3.90]	0.150
Conventional coagulation tests							
PT (s)	10.6 [9.4-12.1]	10.4 [9.1-11.4]	10.6 [9.4-13.5]	10.7 [9.7-13.4]	11.9$^{***\#\#\#}$ [10.2-17.2]	12.7$^{**\#\#\#}$ [9.8-16.9]	<0.0001
APTT (S)	29.8 [25.6-36.7]	30.9 [25.3-51.8]	31.4 [24.2-36.0]	31.1 [22.2-35.3]	32.6* [27.4-39.0]	34.5** [28.0-38.4]	0.001
Fibrinogen (mg/ml)	2.85 [1.78-4.43]	3.57 [2.12-4.73]	3.21 [2.08-5.32]	3.36 [1.98-5.07]	2.90 [1.49-5.91]	2.59 [1.82-4.53]	0.007
FVII (%)	105 [27]	127 [32]	113 [29]	114 [34]	79 [32]$^{\#\#\#}$	62 [25]$^{***\#\#\#}$	<0.0001
FVIII (%)	144 [37]	149 [39]	141 [46]	142 [35]	182 [55]*	154 [48]	0.016
Protein C (%)	114 [61-145]	118 [83-181]	120 [100-182]	125 [86-189]	76$^{\#\#}$ [34-177]	63$^{***\#\#\#}$ [25-105]	<0.0001
Antithrombin (%)	112 [87-132]	108 [91-144]	105 [89-128]	105 [82-160]	86$^{***\#\#\#}$ [34-129]	84$^{***\#\#\#}$ [43-123]	<0.0001
tPA (ng/ml)	4.19 [1.17-32.9]	6.19 [2.27-19.4]	7.20 [2.56-70.7]	6.68 [2.79-23.2]	11.8$^{***\#\#\#}$ [6.62-40.5]	10.7** [3.89-42.4]	<0.0001
PAI-1 (ng/ml)	0.81 [0.09-4.04]	1.43 [0.19-7.01]	2.24 [0.29-6.01]	1.97* [0.32-9.15]	1.73 [0.19-9.12]	0.91 [0.01-3.51]	0.001
D-dimer (ng/ml)	177 [43-559]	263 [49-1199]	241 [93-745]	320* [99-2351]	503$^{***\#\#}$ [164-20398]	891$^{***\#\#}$ [183-4455]	<0.0001
Thrombin generation							
ETP (nM*min)	1211 [866-1518]	1341 [925-1967]	1239 [768-1898]	1180 [798-1390]	1084$^{\#}$ [739-1470]	1044$^{*\#\#\#}$ [905-1150]	0.0002
ETP TM+ (nM*min)	712 [383-1209]	742 [197-1462]	619 [316-1046]	554 [200-921]	652 [261-1164]	794 [390-933]	0.026
Peak (nM)	276 [204-354]	299 [141-408]	266 [126-314]	234$^{*\#\#\#}$ [125-316]	230$^{**\#\#\#}$ [91-287]	218$^{***\#\#\#}$ [199-277]	<0.0001
Peak TM+ (nM)	191 [97-301]	205 [48-367]	159 [76-255]	145$^{**\#}$ [64-245]	161 [63-252]	184 [105-234]	0.004
Velindex (nM/min)	127 [66-213]	126 [38-245]	108 [38-154]	94$^{**\#\#}$ [29-147]	100 [29-140]	102 [68-142]	0.0002
Velindex TM+ (nM/min)	98 [45-204]	102 [20-202]	78 [33-125]	66$^{**\#}$ [28-123]	75 [29-128]	92 [50-126]	0.001
Lag time (min)	1.57 [0.23]	1.79 [0.36]	1.73 [0.33]	1.96 [0.38]**	1.88 [0.39]*	1.77 [0.41]	0.003
Lag time TM+ (min)	1.54 [0.26]	1.75 [0.29]	1.69 [0.35]	1.90 [0.34]**	1.91 [0.36]***	1.80 [0.38]	0.0004
TM-SR	1.28 [0.32]	1.16 [0.32]	1.11 [0.30]	1.04 [0.34]	1.33 [0.39]	1.54 [0.32]$^{\#\#}$	<0.0001
Clot lysis							
Clot lysis time (min)	68.2 [59.1-140]	80.6 [61.3-138]	79.5 [58.5-148]	84.9 [65.2-178]	67.1 [48.6-143]	57.1$^{\#\#}$ [31.6-95.4]	<0.0001
Fibrin structure							
Clot permeability: Ks	6.7*10^{-9} [4.6*10^{-9}-8.7*10^{-9}]	5.7*10^{-9} [3.9*10^{-9}-9.0*10^{-9}]	6.0*10^{-9} [3.9*10^{-9}-8.8*10^{-9}]	5.5*10^{-9} [4.2*10^{-9}-7.6*10^{-9}]	5.3*10^{-9} [3.9*10^{-9}-9.0*10^{-9}]	6.7*10^{-9} [4.7*10^{-9}-9.9*10^{-9}]	0.050
Fiber density (fibers/100µm)	11.5 [9.8-13.9]	14.8*** [11.6-21.2]	14.0** [12.8-17.0]	15.7*** [11.7-20.1]	17.1*** [11.6-23.8]	16.1*** [14.0-21.6]	<0.0001
Fibrinogen carbonyl (nmol/mg)	1.9 [1.6-2.1]	1.8 [1.1-2.1]	2.1 [1.3-2.5]	2.4 [1.1-3.4]	2.5 [1.4-5.5]	3.2$^{**\#\#}$ [1.5-3.6]	0.001

Table 2. Data from all hemostatic tests. NASH: Non-alcoholic steatohepatitis, ASH cirrhosis: alcoholic cirrhosis, MFI: mean fluorescence intensity, sP-selectin: soluble P-selectin, PF4: platelet factor 4, VWF: von Willebrand Factor, ADAMTS13: a disintegrin and metalloproteinase with a thrombospondin type 1 motif member 13, PT: prothrombin time, APTT: activated partial thromboplastin time, F: factor, tPA: tissue plasminogen activator, PAI: plasminogen activator inhibitor, ETP: endogenous thrombin potential, Velindex: velocity index, TM+: in presence of thrombomodulin, TM-SR: thrombomodulin sensitivity ratio, R-time: reaction time, K-time: kinetics time, MA: maximum amplitude, LY30: lysis at 30 minutes. *P<0.05, **P<0.01, ***P<0.001 compared to lean controls. #P<0.05, #P<0.01, #P<0.001 compared to overweight controls. Data are expressed as number (%), mean [SD], or median [range].

Plasma levels of proteins involved in fibrinolysis

Levels of PAI-1 stepwise increased from lean controls to overweight controls to patients with simple steatosis, and were still increased in patients with NASH and NASH cirrhosis (Fig. 4), although levels were only significantly increased in patients with NASH compared to lean controls. Levels of tPA also stepwise increased from healthy controls to patients with NASH cirrhosis, although levels were only significantly increased in patients with NASH cirrhosis compared to lean controls.

The clot lysis time correlated with PAI-1 levels ($r = 0.68$; $p < 0.0001$) and fibrinogen levels ($r = 0.25$; $p = 0.007$) in patients with NAFLD. In addition, within the patients with NAFLD, clot lysis time slightly correlated with BMI ($r = 0.19$; $p = 0.04$), but not with lipid levels (HDL-C, LDL-C, total cholesterol, and TGs), NAS score or individual histological parameters (steatosis, inflammation, and ballooning). Levels of PAI-1 also correlated with BMI ($r = 0.21$; $p = 0.02$), but not with lipid levels. Furthermore, PAI-1 levels increased with increasing grade of NAS score (1.08 ng/ml [0.29–9.15] in patients with NAS <3, 1.94 ng/ml [0.58–7.70] in patients with NAS 3-4, and 2.18 ng/ml [0.19–9.12] in patients with NAS ≥5), although these differences were not significant. PAI-1 levels also appeared to increase with increasing severity of steatosis, inflammation, and ballooning on liver biopsy, although differences did not reach statistical significance (data not shown). Finally, within patients with NAFLD, none of the fibrinolysis parameters correlated with AST, ALT, bilirubin, or CRP.

Discussion

The combined results of this study show that the overall hemostatic status is comparable between patients with NAFLD and controls. Our study, therefore, suggests that the role for hyperactive hemostasis in the increased risk of thrombosis in patients with NAFLD is probably limited. Furthermore, since there were no differences in the coagulation status between patients with NASH- or ASH-related cirrhosis, there is probably also a limited role for hemostasis in the increased thrombotic risk in patients with NASH-related cirrhosis compared to patients with cirrhosis from other etiologies. However, our data show some pro-thrombotic features in patients with NAFLD, including hypofibrinolysis and a pro-thrombotic structure of the fibrin clot, which appear driven by obesity rather than the liver disease itself. We observed a hypofibrinolytic state in patients with non-cirrhotic NAFLD, which appeared to resolve in NASH-related cirrhosis. The hypofibrinolytic state in non-cirrhotic NAFLD may contribute to thrombosis risk in these patients as a hypofibrinolytic state as determined with our clot lysis assay has been demonstrated to form a risk factor for both venous and arterial thrombosis in the general population [26–28]. Furthermore, we observed decreased fibrin clot permeability and increased clot density in patients with NAFLD indicating a pro-thrombotic structure of the fibrin clot in these patients.

Previous studies have shown decreased fibrin clot permeability to be associated with thrombotic diseases [29–31]. We have recently shown that fibrin clot permeability in patients with cirrhosis of varying etiology is markedly reduced despite decreased fibrinogen plasma levels in these patients. The pro-coagulant properties of the fibrin clot in cirrhosis was attributed to increased oxidation of the fibrinogen molecule [25], which is known to result in a more thrombogenic fibrin clot. The thrombogenic nature of fibrin clots in patients with NAFLD, as identified in the present study, is likely attributable to multiple factors including increased oxidation of the fibrinogen molecule and elevated plasma fibrinogen levels. In

addition, in those patients with diabetes, glycation of the fibrinogen molecule contributes to decreased fibrin clot permeability [32].

Previous studies have suggested an increased activity of platelets in patients with the metabolic syndrome [12,13] and in patients with NAFLD [14,33–35], which might contribute to their increased risk of thrombosis. However, results from these studies are inconsistent, most of these studies are limited by their relative small sample size, and use indirect markers of platelet activation (e.g., mean platelet volume). The results of our study show that NAFLD is not associated with platelet hyperactivity measured using a direct flow cytometric assay of platelet activation status. In addition, NAFLD was not associated with changes in pivotal proteins in primary hemostasis. We did observe decreased platelet activatability in patients with cirrhosis, which has been previously shown [17].

Although TEG and thrombin generation testing in the absence of thrombomodulin revealed a slight hypocoagulability in patients with cirrhosis, when tested in the presence of thrombomodulin thrombin generation was comparable to that of lean or overweight controls in patients with cirrhosis, which has been previously shown [36–38]. In extent, thrombin generation was also normal in patients with NAFLD. In contrast, Tripodi et al. concluded that NAFLD is characterized by a pro-coagulant-imbalance, as shown by an increase in ETP-ratio (with-to-without thrombomodulin) in these patients [11]. However, this ratio only represents the capacity of thrombomodulin to downregulate thrombin generation, and is by no means a direct indicator of hemostatic potential. An increase in the ETP-ratio in patients with NAFLD therefore does not explain their increased risk of thrombosis compared to patient with alcoholic/viral cirrhosis. We argue against the use of the ETP-ratio or TM-SR to classify if patient samples are normo- or hypercoagulable, but instead believe that the thrombin generation performed in the presence of thrombomodulin is the most accurate laboratory measure of the coagulant potential of a patients' plasma.

Several studies have shown increased levels of various individual pro-thrombotic factors in patients with NAFLD [7–9,11,39–41]. Although results have been inconsistent, an increase in PAI-1, fibrinogen, and factor VIII and a decrease in antithrombin are most frequently reported. Levels of fibrinogen were also increased in patients with non-cirrhotic NAFLD in this study, although the difference with controls did not reach statistical significance. However, in patients with cirrhosis fibrinogen levels are mostly decreased [42], as also observed in patients with ASH-related cirrhosis in this study. Yet, patients with NASH-related cirrhosis in our study had fibrinogen levels comparable to that of the controls, which likely relates to a relative increase in fibrinogen production due to the fatty liver disease. Levels of factor VIII were increased in patients with NASH-related cirrhosis in this study. In contrast, levels of antithrombin were decreased in patients with cirrhosis, which is in agreement with previous studies [36,38,43]. Finally, we observed increased levels of PAI-1 in patients with NAFLD and levels increased with increasing severity of the disease and increasing severity of steatosis, which has been previously shown [9].

In our study, several hemostatic test results (such as platelet activity, clot lysis time, PAI-1 levels, and fiber density) showed a higher variability in patients than in controls, suggesting individual patients may have a more thrombogenic hemostatic profile. We did not identify any characteristics of individual patients to explain the more extreme values in individual

patients, although our study likely lacks power to identify such characteristics. We did observe relations between BMI and clot lysis time, PAI-1 levels, and fibrin fiber density, which in part have been previously described [27,44].

To conclude, the combined results of this study show that the overall hemostatic status is comparable between patients with NAFLD and controls, which contrasts with previously published results in similar populations. We did identify some pro-thrombotic features in patients with NAFLD, particularly a pro-thrombotic structure of the fibrin clots. Although the discrepancy between our study and previous ones is unclear, we included a well-defined cohort of patients and controls, included patients with established cirrhosis, and performed an exhaustive panel of hemostasis tests including functional assessment of platelets, coagulation, and fibrinolysis. The results of this study suggest that the role for hemostasis in the increased risk of thrombosis in patients with NAFLD and NASH-related cirrhosis is probably limited.

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CHAPTER 8

Differential in vitro inhibition of thrombin generation by anticoagulant drugs in plasma from patients with cirrhosis

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Abstract

Background: Treatment and prevention of thrombotic complications is frequently required in patients with cirrhosis. However anticoagulant therapy is often withheld from these patients, because of the perceived bleeding diathesis. As a result of the limited clinical experience, the anticoagulant of choice for the various indications is still unknown.

Objectives: We evaluated the in vitro effect of clinically approved anticoagulant drugs in plasma from patients with cirrhosis.

Patients/Methods: Thirty patients with cirrhosis and thirty healthy controls were studied. Thrombin generation assays were performed before and after addition of unfractionated heparin, low molecular weight heparin, fondaparinux, dabigatran, and rivaroxaban, to estimate anticoagulant potencies of these drugs.

Results: Addition of dabigatran led to a much more pronounced reduction in endogenous thrombin potential in patients compared to controls (72.6% reduction in patients vs. 12.8% reduction in controls, $P<0.0001$). The enhanced effect of dabigatran was proportional to the severity of disease. In contrast, only a slightly increased anticoagulant response to heparin and low molecular weight heparin and even a reduced response to fondaparinux and rivaroxaban was observed in plasma from cirrhotic patients as compared to control plasma.

Conclusions: The anticoagulant potency of clinically approved drugs differs substantially between patients with cirrhosis and healthy individuals. Whereas dabigatran and, to a lesser extent, heparin and low molecular weight heparin are more potent in plasma from patients with cirrhosis, fondaparinux and rivaroxaban showed a decreased anticoagulant effect. These results may imply that in addition to dose adjustments based on altered pharmacokinetics, drug-specific dose adjustments based on altered anticoagulant potency may be required in patients with cirrhosis.

Introduction

Chronic liver disease has long been considered as the epitome of acquired bleeding disorders, due to clinically observed bleeding complications (e.g. variceal bleeding) in combination with a decreased number and function of platelets, decreased synthesis of coagulation factors by the diseased liver, and hyperfibrinolysis [1]. Conventional coagulation tests such as the prothrombin time (PT) and activated partial thromboplastin time (APTT), designed to assess isolated defects of pro-coagulants, but insensitive for anticoagulant factors, are frequently prolonged in patients with cirrhosis suggesting defective hemostasis and thus a bleeding tendency. Recently, more sophisticated tests of hemostasis that allow assessment of the true balance between pro- and anticoagulant factors, have been used to reassess the hemostatic capacity of patients with liver disease. In particular, thrombin generation testing performed in the presence of thrombomodulin (TM) has demonstrated normal or even superior thrombin generation compared to healthy volunteers [2–7]. These experiments in conjunction with clinical observations have led to the concept of 'rebalanced hemostasis', which suggests hemostatic balance by a concomitant decrease in both pro- and anticoagulant drivers [8,9]. Although the hemostatic system of patients with liver disease is in a 'rebalanced' status, clinical thrombotic events and bleeding complications suggest that this balance is more unstable as compared to the balance in healthy individuals and can be easily tipped over to a hyper- or a hypocoagulable state [10].

Despite historical beliefs that patients with liver disease are 'auto-anticoagulated', thrombotic complications do occur in cirrhotic patients and form evidence for hypercoagulability in these patients [11,12]. Because of the perceived bleeding diathesis of liver disease, prophylactic anticoagulant therapy is presumably underused in patients with cirrhosis. Furthermore, treatment of thrombotic complications is frequently required, as patients with liver disease can suffer from deep vein thrombosis or pulmonary embolism, and portal vein thrombosis [11,12]. Moreover, patients may require anticoagulation for systemic arterial events [12].

Nowadays, there is increasing recognition of various thrombotic complications that may occur in patients with chronic liver disease and therefore an increase in the use of anticoagulant therapy in these patients may be expected. Due to the limited clinical experience, the anticoagulant of choice for the various indications is still unclear. Vitamin K antagonists have major drawbacks when used in cirrhotic patients, as vitamin K antagonist therapy requires monitoring by the international normalized ratio (INR) which is frequently already abnormal in cirrhotic patients. Clinical data on the use of low molecular weight heparin (LMWH) indicate that the drug is safe and effective in both the treatment and prevention of portal vein thrombosis [13,14]. In addition heparins appear safe and effective in prevention of venous thrombosis [15]. However, the mode of administration of these agents as well as the concern for heparin-induced thrombocytopenia (HIT) may limit long-term use. In addition, monitoring of heparins is complicated by the substantial underestimation of heparin levels when tested by an anti-Xa assay [16–18]. Finally, LMWH accumulation is known to occur in patients with renal failure, and thus patients with chronic liver disease and decreased renal function likely require dose adjustments based on altered pharmacokinetics.

There is little published clinical experience with the new oral anticoagulants (NOACs) such as the direct factor Xa and thrombin inhibitors Rivaroxaban and Dabigatran in patients with a chronic liver disease. However, these new agents would be potentially applicable in both

long- and short term anticoagulant strategies in patients with cirrhosis, and such agents have (theoretical) advantages over currently used strategies [19]. Nevertheless, since currently available NOACs such as Rivaroxaban and Dabigatran are cleared by liver and kidneys, it is conceivable that the pharmacokinetics of these drugs is also altered in patients with (advanced) liver disease.

In vitro studies have demonstrated that LMWH has a more profound anticoagulant effect in plasma from patients with cirrhosis as compared to plasma from healthy controls [17]. Possible increased responses to anticoagulant drugs may have important consequences for the dosing regiments in these patients. In this study, we aimed to investigate the efficacy of currently approved and widely used anticoagulant drugs by performing in vitro thrombin generation tests in plasma of patients with cirrhosis.

Patients and Methods

Patients

Thirty adult patients with a previous clinical diagnosis of cirrhosis, who were under routine control for their disease by the department of Hepatology of the UMCG or who were admitted at the Hepatology ward of the UMCG, were included in the study. The patients were classified according to the Child-Pugh classification [20]. Ten patients with Child's A cirrhosis, 10 patients with Child's B cirrhosis, and 10 patients with Child's C cirrhosis were studied. Exclusion criteria were documented history of congenital coagulation disorders, presence of active infection (<2 weeks), presence of acute liver failure, use of anticoagulant drugs in the past 10 days, pregnancy, HIV positivity, and recent (<7 days) transfusion with blood products.

The control group consisted of thirty adult healthy volunteers working at our institution. Exclusion criteria for the control group were documented history of congenital coagulation disorders, documented history of hepatic disease, recent viral infection (<2 weeks), use of anticoagulant drugs in the past 10 days, pregnancy, and HIV positivity.

Ethics statement

This study protocol was approved by the medical ethical committee of the University Medical Center Groningen, Groningen, The Netherlands and written informed consent was obtained from each subject before inclusion in the study. The study was conducted according to the principles expressed in the Declaration of Helsinki.

Plasma samples

Blood samples from each patient and control were drawn by clean vena-puncture and collected into vacuum tubes containing 3.8% trisodium citrate as an anticoagulant, at a blood to anticoagulant ratio of 9:1. Platelet poor plasma was prepared by double centrifugation at 2000 g and 10.000 g respectively for 10 min. Plasma was snap-frozen and stored at 280uC until use.

Addition of anticoagulants to plasma samples

The following anticoagulants were added to plasma samples of every cirrhotic patient and control. The mentioned concentrations represent final concentrations in plasma.

- Unfractionated heparin (Leo Pharma, Denmark), 0.1 U/ml
- The LMWH Clexane (Sanofi-Aventis BV, Gouda, the Netherlands), 0.2 U/ml
- Fondaparinux (Arixtra) (GlaxoSmithKline BV, Zeist, the Netherlands), 0.5 mg/ml
- Dabigatran (Alsachim, Illkirch Graffenstaden, France), 300 ng/ml
- Rivaroxaban (Alsachim, Illkirch Graffenstaden France), 25 ng/ml

The final concentrations of the various drugs were based on initial experiments in which drugs were added in various concentrations to pooled normal plasma after which thrombin generation was performed as described in the next paragraph. Those drugs concentrations which gave appreciable (but not maximal) inhibition of thrombin generation in pooled normal plasma were selected so it would be possible to detect both increased and decreased drug effects in patients compared to controls.

Thrombin generation

The thrombin generation test was performed using platelet-poor plasma (PPP) with the fluorimetric method described by Hemker, Calibrated Automated ThrombographyH (CAT) [21]. Coagulation was activated using commercially available reagents containing recombinant tissue factor (TF, final concentration 5 pM), phospholipids (final concentration 4 mM), in the presence or absence of soluble thrombomodulin (TM, the concentration of which is not revealed by the manufacturer). These reagents were purchased from Thrombinoscope BV, Maastricht, The Netherlands. Thrombin Calibrator (Thrombinoscope BV) was added to calibrate the thrombin generation curves. A fluorogenic substrate with CaCl2 (FluCa-kit, Thrombinoscope BV, Maastricht, The Netherlands) was dispensed in each well to allow a continuous registration of thrombin generation. Fluorescence was read in time by a fluorometer, Fluoroskan AscentH (ThermoFisher Scientific, Helsinki, Finland). All procedures were according to the protocol suggested by Thrombinoscope B.V.

The anticoagulant potency of the different drugs was expressed as the percentual reduction of endogenous thrombin potential (ETP), peak, or velocity index, and the percentual increase in lag time after the addition of anticoagulants. These percentages were compared between patients and controls.

Routine coagulation laboratory tests

The INR was assessed with commercially available methods on an automated coagulation analyzer (ACL 500 TOP) with reagents (Recombiplastin 2G) and protocols from the manufacturer (Instrumentation Laboratory, Breda, the Netherlands). Levels of factor (F) VIII, II, and X, and antithrombin (AT) were measured on an automated coagulation analyzer (ACL 500 TOP) with reagents and protocols from the manufacturer (Recombiplastin 2G for FII and FX, Hemosil (R) SynthASil for FVIII, and Liquid Antithrombin reagent for AT) (Instrumentation Laboratory). Total protein S antigen was assayed by enzyme-linked immunosorbent assay (ELISA) using antibodies from DAKO (Glostrup, Denmark). Free protein S was measured by precipitating the C4b-binding protein-bound fraction with polyethylene glycol 8000 and measuring the concentration of free protein S in the supernatant. Protein C was determined using the Coamatic protein C activity kit from Chromogenix (Mölndal, Sweden).

Statistical analysis

Data are expressed as means (with standard deviations (SDs)), medians (with interquartile ranges), or numbers (with percentages) as appropriate. Means of two groups were compared by Student's t-test or Mann-Whitney U test as appropriate. Multiple groups were compared using One-way ANOVA (with the Bonferroni posttest) or Kruskal-Wallis H test (with Dunn's posttest) as appropriate. Spearman's correlation coefficient was used to assess correlation between continuous variables. P values of 0.05 or less were considered statistically significant. GraphPad Prism (San Diego, USA) and IBM SPSS Statistics 20 (New York, USA)) were used for analyses.

Characteristics	Cirrhotic patients Child A n=10	Child B n=10	Child C n=10	P-value
MELD score	8.0 [6.0-10.0]	11.5 [8.0-19.0]	15.5 [6.0-19.0]	.002
Age (yrs)	56.0 [14.2]	50.5 [12.5]	54.9 [8.3]	.560
Sex (male)	4 (40)	6 (60)	8 (80)	.248
BMI	26.0 [18.8-31.4]	26.5 [22.0-41.4]	26.3 [22.2-36.3]	.705
Smoking (number)	4 (40)	1 (10)	4 (40)	.297
Alcohol (U per week)	0 [0-1]	0 [0-7]	0 [0-490]	.670
Etiology of liver disease				
Alcoholic	2 (20)	1 (10)	10 (100)	<.001
HCV	1 (10)	0 (0)	0 (0)	1.000
NASH	1 (10)	2 (20)	0 (0)	.754
Hemochromatosis	0 (0)	1 (10)	0 (0)	1.000
PBC	1 (10)	0 (0)	0 (0)	1.000
PSC	2 (20)	2 (20)	0 (0)	.507
Auto-immune	3 (30)	1 (10)	0 (0)	.286
Alcoholic + NASH	0 (0)	1 (10)	0 (0)	1.000
Unknown	0 (0)	2 (20)	0 (0)	.310
Co-morbidity				
Cardiovascular	3 (30)	2 (20)	0 (0)	.321
DM	2 (20)	1 (10)	1 (10)	1.000
Plasma levels				
Serum bilirubin (µmol/L)	15 [5-35]	40 [18-61]	82 [46-131]	<.0001
Serum albumin (g/L)	36 [28-44]	33 [27-63]	27 [25-91]	.003
Serum creatinin (µmol/L)	69 [23]	72 [32]	70 [20]	.966
Hemoglobin (mmol/L)	8.2 [1.4]	7.2 [0.9]	6.5 [0.8]	.005
Leukocytes (10^9/L)	6.5 [4.5]	5.1 [2.1]	7.4 [5.3]	.463
Platelets (10^9/L)	112 [16-258]	86 [28-471]	76 [44-165]	.565

Table 1. Demographic and clinical characteristics of the study population.
HCV: Hepatitis C virus, NASH: Non-alcoholic steatohepatitis, PBC: Primary biliary cirrhosis, PSC: Primary sclerosing cholangitis, DM: Diabetes Mellitus.
Data are expressed as number (%), mean [SD], or median [range].

Results

Patient characteristics

The main characteristics of the study population are reported in Table 1. Thirty patients with cirrhosis (18 males and 12 females) were included, and they were categorized according to the severity of liver disease as expressed by the Child Pugh classes (10 Child A, 10 Child B and 10 Child C patients). Thirty healthy subjects (14 males and 16 females) were included as controls. The most common etiology of liver disease was alcoholic, especially in the Child class C patients.

INR, FVIII, FII, FX, AT, protein S and protein C levels are shown in Table 2. Patients with cirrhosis showed a statistically significant prolongation of INR and a decrease in all measured coagulation proteins, except for FVIII (which was increased), as compared to controls. The reduction in levels of plasmatic factors was proportional to the severity of liver disease.

	Cirrhotic patients			Healthy	P-value
	Child A	Child B	Child C	controls	
INR	1.1 [0.9-1.2]	1.2 [1.0-2.0]	1.5 [1.4-1.7]	1.0 [0.9-1.1]	<.0001
FVIII (%)	132 [92-185]	129 [91-254]	140 [108-199]	94 [56-138]	<.0001
FII (%)	83 [50-104]	69 [43-98]	42 [27-71]	107 [88-127]	<.0001
FX (%)	90 [74-136]	81 [41-193]	56 [45-69]	106 [80-147]	<.0001
AT (%)	80 [42-107]	50 [27-113]	40 [23-45]	106 [87-126]	<.0001
Protein C (%)	71 [35-105]	53 [22-155]	27 [12-44]	109 [87-168]	<.0001
Protein S total (%)	76 [61-109]	66 [35-200]	57 [52-78]	90 [68-121]	.002
Protein S free (%)	75 [59-130]	69 [45-105]	82 [56-110]	99 [71-136]	.002

Table 2. Coagulation parameters in cirrhotic patients and controls.
INR: International normalized ratio, AT: Antithrombin.
Data are expressed as median [range].

Thrombin generation

When thrombin generation was performed without addition of any anticoagulant, the endogenous thrombin potential (ETP) and peak thrombin generation in plasma from cirrhotic patients were comparable to that of healthy controls, both in absence and presence of TM. The ETP in the presence of TM was slightly, but significantly higher in patients compared to controls. Values in the absence of TM were: ETP 872 ± 260 nM*min (mean ± SD), peak 169 ± 41 nM in patients vs. ETP 945 ± 268 nM*min, peak 188 ± 49 nM in healthy controls, P =0.28 for ETP, P =0.11 for peak; in the presence of TM: ETP 677 ± 304 nM*min, peak 146 ± 48 nM in patients vs. ETP 510 ± 259 nM*min, peak 131 ± 53 nM in controls, P= 0.03 for ETP, P= 0.26 for peak.

To study the anticoagulant potency of antithrombotic drugs in plasma from cirrhotic patients, percentual reductions in ETP, peak, velocity index, and percentual increases in lag time after the addition of the different anticoagulants were calculated and compared between plasma from cirrhotic patients and controls. All data are shown in table 3. In figure 1, the reduction in ETP performed in the presence of TM after the addition of the different anticoagulants in plasma from patients and controls is shown. A detailed analysis of the results for each drug is given below.

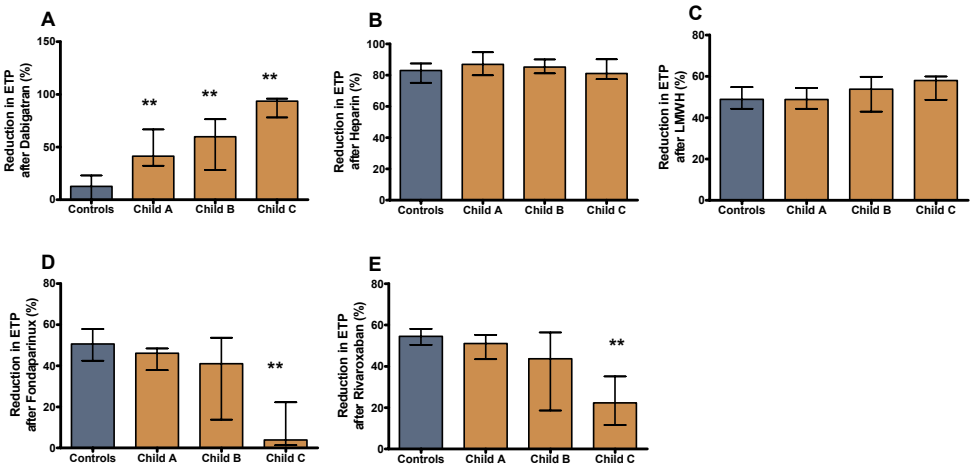

Figure 1. Reductions in ETP after the addition of the different anticoagulant drugs.
Reduction in ETP after the addition of (A) dabigatran, (B) heparin, (C) LMWH, (D) Fondaparinux, and (E) rivaroxaban in plasma from patients with Child A, B, and C cirrhosis and healthy controls.
Bars indicate medians with the error bars representing interquartile ranges. ** P<0.01 compared to controls.

Dabigatran

When dabigatran was added to plasma of healthy controls, the ETP was reduced by 45.4 [39.4–47.7]% (median [IQR]) and peak thrombin generation by 29.8 [22.0–34.9]%. In contrast, the ETP and peak hardly decreased in the presence of TM (12.8 [210.5–23.0]% reduction in ETP and 0.2 [224.3–18.3]% reduction in peak). The lag time was substantially prolonged after addition of dabigatran both in absence and presence of TM (Table 3).

Addition of dabigatran led to a much more pronounced reduction in peak thrombin generation in patients compared to controls, both in absence (83.1 [64.5–96.6]% reduction in patients vs. 29.8 [22.0–34.9]% reduction in controls, P<0.0001) and the presence of TM (82.9 [56.8–96.3]% reduction in patients vs. 0.2 [224.3–18.3]% reduction in controls, P<0.000001). The decrease in thrombin generation with dabigatran mirrored the severity of liver disease. Child class C patients exhibited the most pronounced reduction in peak thrombin generation, with a median reduction of 97.1 [92.1–97.9]% when tested in the presence of TM.

Interestingly, reductions in thrombin generation by dabigatran when tested in the presence of TM strongly correlated with plasma prothrombin (FII) levels (r = - 0.80, P<0.0001) (Figure 2).

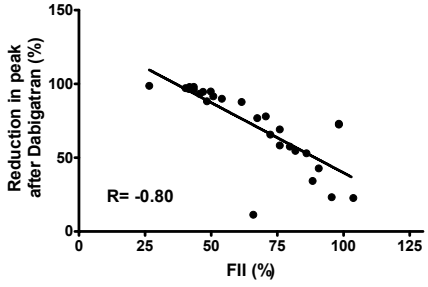

Figure 2. Correlation between FII levels and reductions in thrombin generation after dabigatran. Correlation between plasmatic FII levels and percentual reduction of peak thrombin values when tested in the presence of TM after addition of 300 ng/ml dabigatran in plasma from patients with cirrhosis.

Heparin

After the addition of unfractionated heparin the reduction of thrombin generation was more profound in plasma from cirrhotic patients compared to controls, with a median ETP reduction of 62.0 [54.1–76.8]% in patients vs. 46.0 [32.8–57.2]% in controls (P= 0.0003) (Table 3). However, in the presence of TM the reductions in ETP were not significantly different in plasma from cirrhotic patients compared to controls. The decrease in peak thrombin generation was comparable between cirrhotic patients and controls (87.4 [78.6–93.0]% in patients vs. 87.3 [78.9–91.3]% in controls, P= 0.46, when tested in the presence of TM).

Low molecular weight heparin

After the addition of LMWH the decrease in the ETP in the absence of TM was more pronounced in plasma from cirrhotic patients compared to controls (42.1 [37.5–51.5]% reduction in patients vs. 32.8 [29.8–39.0]% reduction in controls, P<0.001) (Table 3). The reduction in peak thrombin generation by LMWH was not significantly different in plasma from cirrhotic patients (37.0 [32.4–43.5]% reduction) compared to controls (43.3 [33.1–52.1]% reduction) (P= 0.14). Moreover, when thrombin generation was performed in the presence of TM there was no difference in ETP reduction in plasma from patients compared to controls (52.3 [46.3–60.0]% reduction in patients vs. 48.9 [44.3–54.8]% reduction in controls, P =0.05).

Fondaparinux

Addition of fondaparinux in the absence of TM hardly affected the ETP in both patients and controls. The reduction in thrombin generation after the addition of fondaparinux was comparable between plasma from patients with Child A and B cirrhosis (49.0 [44.1–55.3]% and 44.2 [19.4–58.7]% reduction in peak thrombin generation in the presence of TM, respectively) and in plasma from healthy controls (54.4 [47.7–62.3]% reduction in peak thrombin generation). A reduced anticoagulant response to fondaparinux was seen in plasma from patients with Child C cirrhosis, with only 12.9 [8.6–22.5]% reduction in peak thrombin generation when tested in presence of TM (P<0.001). In addition, when tested in the presence of TM, the prolongation of the lag time was more extensive in plasma from Child C patients (25.6 [25.0–38.1]%) as compared to controls (19.8 [12.3–25.6]%; P<0.01).

Interestingly, reductions in peak thrombin generation by fondaparinux when tested in the presence of TM strongly correlated with both plasma antithrombin (AT) levels (r = 0.76, P<0.0001) and plasma factor X (FX) levels (r = 0.65, P<0.0001) (Figure 3).

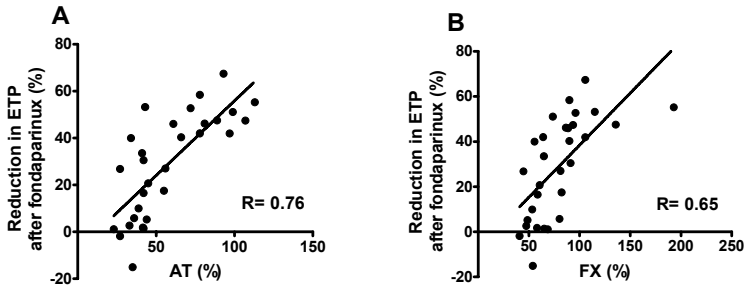

Figure 3. Correlation between AT/FX levels and reductions in thrombin generation after fondaparinux. Correlation between the percentual reduction in ETP when tested in the presence of TM after addition of 0.5 mg/ml fondaparinux and (A) plasmatic AT levels and (B) plasmatic FX levels in plasma from patients with cirrhosis.

Rivaroxaban

Addition of rivaroxaban in the absence of TM hardly affected the ETP in both patients and controls. In contrast, peak thrombin generation was markedly reduced by rivaroxaban in both patients and controls, whereas lag times were prolonged. A reduced response to rivaroxaban was observed in plasma from cirrhotic patients as compared to control plasma, 46.5 [27.1–60.5]% peak reduction in patients vs. 61.8 [55.7–64.3]% peak reduction in controls in the presence of TM (P= 0.0005). In plasma from Child class C patients the lowest reduction in peak thrombin generation was observed, with a median reduction of 28.3 [21.1–39.5]%.

Interestingly, reductions in peak thrombin generation by rivaroxaban in the presence of TM correlated with plasma factor X (FX) levels (r = 0.61, P= 0.0004) (Figure 4).

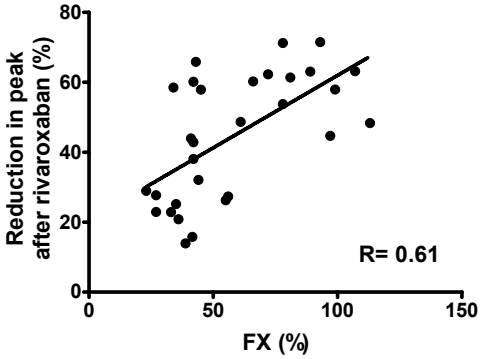

Figure 4. Correlation between FX levels and reductions in thrombin generation after rivaroxaban. Correlation between plasmatic FX levels and the percentual reduction in peak thrombin generation when tested in the presence of TM after addition of rivaroxaban at 25 ng/ml in plasma from patients with cirrhosis.

	Dabigatran							
	TM- ETP	Peak	Lag-time	Velindex	TM+ ETP	Peak	Lag-time	Velindex
Controls	45.4 [39.4–47.7]	29.8 [22.0–34.9]	207.8 [143.0–267.1]	3.8 [-11.1–17.3]	12.8 [-10.5–23.0]	0.2 [-24.3–18.9]	199.7 [141.5–258.5]	-15.7 [-46.2–8.9]
All patients	75.2 [62.5–92.2]**	83.1 [64.5–96.6]**	165.4 [133.2–299.4]	84.9 [63.3–98.3]**	72.6 [39.1–90.9]**	82.9 [56.8–96.3]**	165.4 [136.4–301.8]	90.3 [62.1–98.2]**
Child A	64.3 [54.4–70.1]**	67.6 [46.8–79.9]**	245.3 [164.3–393.0]	66.7 [29.4–82.3]**	41.4 [32.4–66.8]**	55.3 [31.5–79.6]**	276.0 [154.3–402.8]	66.1 [26.8–83.0]**
Child B	67.1 [60.5–79.3]**	69.8 [63.4–92.5]**	160.1 [125.6–436.2]	72.7 [57.5–95.0]**	59.9 [28.3–76.4]**	72.7 [57.4–92.3]**	155.7 [136.0–493.9]	89.8 [57.6–95.2]**
Child C	93.7 [81.2–96.4]**	97.2 [92.9–97.8]**	152.1 [84.8–185.0]	98.3 [95.3–98.5]**	93.7 [78.1–96.0]**	97.1 [92.1–97.9]**	155.0 [75.2–175.9]	98.5 [94.8–98.7]**
	Heparin							
	TM- ETP	Peak	Lag-time	Velindex	TM+ ETP	Peak	Lag-time	Velindex
Controls	46.0 [32.8–57.2]	81.3 [64.0–86.9]	50.0 [33.5–67.6]	91.3 [84.9–95.9]	83.0 [75.1–87.5]	87.3 [78.9–91.3]	39.2 [19.8–47.3]	88.3 [79.8–92.5]
All patients	62.0 [54.1–76.8]**	82.3 71.3–92.6]	58.4 [29.8–78.0]	92.8 [85.5–96.3]	84.6 [79.4–91.0]	87.4 [78.6–93.0]	39.9 [16.9–69.1]	90.6 [84.0–95.5]
Child A	59.8 [46.2–81.1]	82.5 [76.3–94.3]	43.7 [29.8–89.9]	93.5 [90.4–97.4]	86.9 [79.9–94.7]	89.9 [83.0–96.5]	30.0 [19.2–68.0]	92.9 [85.7–97.4]
Child B	62.0 [46.7–82.0]	81.9 [63.5–93.8]	67.2 [21.6–78.3]	92.6 [82.4–98.1]	85.2 [81.1–90.0]	87.4 [81.8–91.8]	45.0 [7.2–70.0]	90.6 [84.8–92.8]
Child C	65.5 [57.2–71.2]*	76.1 [71.3–89.8]	59.1 [32.0–82.4]	87.1 [84.9–95.8]	81.1 [77.5–90.1]	81.1 [75.8–92.7]	45.0 [19.0–72.8]	86.9 [79.7–95.5]
	LMWH							
	TM- ETP	Peak	Lag-time	Velindex	TM+ ETP	Peak	Lag-time	Velindex
Controls	32.8 [29.8–39.0]	43.3 [33.1–52.1]	10.5 [6.9–17.7]	52.9 [42.1–61.3]	48.9 [44.3–54.8]	47.5 [41.1–53.5]	12.1 [0.0–19.8]	49.6 [38.1–56.2]
All patients	42.1 [37.5–51.5]**	37.0 [32.4–43.5]	7.7 [0.0–21.2]	37.1 [33.0–47.5]**	52.3 [46.3–60.0]	41.6 [34.8–46.6]	10.4 [0.0–20.8]	40.0 [31.9–48.2]*
Child A	38.8 [31.8–42.2]	37.1 [30.6–45.0]	6.6 [0.0–21.2]	43.0 [30.8–50.0]	48.8 [44.3–54.3]	43.5 [36.5–49.9]	10.9 [4.4–19.8]	43.4 [36.5–52.0]
Child B	41.3 [37.1–49.1]]*	37.9 [31.9–49.0]	7.7 [0.0–25.6]	39.9 [34.5–51.3]	53.7 [42.9–59.8]	42.0 [34.3–50.1]	0.0 [0.0–25.6]	42.5 [26.5–51.7]
Child C	51.9 [42.2–55.9]**	36.2 [32.8–41.0]	12.8 [0.0–19.4]	34.1 [30.3–37.5]**	58.0 [48.6–59.9]	39.9 [34.5–45.8]	17.3 [0.0–25.6]	38.4 [27.0–43.1]
	Fondaparinux							
	TM- ETP	Peak	Lag-time	Velindex	TM+ ETP	Peak	Lag-time	Velindex
Controls	5.2 [0.8–20.2]	37.3 [17.3–49.2]	28.2 [21.4–35.6]	60.5 [43.0–67.3]	50.6 [42.4–57.9]	54.4 [47.7–62.3]	19.8 [12.3–25.6]	62.2 [52.8–67.7]
All patients	-2.0 [-9.3–5.5]**	14.5 [7.8–30.8]**	26.9 [25.2–39.5]	38.3 [24.4–55.8]**	32.0 [5.6–47.4]**	36.8 [14.6–52.3]**	25.6 [19.8–28.9]**	42.8 [21.8–60.1]**
Child A	-0.2 [-4.0–6.2]	27.5 [16.2–37.7]	39.2 [30.9–40.5]	54.6 [39.0–57.1]	46.1 [37.8–48.4]	49.0 [44.1–55.3]	23.5 [19.8–29.0]	55.5 [52.3–61.5]
Child B	-2.0 [-6.5–14.6]	18.1 [8.3–40.2]	25.6 [18.9–37.4]	45.1 [26.2–61.2]	41.0 [13.8–53.7]	44.2 [19.4–58.7]	25.6 [15.8–25.6]	53.1 [26.1–64.2]
Child C	-8.0 [-11.2–1.4]**	8.5 [5.2–12.2]**	25.6 [25.5–35.7]	26.7 [17.0–31.0]**	3.9 [1.4–22.2]**	12.9 [8.6–22.5]**	25.6 [25.0–38.1]**	20.9 [13.0–33.2]**
	Rivaroxaban							
	TM- ETP	Peak	Lag-time	Velindex	TM+ ETP	Peak	Lag-time	Velindex
Controls	3.2 [-1.5–7.9]	47.6 [39.0–55.6]	59.5 [49.6–64.8]	75.1 [67.6–80.4]	54.5 [50.4–58.1]	61.8 [55.7–64.3]	39.5 [26.2–48.2]	68.4 [64.0–71.5]
All patients	2.5 [-4.6–6.2]	30.7 [15.6–42.9]**	64.2 [50.1–75.2]	55.2 [38.9–69.1]**	39.3 [19.7–53.2]**	46.5 [27.1–60.5]**	48.2 [36.7–56.7]*	57.3 [35.7–70.4]**
Child A	1.0 [-4.1–5.8]	39.0 [26.9–47.7]	60.8 [56.2–75.5]	66.3 [51.3–77.5]	51.1 [43.4–55.2]	60.2 [47.7–63.1]	47.5 [39.5–50.3]	68.5 [56.6–72.6]
Child B	4.3 [-5.3–9.5]	32.9 [15.3–47.1]	66.9 [57.3–76.0]	61.6 [38.2–72.1]*	43.7 [18.6–56.3]	51.0 [26.2–63.2]	50.4 [30.8–56.7]	59.6 [35.1–71.7]
Child C	-0.2 [-7.3–6.5]	17.1 [15.0–23.3]**	50.6 [42.3–81.2]	38.5 [31.4–51.2]**	22.3 [11.6–35.1]**	28.3 [21.1–39.5]**	49.3 [36.7–77.0]	38.4 [30.0–49.8]**

Table 3. Inhibition of in vitro thrombin generation after addition of various anticoagulant drugs to plasma taken from patients with cirrhosis or plasma from healthy controls. Shown are the percentual inhibition of the ETP, peak, or velocity index, and the percentual increase in the lag time.
TM: thrombomodulin, ETP: Endogenous thrombin potential, Velindex: velocity index, LMWH: low molecular weight heparin. Data are expressed as median percentages with interquartile range.
* P<0.05, ** P< 0.01 compared to controls

Discussion

We observed a profoundly increased anticoagulant response to the direct thrombin inhibitor dabigatran in plasma from patients with cirrhosis compared to controls. The enhanced effect of dabigatran on thrombin generation was proportional to the severity of liver disease. Furthermore, we observed a modestly increased anticoagulant response to heparin and low molecular weight heparin in patients compared to controls, but only when tested in the absence of TM. In addition, a reduced response to fondaparinux and rivaroxaban was observed in plasma from patients with cirrhosis, and the reduced effect was also proportional to the severity of disease.These data show that the anticoagulant potency of the different drugs differs substantially between cirrhotic patients and controls. In addition, the difference in anticoagulant potency between patients and controls differs substantially between the different drugs that were tested. The different drugs that were tested may also have an altered pharmacokinetics in patients with chronic liver disease, in particular in patients with advanced disease and in patients with concomitant renal failure. Therefore, dose adjustments based on both pharmacokinetics and altered anticoagulant potency of the specific drug may be required. Monitoring assays taking both drug level and drug potency would thus ideally be required for patients with liver diseases.

Although there is increasing clinical experience with heparins in patients with cirrhosis, clinical experience with NOACs in these patients is lacking. As we foresee that there might be an interest in using NOACs in patients with liver disease that require long-time anticoagulant treatment [19], we believe it is vital to understand the potentially altered behavior of these drugs in patients with liver disease, in addition to altered pharmacokinetics. We have recently demonstrated that, in contrast to heparins, monitoring of plasma levels of NOACs is possible in patients with liver disease [18], which will enable careful monitoring of these drugs. How altered drug potency needs to be assessed in clinical practice requires further study, but one could imagine that dose-adjustments based on plasma levels of FII (for Dabigatran) or FX (for Rivaroxaban) may be applicable given the linear relation between factor levels and anticoagulant potency (figures 2 and 4).

Our results extend recent observations by Senzolo et al. [17] on the increased anticoagulant response to LMWH in patients with cirrhosis. Our results, however, showed a more modest increase in anticoagulant potency of LMWH in patients with cirrhosis as compared to the data by Senzolo et al, and we failed to detect a difference in anticoagulant potency when thrombin generation was tested in the presence of TM. Indeed, clinical data on the use of heparins indicate that the drug is safe and effective in both the treatment and prevention of portal vein thrombosis [13,14,22]. The partially divergent results between our study and that of Senzolo et al. may be explained by the differences in LMWH dosage used, differences in patient characteristics, and slight differences in methodology. In fact, research has shown that thrombin generation is influenced substantially by pre-analytical conditions [23], among which the protocol for centrifugation of blood to obtain platelet poor plasma. Senzolo et al. prepared the platelet poor plasma by double centrifugation at 2000 g for 10 min. In our study the second centrifugation step was set at 10.000 g as recommended [23]. Another possible critical difference in the method was the type and concentration of thrombomodulin used in the thrombin generation experiments.

Various anticoagulant drugs show different effects on the thrombin generation curves [24]. For

example, direct factor Xa inhibitors substantially reduce the peak thrombin generation, while hardly affecting the endogenous thrombin potential, which was also observed with the direct factor Xa inhibitor rivaroxaban in this study. Therefore it has yet to be studied which parameter (ETP, peak, lag time, or velocity index) forms the best representation of the anticoagulant effect of a specific drug. In addition, the effects of a drug on the various parameters of the thrombin generation curve are not per definition concordant. For example, the reductions in peak and ETP after addition of fondaparinux and rivaroxaban were less pronounced in plasma from Child C cirrhotic patients compared to controls, which may imply increase in the dosages. However, Child class C patients exhibited the greatest prolongation of the lag time after the addition of both fondaparinux and rivaroxaban, which may imply that in patients with cirrhosis the dosage should be decreased.

We chose the concentrations of the various drugs to obtain appreciable (but not maximal) inhibition of thrombin generation in controls. The drug levels therefore do not necessarily represent peak levels found in clinical practice, although the levels we chose are all compatible with drugs levels at some stage of therapy. We would like to stress that the aim of our study was to provide proof of concept of differential drug potency in patients with liver disease rather than an exact estimate of drug potencies at clinically relevant plasma levels.

In conclusion, using in vitro thrombin generation assays, we observed a substantially increased anticoagulant response to dabigatran and a modestly increased anticoagulant response to heparin and LMWH in plasma from patients with cirrhosis. In addition, a reduced response to fondaparinux and rivaroxaban was observed in plasma from patients with cirrhosis. These results may imply that drug-specific dose adjustments may be required for patients with cirrhosis, in particular with dabigatran. However since the pharmacokinetics of the drugs may also be altered in these patients, the final dosing regimen should ideally take both the pharmacokinetics of the drug and the altered anticoagulant potency into account. Clinical studies on the in vivo effect of the available anticoagulant drugs in cirrhotic patients with thrombosis are needed to further validate this hypothesis.

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CHAPTER
8

APPENDIX TO CHAPTER 8

Decreased in vitro anticoagulant potency of Rivaroxaban and Apixaban in plasma from patients with cirrhosis

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To the Editor

There is increasing interest in treatment and prevention of cirrhotic portal vein thrombosis (PVT) with anticoagulant drugs [1]. Two recent reports in Hepatology suggested efficacy and safety of new-generation oral anticoagulant drugs (the direct factor Xa inhibitors, Rivaroxaban and Apixaban) in treatment of PVT in patients with compensated cirrhosis [2,3]. These new drugs have practical advantages over traditional anticoagulants [4]. We recently showed altered in vitro potency of different anticoagulant drugs in patients with cirrhosis, compared to patients with intact liver function [5]. A theoretical risk for excessive anticoagulation when using these drugs in patients with cirrhosis and concomitant alterations in their hemostatic system exists.

We previously demonstrated a decreased in vitro anticoagulant effect of Rivaroxaban in patients with cirrhosis. Using thrombomodulin-modified thrombin generation testing, we examined the in vitro anticoagulant potency of Apixaban, which we compared to the anticoagulant potency of Rivaroxaban. This study protocol was approved by the medical ethical committee of the University Medical Center Groningen (Groningen, The Netherlands), and written informed consent was obtained from each subject before inclusion in the study. We added vehicle, 25 ng/mL of Apixaban, or 50 ng/mL of Rivaroxaban to plasma samples of 11 healthy individuals and 14 patients with cirrhosis (9 patients with Child B cirrhosis and 5 with Child C cirrhosis). We performed thrombin generation tests in the presence of thrombomodulin and calculated the percentual decrease in total thrombin generation by the two anticoagulant drugs, as described previously [5]. Whereas a fixed dose of the drugs decreased total thrombin generation in healthy volunteers by 55 ± 6% (Rivaroxaban, mean ± standard deviation) and 51 ± 4% (Apixaban), the mean decrease in thrombin generation in patients was significantly lower (30 ± 9% for Rivaroxaban, $P<0.0001$ [t test]; 32 ± 10% for Apixaban, $P<0.0001$).

In conclusion, the in vitro anticoagulant potency of Apixaban is substantially reduced in patients with moderate and advanced cirrhosis, similar to the reduced potency of Rivaroxaban, which we previously reported. These results suggest that anticoagulant treatment with these direct factor Xa inhibitors will likely not result in over-anticoagulation, with a potentially increased bleeding risk, provided drug levels remain in the target range. Careful monitoring of drug levels, for example, by anti-Xa testing [6] may be required.

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CHAPTER
8

CHAPTER 9

Routine coagulation assays underestimate levels of antithrombin-dependent drugs but not of direct anticoagulant drugs in plasma from patients with cirrhosis

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Summary

There is increasing recognition that thrombotic complications may occur in patients with cirrhosis, and literature on antithrombotic treatment in these patients is rapidly emerging. Due to extensive haemostatic changes in patients with cirrhosis, careful monitoring of anticoagulant therapy may be required. Recent data suggest that plasma levels of low molecular weight heparin (LMWH) are substantially underestimated by the anti-activated factor X (anti-Xa) assay in patients with cirrhosis. We studied the in vitro recovery of antithrombin (AT)-dependent and –independent anticoagulant drugs in plasma from 26 patients with cirrhosis and 30 healthy controls and found substantially reduced anti-Xa levels when AT-dependent anticoagulant drugs were added to the plasma of patients with cirrhosis. LMWH (0.2 U/ml) had the poorest recovery in plasma from patients with cirrhosis (0.13 ± 0.06 U/ml, compared to 0.23 ± 0.03 U/ml in controls, $P < 0.0001$), followed by unfractionated heparin and fondaparinux. In contrast, the recovery of rivaroxaban and dabigatran was identical between patients and controls. These data suggest that the anti-Xa assay cannot be used to monitor AT-dependent anticoagulant drugs in patients with cirrhosis, as it substantially underestimates drug levels. The direct factor Xa and IIa inhibitors, however, may be monitored through the respective anti-Xa and anti-IIa assays in patients with cirrhosis.

Introduction

In patients with chronic liver disease the haemostatic system is considered to be in a 'rebalanced' status, due to a concomitant decrease in pro- and anti-haemostatic systems [1]. However, the relatively high incidence of thrombotic events and bleeding complications in these patients suggests that this balance is less stable than that in healthy individuals and that it can be easily tipped over to a hyper- or a hypocoagulable state [2]. In fact, treatment of thrombotic complications is frequently required, as patients with liver disease can suffer from deep vein thrombosis, pulmonary embolism or portal vein thrombosis [3,4]. Furthermore, patients may require anticoagulation because of concomitant cardiovascular disease[3]. Nowadays, there is increasing recognition of the various thrombotic complications that may occur in patients with chronic liver disease and therefore the increased use of anticoagulant therapy in these patients may be expected [5]. Due to the limited clinical experience, the anticoagulant of choice for the various indications is still unclear.

Vitamin K antagonists and/or heparins are widely used in the prevention or treatment of thrombosis, but both drug classes have drawbacks in patients with liver diseases. Vitamin K antagonist therapy requires monitoring by the international normalized ratio (INR). However, as the INR is frequently already prolonged in patients with cirrhosis in the absence of anticoagulant therapy, the target INR is unclear. In addition, there is major inter-laboratory variability in the INR measurement in patients with cirrhosis, making the test results inherently unreliable in these patients [6].

A problem with monitoring unfractionated heparin (UFH) is that the activated partial thromboplastin time (APTT), which is instrumental in dosing this agent, is already prolonged in many patients with chronic liver disease, and therefore APTT target ranges for these patients are unclear.

Low molecular weight heparin (LMWH) and fondaparinux do not require laboratory monitoring in the general population, except in patients with extreme obesity and with renal dysfunction. Patients with cirrhosis may also require laboratory monitoring of these agents, for example because of concomitant renal failure. LMWH can be monitored with anti-activated factor X (anti-Xa) assays, but these appear to be unreliable in patients with cirrhosis. It has been shown that, after the administration of a standard prophylactic or therapeutic dose of LMWH to patients with cirrhosis, anti-Xa levels fall below the recommended ranges for optimal anticoagulant control [7]. However, the decreased anti-Xa levels in these patients appear to be a laboratory anomaly and not a true indication of anticoagulant effect [8,9]. In fact, anti-Xa values have been shown to correlate positively with AT levels [7], which are reduced in patients with cirrhosis. Despite reduced anti-Xa values, LMWH was shown to be safe and effective in patients with cirrhosis [7,10,11]. Whether similar monitoring problems also occur with fondaparinux has not yet been assessed.

New antithrombotic agents, such as the direct factor Xa inhibitor rivaroxaban and the direct thrombin inhibitor dabigatran, have theoretical advantages over heparins: fewer (fatal) bleeding events, rapid onset of action, fewer drug-drug interactions, and oral mode of administration [12]. In addition, both agents do not require laboratory monitoring in the general population. Although patients with liver disease have been excluded from clinical trials on the new oral anticoagulants, they have theoretical advantages over currently used drugs, in

particular the oral mode of administration. Since LMWH is currently used for months or years to treat or prevent portal vein thrombosis, it is conceivable that the new oral anticoagulants will be considered in the future to avoid prolonged subcutaneous LMWH administration [5]. However, the current package insert for rivaroxaban indicates that it is contra-indicated for patients with cirrhosis, and other theoretical disadvantages of the new oral anticoagulants require attention [5]. Monitoring of these drugs may be required in patients with liver disease due to the possible altered clearance and the increased extravascular volume of patients with cirrhosis. No clinical studies on the efficacy and safety of these agents in cirrhotic patients have been performed yet. Furthermore, laboratory methodologies for monitoring these new drugs are still in development, and validation in patients with chronic liver disease will be required.

Thus, monitoring of anticoagulant drugs may be required in patients with cirrhosis, however, this appears to be difficult, due to the haemostatic changes associated with the underlying liver disease. The aim of this study was to determine whether different anticoagulant drugs could be reliably monitored using the anti-Xa or anti-activated factor II (anti-IIa) tests in plasma from patients with cirrhosis.

Patients and methods

Patients

Twenty-six adult patients with liver cirrhosis, who were seen on an out-patient basis or were admitted to the hospital, were included in the study. Patients were classified according to the Child-Pugh classification [13]. Ten patients were classified as Child A, ten as Child B, and six patients as Child C cirrhosis. Exclusion criteria were a documented history of congenital coagulation disorders, presence of active infection (<2 weeks), presence of acute liver failure, use of anticoagulant drugs in the past 10 d, pregnancy, human immunodeficiency virus (HIV) positivity and recent (<7 d) transfusion with blood products.
The control group consisted of 30 adult healthy volunteers working at our institution. Exclusion criteria for the control group were a documented history of congenital coagulation disorders, documented history of hepatic disease, recent viral infection (<2 weeks), use of anticoagulant drugs in the past 10 d, pregnancy and HIV positivity.

This study protocol was approved by the local medical ethical committee and informed consent was obtained from each subject before inclusion in the study.

Plasma samples

Blood samples were drawn by venepuncture and collected into vacuum tubes containing 3.8% trisodium citrate as an anticoagulant, at a blood to anticoagulant ratio of 9:1. Platelet poor plasma was prepared by double centrifugation at 2000 g and 10 000 g respectively for 10 min. Plasma was snap-frozen and stored at - 80°C until use.

Addition of anticoagulants to plasma samples

The following anticoagulants were added to plasma samples of cirrhotic patients and control. The indicated concentrations represent final concentrations in plasma.
1. UFH (Leo Pharma, Ballerup, Denmark), 0.3 U/ml
2. LMWH (Clexane; Sanofi-Aventis BV, Gouda, the Netherlands), 0.2 U/ml

3. Fondaparinux (Arixtra; GlaxoSmithKline BV, Zeist, the Netherlands), 0.5 µg/ml
4. Dabigatran (Alsachim, Illkirch Graffenstaden, France), 0.3 µg/ml
5. Rivaroxaban (Alsachim, Illkirch Graffenstaden France), 100 ng/ml

Anti-Xa/anti-IIa assay and APTT

Anti-Xa and anti-IIa values were all measured using the ACL 500 TOP analyser (Instrumentation Laboratory, Breda, the Netherlands). Anti-Xa values were measured in the plasma samples after the addition of UFH, LMWH, or fondaparinux with the Biophen Heparin (LRT) kit (Hyphen Biomed, Neuville Sur Oise, France), the Biophen heparin calibrator (for UFH and LMWH) or the Biophen arixtra calibrator (for fondaparinux), both purchased from Hyphen Biomed. No exogenous antithrombin is added in these assays. We repeated anti-Xa measurements in a limited set of plasma samples (10 patients, 10 controls), which we spiked with LMWH using the same kit, to which was added exogenous antithrombin (Hyphen Biomed, 75 lg/ml, final concentration) to assess the effect of low endogenous antithrombin levels on the outcome of the assay. Anti-Xa values, after the addition of rivaroxaban, were measured using the Biophen DiXal kit (Hyphen Biomed). A calibration curve was constructed by adding rivaroxaban to pooled normal plasma (obtained by combining plasma from >200 individuals). Anti-IIa values in the plasma samples were measured after addition of dabigatran using the Hemoclot thrombin inhibitors kit (Hyphen Biomed). A calibration curve was constructed by adding dabigatran to pooled normal plasma.

UFH activity was estimated by determination of the APTT on the ACL 500 TOP analyser, using reagents (HemosIL SynthASil) and protocols from the manufacturer (Instrumentation Laboratory, Breda, The Netherlands).

Routine coagulation laboratory tests

The INR was assessed with commercially available methods on an automated coagulation analyser (ACL 500 TOP) with reagents (Recombiplastin 2G) and protocols from the manufacturer (Instrumentation Laboratory, Breda, the Netherlands). Levels of factor (F) VIII, FII, FX and antithrombin (AT) were measured on an automated coagulation analyser (ACL 500 TOP) with reagents and protocols from the manufacturer (Recombiplastin 2G for FII and FX, HemosIL SynthASil for FVIII, and Liquid Antithrombin reagent for AT, all from Instrumentation Laboratory).

Statistical analysis

Data are expressed as mean ± standard deviation (SD), medians (with ranges), or numbers (with percentages) as appropriate. Means of two groups were compared by Student's t-test or Mann-Whitney U test as appropriate. Multiple groups were compared using One-way analysis of variance (ANOVA) (with the Bonferroni post-test) or Kruskal-Wallis H test (with Dunn's post-test) as appropriate. Spearman's correlation coefficient was used to assess correlation between continuous variables. P values of 0.05 or less were considered statistically significant. GraphPad Prism (San Diego, USA) and IBM SPSS Statistics 20 (New York, USA) was used for all analyses.

Results

Patient characteristics

The main characteristics of the study population are reported in Table 1. Twenty-six patients with cirrhosis (14 males and 12 females) were included, and they were categorized according to the severity of liver disease as expressed by the Child Pugh score (10 Child A, 10 Child B and 6 Child C patients). The most common aetiology of liver disease was alcoholic, especially in the Child class C patients. None of the patients used pro- or anti-coagulant drugs. Thirty healthy subjects (14 males and 16 females) were included as normal controls.

The baseline INR and plasma levels of FVIII, FII, FX, and AT are shown in Table 2. Patients with cirrhosis showed a statistically significant prolongation of the INR and APTT, and a decrease in all measured coagulation proteins, except for FVIII (which was increased), as compared to controls. The reduction in levels of plasmatic factors was proportional to the severity of liver disease.

Characteristics	Cirrhotic patients Child A n=10	Child B n=10	Child C n=6	P-value
MELD score	8.0 [6.0-10.0]	11.5 [8.0-19.0]	16.5 [12.0-19.0]	.0006
Age (yrs)	56.0 [14.2]	50.5 [12.5]	59.0 [4.7]	.367
Sex (male)	4 (40)	6 (60)	4 (67)	.682
BMI	25.4 [4.0]	28.4 [6.0]	28.4 [4.2]	.337
Smoking (number)	4 (40)	1 (10)	1 (17)	.368
Alcohol (U per week)	0 [0-1]	0 [0-7]	0 [0-0]	.731
Etiology of liver disease				
Alcoholic	2 (20)	1 (10)	6 (100)	.001
HCV	1 (10)	0 (0)	0 (0)	1.000
NASH	1 (10)	2 (20)	0 (0)	.769
Hemochromatosis	0 (0)	1 (10)	0 (0)	1.000
PBC	1 (10)	0 (0)	0 (0)	1.000
PSC	2 (20)	2 (20)	0 (0)	.639
Auto-immune	3 (30)	1 (10)	0 (0)	.403
Alcoholic + NASH	0 (0)	1 (10)	0 (0)	1.000
Unknown	0 (0)	2 (20)	0 (0)	.323
Co-morbidity				
Cardiovascular	3 (30)	2 (20)	0 (0)	.391
Diabetes Mellitus	2 (20)	1 (10)	0 (0)	.769
Laboratory variables				
Serum bilirubin (µmol/L)	15 [5-35]	40 [18-61]	93 [63-131]	.0001
Serum albumin (g/L)	36 [28-44]	33 [27-63]	26 [25-29]	.002
Serum creatinin (µmol/L)	69 [23]	72 [32]	75 [17]	.920
Hemoglobin (mmol/L)	8.2 [1.4]	7.2 [0.9]	6.2 [0.8]	.005
Leukocytes (10^9/L)	6.5 [4.5]	5.1 [2.1]	5.4 [2.1]	.591
Platelets (10^9/L)	112 [16-258]	86 [28-471]	73 [44-114]	.482

Table 1. Demographic and clinical characteristics of the study population.
HCV: Hepatitis C virus, NASH: Non-alcoholic steatohepatitis, PBC: Primary biliary cirrhosis, PSC: Primary sclerosing cholangitis.
Data are expressed as number (%), mean [SD], or median [range].

	Cirrhotic patients			Healthy	P-value
	Child A	Child B	Child C	controls	
INR	1.1 [0.9-1.2]	1.2 [1.0-2.0]	1.5 [1.4-1.7]	1.0 [0.9-1.1]	<.0001
APTT	40.3 [5.9]	38.1 [4.4]	37.0 [3.7]	33.8 [3.8]	.0006
FVIII (%)	132 [92-185]	129 [91-254]	140 [108-171]	94 [56-138]	<.0001
FII (%)	83 [50-104]	69 [43-98]	42 [40-71]	107 [88-127]	<.0001
FX (%)	90 [74-136]	81 [41-193]	56 [49-69]	106 [80-147]	<.0001
AT (%)	80 [42-107]	50 [27-113]	40 [23-44]	106 [87-126]	<.0001

Table 2. Coagulation parameters in cirrhotic patients and normal controls.
INR: International normalized ratio, APTT: Activated partial thromboplastin time, AT: Antithrombin.
Data are expressed as mean [SD], or median [range].

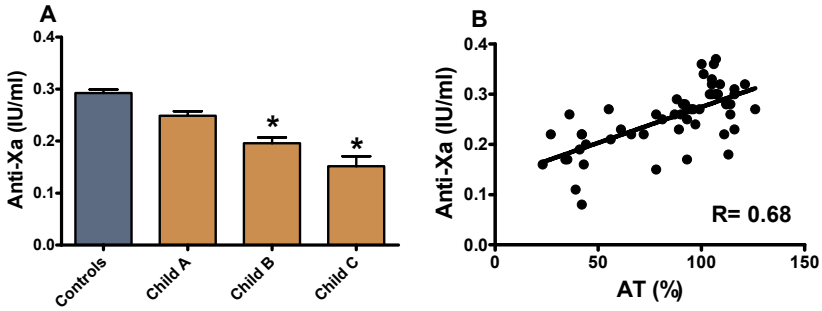

Figure 1. (A) Mean anti-Xa activity in plasma from healthy controls and patients with Child A, Child B, and Child C cirrhosis after addition of 0.3 U/ml unfractionated heparin (UFH). Error bars indicate the standard error of the mean (SEM). (B) Correlation between antithrombin (AT) levels and anti-Xa values after addition of 0.3 U/ml UFH in plasma from patients with cirrhosis.
*P < 0.001 compared to controls.

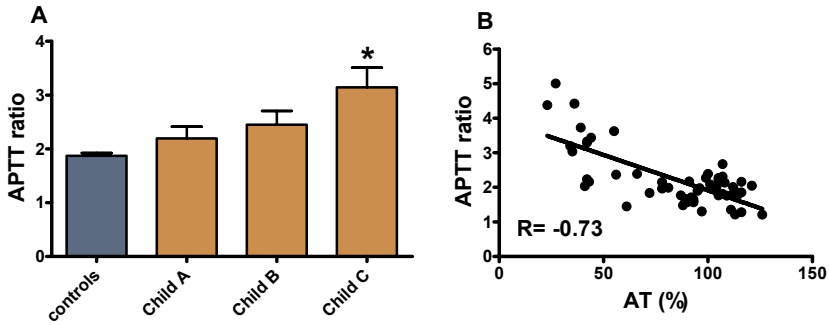

Figure 2. (A) Mean activated partial thromboplastin time (APTT) ratios after the addition of 0.3 U/ml unfractionated heparin (UFH) in plasma from healthy controls and patients with Child A, Child B, and Child C cirrhosis. Error bars indicate the standard error of the mean (SEM). (B) Correlation between antithrombin (AT) levels and APTT ratios in plasma from patients with cirrhosis.
APTT ratios were defined as (APTT in presence of UFH/APTT in absence of UFH). *P < 0.01 compared to controls.

Recovery of anticoagulant drugs assessed by anti-Xa and anti-IIa activity

UFH. When 0.3 U/ml UFH was added to the plasma of the controls, the mean anti-Xa level was 0.29 ± 0.04 U/ml. In contrast, plasma from patients with cirrhosis spiked with the same amount of heparin resulted in a mean anti-Xa level of only 0.21 ± 0.05 U/ml. The reduced recovery

in the plasma of cirrhotic patients was statistically significant (P < 0.0001) and correlated with the severity of liver disease (Figure 1A). Furthermore, we observed a significant positive correlation between AT levels and anti-Xa values in plasma from cirrhotic patients (r = 0.68, P < 0.0001; Figure 1B).

When UFH activity was determined by the APTT, we observed a significantly greater prolongation of the APTT in plasma from patients compared to controls. The APTT in patients increased from 38.7 ± 4.9 to 104.6 ± 49.1 s after addition of 0.3 U/ml of UFH and from 33.8 ± 3.8 to 63.8 ± 15.0 s in controls, resulting in APTT ratios (APTT with UFH/APTT without UFH) of 2.6 ± 1.0 and 1.9 ± 0.3, respectively (P = 0.0009). The increased effect of heparin on the APTT became more pronounced with increasing severity of liver disease (Figure 2A). Interestingly, the response to heparin (expressed as the APTT ratio) negatively correlated with the AT levels (r = -0.73, P < 0.0001; Figure 2B).

Low molecular weight heparin. When 0.2 U/ml LMWH was added to the plasma of the controls, the mean anti-Xa level was 0.23 ± 0.03 U/ml (mean ± SD). In contrast, when plasma from patients with cirrhosis was spiked with the same amount of LMWH, the mean anti-Xa level detected was only 0.13 ± 0.06 U/ml. The reduced recovery in plasma from cirrhotic patients, compared to normal controls was statistically significant (P < 0.0001) and correlated with the severity of liver disease (Figure 3A).

Furthermore, we observed a significant positive correlation between AT levels and anti-Xa values in plasma from cirrhotic patients (r = 0.71, P = 0.0002; Figure 3B).

To assess whether the reduced recovery in patients was due to the decreased AT levels, we tested the effect of addition of exogenous AT (75 µg/ml, final concentration) to the plasma in 10 patients and 10 controls. When anti-Xa levels were set at 100% in controls, the anti-Xa level in the absence of exogenously added AT in patients was only 64 ± 10%, P < 0.0001. In contrast, when exogenously added antithrombin was present, the recovery in patients was 97 ± 8%, P = 0.56).

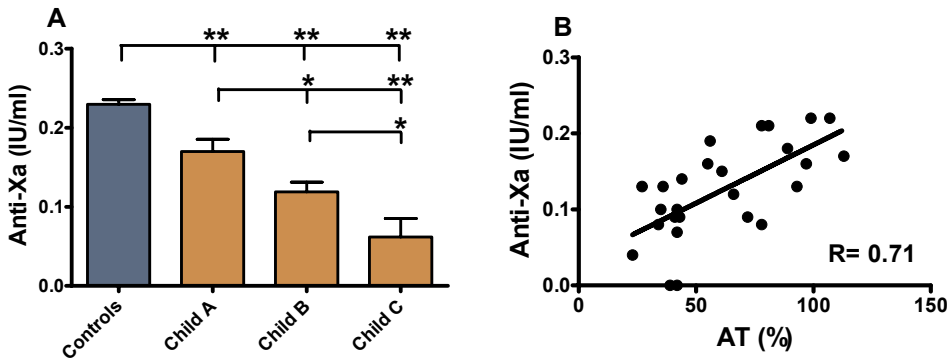

Figure 3. (A) Mean anti-Xa activity in plasma from healthy controls and patients with Child A, Child B, and Child C cirrhosis after addition of 0.2 U/ml low molecular weight heparin (LMWH). Error bars indicate the standard error of the mean (SEM). (B) Correlation between antithrombin (AT) levels and anti-Xa values after addition of 0.2 U/ml LMWH in plasma from patients with cirrhosis. *P < 0.05; **P < 0.001.

Fondaparinux. When 0.5 µg/ml fondaparinux was added to the plasma of the controls, mean anti-Xa levels were 0.59 ± 0.04 µg/ml. Mean anti-Xa levels were significantly lower in plasma from patients with cirrhosis (0.50 ± 0.07 µg/ml, P < 0.0001). The reduced recovery in cirrhosis plasma correlated with the severity of liver disease (Figure 4A).
Again, we observed a significant positive correlation between AT levels and anti-Xa values in plasma from cirrhotic patients (r = 0.86, P < 0.0001; Figure 4B).

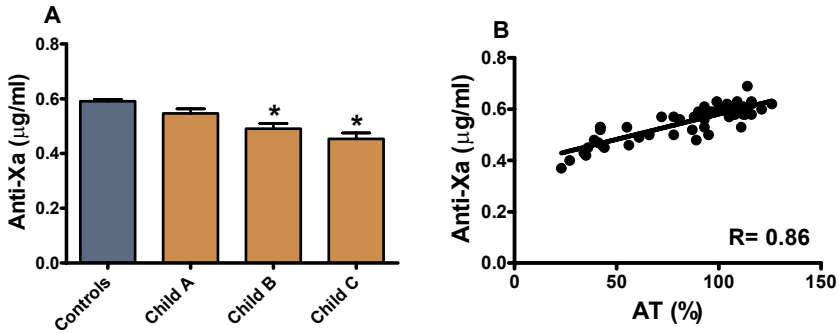

Figure 4. (A) Mean anti-Xa activity in plasma from healthy controls and patients with Child A, Child B, and Child C cirrhosis after addition of 0.5 µg/ml fondaparinux. Error bars indicate the standard error of the mean (SEM). (B) Correlation between antithrombin (AT) levels and anti-Xa values after addition of 0.5 µg/ml fondaparinux in plasma from patients with cirrhosis. *P < 0.001 compared to controls.

Rivaroxaban. When 100 ng/ml rivaroxaban was added to the plasma samples, mean anti-Xa levels were comparable between patients and normal controls (85.0 ± 14.2 vs. 88.0 ± 11.4 ng/ml; P = 0.38) (Figure 5).

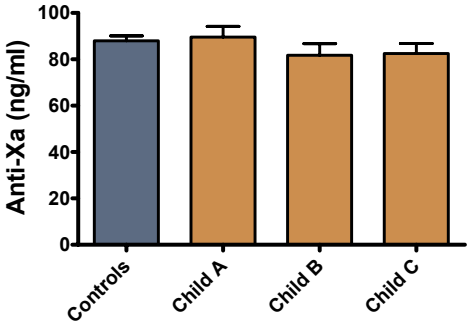

Figure 5. Mean anti-Xa activity in plasma from healthy controls and patients with Child A, Child B, and Child C cirrhosis after addition of 100 ng/ml rivaroxaban. Error bars indicate the standard error of the mean (SEM).

Dabigatran. After the addition of 0.3 µg/ml dabigatran, anti-IIa levels were 0.33 ± 0.01 µg/ml and 0.31 ± 0.02 µg/ml in plasma from patients and normal controls, respectively. The observed anti-IIa levels were slightly higher in plasma from patients compared to controls (P = 0.0003). However, when separating patients according to the Child-Pugh classification, the anti-IIa levels were only significantly higher in the patients with Child C cirrhosis (0.33 ± 0.01 µg/ml; P < 0.05), compared to controls (Figure 6).

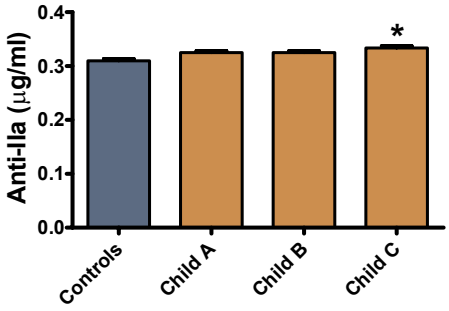

Figure 6. Mean anti-IIa activity in plasma from healthy controls and patients with Child A, Child B, and Child C cirrhosis after addition of 03 lg/ml dabigatran. Shown are means. Error bars indicate the standard error of the mean (SEM).
*P < 0.05 compared to controls.

Discussion

We observed a reduced recovery of AT-dependent anticoagulants assessed by anti-Xa levels when drugs were added to the plasma of patients with cirrhosis, compared to the recovery in plasma from healthy individuals. The addition of LMWH led to the most pronounced underestimation of drug levels, followed by UFH and fondaparinux. In contrast, comparable anti-Xa and anti-IIa levels were measured in plasma from patients and controls after the in vitro addition of direct FXa (rivaroxaban) and direct FIIa inhibitors (dabigatran).

We [8] and others [7,9] have previously demonstrated that the anti-Xa assay underestimates the LMWH mass present in plasma from patients with cirrhosis. In addition, this current study showed that the anti-Xa assay also underestimates the masses of other AT-dependent anticoagulant drugs (UFH and fondaparinux) in plasma from patients with cirrhosis.

The reduced recovery of heparins correlated with the severity of liver disease, as assessed by the Child-Pugh score. Accordingly, and as a potential explanation for this phenomenon, a positive correlation between AT levels and anti-Xa values was observed. The reduced recovery of AT-dependent anticoagulants thus appears to be a direct consequence of the acquired AT deficiency of patients with liver disease [14]. Indeed, when exogenous AT was added to the anti-Xa assay, the reduced recovery of the LMWH in patients compared to controls was fully blunted. Furthermore, other studies have also shown decreased anti-Xa values in patients with AT deficiency treated with UFH or LMWH [15-17]. In addition, in neonates, who have reduced plasma levels of AT, the anti-Xa assays have also been shown to be unreliable for this reason [18].

We observed a more pronounced increase in the APTT after the addition of UFH in plasma from patients with cirrhosis in comparison to the controls, suggesting an enhanced anticoagulant effect of UFH in cirrhosis. In clinical practice, the APTT would thus suggest that dose reductions are required in patients with cirrhosis. In contrast, recovery of UFH in the anti-Xa assay is reduced in cirrhosis, which, in clinical practice, may lead to dose escalations to reach a desired anti-Xa level. Importantly, both tests are used in clinical practice to monitor UFH.

Based on the data of this study, we strongly suggest that anti-Xa levels should not be relied upon for monitoring heparins (that exert their effect through AT) in patients with cirrhosis, unless an anti-Xa test with exogenous antithrombin is available. However, many clinical laboratories

may not offer such a modified anti-Xa test. Novel monitoring methods (such as, for example, thrombin generation tests) may provide better monitoring options in patients with cirrhosis, but unfortunately such methods are not yet available in routine diagnostic laboratories. Indeed, in vitro studies using thrombin generation tests have demonstrated that LMWH has a more profound anticoagulant effect in plasma from patients with cirrhosis as compared to plasma from healthy controls [9]. Furthermore, we have also recently demonstrated that the anticoagulant potency of clinically approved drugs differs substantially between patients with cirrhosis and healthy individuals, using thrombin generation tests [19]. Whereas dabigatran and, to a lesser extent, heparin and LMWH are more potent in plasma from patients with cirrhosis, fondaparinux and rivaroxaban showed a decreased anticoagulant effect. Thus, although anti-Xa levels underestimate drug levels in cirrhotic patients treated with UFH or LMWH (which may prompt dose escalations), thrombin generation tests suggest that UFH or LMWH are slightly more potent in patients with cirrhosis compared to individuals with intact liver function. In other words, dose escalations instigated by a low anti-Xa level will potentially lead to a substantial bleeding risk.

Although our study shows that heparin monitoring in patients with cirrhosis may be improved by using anti-Xa assays to which exogenous AT is added, further studies on the performance of such assays in patients with cirrhosis are required. The direct FXa and FIIa inhibitors, however, may be monitored through the respective anti-Xa and anti-IIa assays, as comparable anti-Xa and anti-IIa levels were observed after the addition of rivaroxaban and dabigatran, respectively, in plasma from patients and controls.

Due to the limited clinical experience, the anticoagulant of choice and the dosages for the various indications is still unclear. Nevertheless, (theoretical) advantages and disadvantages of the available drugs in patients with cirrhosis have been recognized, which may facilitate a rational choice for a drug in a specific clinical situation [5]. The results of the present study will assist clinicians and clinical laboratories in interpreting anti-Xa or anti-IIa test results when applied in patients with cirrhosis treated with established or new anticoagulant drugs.

In conclusion, routine coagulation assays underestimate levels of antithrombin-dependent drugs, but not of direct anticoagulant drugs in plasma from patients with cirrhosis. This finding has practical consequences for monitoring heparins in these patients. Clinical (dose-finding) studies on the monitoring, efficacy and safety of heparins are urgently required to improve antithrombotic therapy in the patients with cirrhosis.

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APPENDIX TO CHAPTER 9

Issues With Monitoring of Unfractionated Heparin in Cirrhosis

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To the Editor

Fuentes et al report a retrospective analysis on bleeding events and monitoring of unfractionated heparin in patients with cirrhosis compared with matched controls [1]. We would like to comment on a number of issues related to the results described in this publication.

First, this study suggests that patients with cirrhosis are at greater risk for bleeding compared with matched controls when treated with unfractionated heparin. Patients with cirrhosis frequently have substantial alterations in their hemostatic system. The net result of these changes is considered to be a rebalanced hemostatic status, which is much more fragile to disruption compared with the hemostatic balance in healthy individuals [2]. As a consequence, it is of utmost importance to avoid excessive anticoagulation in patients with such a delicate hemostatic balance to avoid bleeding [3]. It may thus not come as a surprise that patients with cirrhosis seem to be at a greater risk for anticoagulant-associated bleeding as compared with matched controls. The findings by Fuentes et al, however, contrast with several recent studies, which have suggested that low-molecular-weight and unfractionated heparin to have a favorable safety profile in patients with cirrhosis [4–9]. Their study concluded that patients with cirrhosis have an increased anticoagulant-associated bleeding risk on basis of an increase in administered blood products. This conclusion may not be justified, as it is unclear whether these products were actually administered to actively bleeding patients. It is well known that a substantial proportion of blood products in the cirrhotic population are administered prophylactically [10]. As patients with cirrhosis often have abnormal routine hemostatic test results that are suggestive of a bleeding tendency (such as a prolonged prothrombin time or a decreased platelet count), prophylactic administration of fresh frozen plasma or platelet concentrates is still common practice, despite increasing awareness that these abnormal routine coagulation tests do not predict a bleeding risk [11].

Second, we wonder why these patients all received unfractionated (instead of low-molecular weight) heparin. Low-molecular-weight heparin is the drug of choice for at least some of the indications listed (e.g., treatment of acute venous thromboembolism of the leg or acute pulmonary embolism [12]). In addition, given the favorable published data on low-molecular-weight heparin in patients with cirrhosis, this drug may be preferable over unfractionated heparin, which has a less established safety profile in patients with cirrhosis. We do acknowledge that concomitant renal failure, in which accumulation of low-molecular-weight heparin is known to occur, may lead to the decision to use unfractionated and not low-molecular-weight heparin.

Third, the authors note discrepancies between activated partial thromboplastin time (APTT) and anti-Xa monitoring of low-molecular-weight heparin. We would like to comment that both tests fail to accurately measure drug levels in patients with cirrhosis, as we have recently shown [13]. With in vitro experiments in which we added a known concentration of unfractionated heparin to plasma from healthy individuals or plasma from patients with cirrhosis, we demonstrated that the anti-Xa tests underestimate drug levels whereas the APTT gives an overestimation. Monitoring of heparin by APTT or anti-Xa tests is indirect. These tests do not assess drug levels directly but rather estimate drug levels based on the anticoagulant action of the drug. Heparins exert an anticoagulant effect by enhancing the anticoagulant

effect of the endogenous anticoagulant antithrombin, and it is the heparin-enhanced antithrombin effect that is assessed by the APTT and anti-Xa assays. The decreased antithrombin levels in plasma from patients with cirrhosis lead to falsely elevated APTT and falsely decreased anti-Xa levels in heparin-treated patients, and both tests thus seem unsuitable for heparin monitoring in patients with cirrhosis [13].

Although the anti-Xa and APTT tests are designed to get an estimate of drug levels in plasma, the clinically relevant parameter is the "true" anticoagulant effect, which may, for example, be estimated by thrombin generation testing. We have investigated the functional effects of unfractionated and low-molecular-weight heparin in plasma from healthy individuals and plasma from patients with cirrhosis using thrombin generation testing [14]. When a known concentration of (low-molecular-weight) heparin was added to plasma, thrombin generation was inhibited to a larger extent in patients with cirrhosis compared with the decrease in healthy individuals, indicating that the anticoagulant potency of heparins is not necessarily equal in plasma from patients with cirrhosis and healthy individuals. The differences in anticoagulant potency, however, were not present when thrombin generation was tested in the presence of thrombomodulin, which allows activation of the endogenous anticoagulant protein C system. In other words, depending on the exact experimental conditions, heparins exert an equal to enhanced anticoagulant activity in plasma from patients with cirrhosis. This enhanced drug potency contrasts with the anti-Xa assay, which at equal drug levels gives a much lower value in patients with cirrhosis and thus suggests inadequate anticoagulation. The problems with monitoring of heparins and the altered potency of heparins in plasma from patients with cirrhosis are related to the antithrombin deficiency that is common in these patients, which is understandable because heparin exerts its anticoagulant effect by enhancing the anticoagulant action of endogenous antithrombin.

Finally, we agree with the authors that studies on safety and efficacy of heparin anticoagulation specifically in patients with cirrhosis are warranted. These studies should also take improvements in laboratory monitoring in consideration. Ideally, heparin monitoring tests for patients with cirrhosis should take both drug levels and drug effect into account. Given the drawbacks of heparin use in patients with cirrhosis, there may be opportunities for new (antithrombin-independent) oral anticoagulants [3]. Recently, the successful use of rivaroxaban and apixaban in the treatment of cirrhotic portal vein thrombosis has been reported [15,16]. Although monitoring of these drugs in cirrhosis seems to be much more accurate compared with monitoring of heparins, prospective studies on efficacy and safety are essential before such drugs can be implemented routinely in hepatology clinics.

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CHAPTER
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CHAPTER 10

General Discussion

The concept of a rebalanced hemostasis in patients with liver disease is increasingly gaining acceptance. This balance is perceived to be more unstable than in individuals with intact liver function, with the risk of both bleeding and thrombotic complications. However, it is still unclear which patients are at risk for bleeding or thrombotic complications and how we can predict those risks. Furthermore, the hemostatic system in patients with liver disease is still incompletely understood, especially in the increasing patient population with a fatty liver disease. Due to the increase in patients with NAFLD and aging of the patient population, thrombotic complications are likely to be increasingly diagnosed in patients with liver disease. However, little research has been done on the efficacy and safety of the available anticoagulant drugs in patients with liver disease. Therefore, guidelines on the anticoagulant drug of choice and dosing for the various indications are still lacking. The aim of this thesis was to gain a better understanding of the hemostatic system in patients with (fatty) liver disease and to study the efficacy and monitoring of anticoagulant drugs in plasma from patients with liver disease. Results of this thesis will contribute to better strategies to prevent or treat thrombosis in patients with liver disease in the future. In this chapter, the results of this thesis are discussed in the context of the current literature on the hemostasis in liver disease and the prevention or treatment of thrombotic complications.

Hemostatic changes in liver disease

As outlined in chapter 2, there are changes in all phases of the hemostatic system in patients with liver disease. Most important alterations of the primary hemostasis in patients with liver disease include a reduced platelet count [1], elevated levels of von Willebrand factor (VWF) [2], and low levels of ADAMTS13 [3]. In secondary hemostasis, most procoagulant and anticoagulant proteins are reduced. Finally, in the fibrinolytic system, levels of plasminogen, plasmin inhibitor, thrombin activatable fibrinolysis inhibitor (TAFI), and factor XIII are frequently reduced [4,5], and levels of tissue-type plasminogen activator (t-PA) and plasminogen activator inhibitor-1 (PAI-1) are frequently elevated [6] in patients with chronic liver disease.

If we focus on the secondary hemostasis in patients with liver disease, it is well known that levels of coagulation factors V, VII, IX, X, XI, and prothrombin are reduced, because the liver is the site of synthesis of these proteins [7]. In contrast, levels of factor VIII are often elevated [8]. The decreased production of procoagulant proteins is mostly counterbalanced by decreased production of anticoagulant proteins, such as protein C and antithrombin [9]. Also protein S levels are reduced in patients with cirrhosis [10,11]. Tissue factor pathway inhibitor (TFPI) is not produced by the liver, but the microvascular endothelium is thought to be the principal source of TFPI [12]. It has recently been established that protein S acts as a cofactor for TFPI in the down regulation of thrombin generation [13]. As a result, both acquired and congenital protein S deficiencies are associated with a concomitant TFPI deficiency [14]. In chapter 3, we studied protein S and TFPI plasma levels in patients with cirrhosis, and found that patients with cirrhosis have an acquired protein S deficiency, which is, however, not accompanied by a decrease in TFPI plasma levels. This indicates that the decrease in TFPI levels in conjunction with acquired protein S deficiency as described previously [14] does not occur in patients with chronic liver diseases. A possible explanation might be that TFPI release in patients with cirrhosis is substantially increased due to continuous activation of the endothelium, but that this increased TFPI release is masked, in part, by the protein S deficiency. Indeed, in both chronic and acute liver diseases, continuous activation of the endothelium is common, resulting in increased plasma levels of endothelial-derived proteins,

such as VWF [2,15]. The combination of increased TFPI release, due to continuous endothelial cell activation, and protein S deficiency may thus result in normal TFPI levels. Despite normal TFPI plasma levels, patients with cirrhosis did show a reduced activity of the TFPI-protein S system when compared to healthy controls. Recently, studies have proposed that the increase in factor VIII in combination with decreased protein C is responsible for a hypercoagulable state contributing to thrombotic complications in patients with cirrhosis [16]. However, in the study of chapter 3 we have shown that the impaired activity of the TFPI-protein S system in patients with cirrhosis may also contribute to this hypercoagulable state increasing the risk of thrombotic complications in these patients.

Laboratory tests of hemostasis in patients with liver disease

Since patients with liver disease can suffer from both thrombotic and bleeding complications, there is a frequent clinical need for predicting the risk of bleeding or thrombosis in individual patients with liver disease. However, current available clinical tests fail to accurately predict those risks in patients with liver disease.

The standard coagulation profile processed in most clinical laboratories includes the prothrombin time (PT), international normalized ratio (INR), and activated partial thromboplastin time (APTT). In fact, many consider the PT the test of choice for diagnosing either inherited or acquired coagulopathies, in addition to the monitoring of vitamin K antagonists. However, these conventional coagulation tests fail to assess all the changes in the coagulation in patients with a liver disease. This can be explained by two considerations. First, the PT and APTT only measure the first 5% of thrombin being generated, since this is enough for plasma to start clotting [17]. Second, in the PT and APTT the anticoagulant factor protein C, whose activation requires thrombomodulin, cannot be properly activated [18]. Thrombomodulin is a protein located on the endothelial cells, and plasma and reagents needed to perform the PT or APTT do not contain sufficient amounts of this protein. The PT and APTT are, therefore, sensitive to changes in the procoagulant proteins, but not to changes in the anticoagulant proteins. Since in patients with liver disease both pro- and anticoagulant proteins are decreased, these conventional coagulation tests cannot reliably predict bleeding risk in these patients. Indeed, multiple studies have shown that the PT is a poor predictor of bleeding following liver biopsy as well as other surgical procedures, and that the PT does not correlate with severity of gastrointestinal bleeding in patients with cirrhosis [19-21].

To better resemble the hemostatic system in patients with chronic liver disease, other tests have been recently studied. These include thrombin generation, thromboelastography (TEG), and thromboelastometry. The TEG uses whole blood to measure speed and strength of clot formation continuously. It can, thus, theoretically analyze all components of the hemostatic system [22]. Both TEG and rotational thromboelastometry have been routinely used to guide transfusion of platelet concentrates, factor repletion, and fibrinolytic therapy during liver transplantation [23]. In fact, a recent randomized controlled trial reported a significant lower use of blood products without an increase in bleeding complications by using TEG-guided transfusion strategy compared to transfusion guided by INR and platelet count [24]. However, most parameters of the test are not standardized yet and TEG thresholds remain unclear. Furthermore, to date no studies have examined the use of TEG in predicting bleeding or thrombosis in nonsurgical patients with liver disease. Other drawbacks of TEG include pre-

analytic and analytic variables that impact test reliability and reproducibility, and the various methods to perform the test which results poorly correlate [25-27].

Another test that has been increasingly used to reassess the hemostatic capacity of patients with liver disease is the thrombin generation test. The thrombin generation assay evaluates thrombin generation (resulting from the action of the procoagulants) and decay (resulting from the action of the anticoagulants). In the assay, coagulation of test plasma is activated by small amounts of tissue factor and phospholipids, and the formation of thrombin is continuously monitored by means of a thrombin-specific fluorogenic substrate. There are several parameters that can be derived from the thrombin generation curve, but the endogenous thrombin potential (ETP) is the most used, which refers to the total amount of thrombin generated during the test. When thrombomodulin is added to the test plasma, the thrombin generation test is sensitive to all anticoagulant proteins in the plasma and, thus, measures the true balance between the pro- and anticoagulant factors. Indeed, thrombin generation testing in the presence of thrombomodulin has demonstrated normal or even superior thrombin generation in patients with liver disease [28-32]. Some studies have also shown that thrombin generation testing is useful in identifying patients with an increased risk of venous thrombosis or portal vein thrombosis [33-36]. However, the thrombin generation test is not widely available, the test is not standardized yet, and currently the test is too complicated for routine use in diagnostic laboratories.

The Thrombodynamics assay is a newly developed plasma-based global hemostasis assay, which continuously monitors clot growth in non-stirred plasma. Whereas in other coagulation tests (such as the PT or thrombin generation test) clotting is activated by homogenously dissolved tissue factor, in the Thrombodynamics test clotting is activated by a surface with immobilized tissue factor [37,38]. Therefore, this spatial clot growth assay better resembles in vivo clot formation. The spatial clot growth assay has been successfully used to assess the hemostatic system in various indications [37-40], including in patients with hemophilia [41]. In chapter 4, we also show preserved clot formation in plasma from patients with cirrhosis using the novel Thrombodynamics assay.

Advantages of this assay include the stability of the assay (provided a strict blood processing protocol is followed) and the easy use and clear methods of the assay. Furthermore, the test provides clear reference values for the various parameters of clot growth that can be measured. The Thrombodynamics assay may hold promise in the prediction of bleeding or thrombotic risk in patients with a liver disease. However, currently the test is for research use only and should not be used in diagnostic procedures. Future studies to assess the clinical value of this novel test in predicting hemostatic abnormalities in patients with liver disease are needed.

Finally, it should be noticed that it may also be possible that detailed clinical assessment, for example by standardized bleeding scores or thrombosis risk assessment, could be able to identify those patients who are at risk for either bleeding or thrombosis [42]. However, data on the use of such clinical scores in patients with liver disease are scarce. Nevertheless, one recent study evaluated the Padua Predictor Score (PPS) as a risk-stratification tool for the development of venous thromboembolism (VTE) in patients with chronic liver disease [43].

The PPS was significantly associated with risk of VTE and, thus, appears to be an effective assessment tool in patients with chronic liver disease. However, this was a retrospective study and prospective studies would be needed to confirm this finding.

Bleeding risk in surgical patients

Patients with a liver disease are not as prone to bleeding complications as clinicians formerly thought. As mentioned in the previous section, routine coagulations tests cannot be reliably used to assess a bleeding tendency in these patients. Furthermore, many patients can currently undergo a liver transplantation with minimal or even without transfusion of blood products [44-47]. Such a major surgical procedure would never be possible in a patient with a true coagulopathy, such as in hemophilia, without correction of the coagulopathy with factor concentrates or blood product transfusion. There is no evidence for the benefits of prophylactic blood product transfusion in perioperative medicine in patients with liver disease. Despite the lack of benefits, prophylactic use of blood products prior to smaller invasive procedures in these patients is still common practice. However, the side effects, such as risk of infection, risk of transfusion related acute lung injury, and transfusion associated circulatory overload with an increasing risk of bleeding, are increasingly acknowledged. In chapter 2 we, therefore, argue against routinely correcting a prolonged PT or INR with transfusion of blood products prior to procedures in patients with liver disease. Instead, only those patients with significant bleeding should be treated. Moreover, risk factors for bleeding, such as portal hypertension, endothelial dysfunction, bacterial infection, and renal failure, should also be addressed when preventing (re)bleeding.

Previously, patients following liver surgery were also perceived to be in a hypocoagulable state due to a prolonged PT or APTT [48-50]. However, we now know that these conventional coagulation tests are not reliable for the assessment of the overall hemostatic status in these patients. In fact, in chapter 5 we showed a hypercoagulability of plasma from patients following liver resection using the thrombin generation test in the presence of thrombomodulin. This hypercoagulable state following liver resection is also clinically evidenced by studies showing that the occurrence of venous thromboembolism following liver surgery is common [51] and, in fact, the risk increases with the extent of hepatectomy [52,53]. Furthermore, several recent studies also found a normal to hypercoagulable state following liver surgery using thromboelastography [50,54,55]. However, in chapter 5 we also provide a mechanistic explanation for the hypercoagulable state following liver resection. Specifically, the hypercoagulable state was associated to a profound thrombomodulin resistance, meaning that thrombin generation only slightly decreased by the addition of thrombomodulin in plasma from patients following liver resection compared to a substantial decrease in plasma from healthy controls.

This thrombomodulin resistance was likely attributable to decreased levels of protein C and elevated levels of factor VIII. Combined with the decreased levels of antithrombin, the net effect is an increased thrombin generation when tested in the presence of thrombomodulin following liver resection. This decrease in coagulation factors might, in part, be explained by the decreased synthetic capacity of the liver remnant. Consumption of coagulation proteins as a result of surgical damage may also play a role. This might explain why in patients following pancreatic resection in our study also certain coagulation proteins decreased. Furthermore, this consumption theory may be supported by the finding that the risk of venous

thromboembolism increases with the extent of the hepatectomy [52,53], and that extended operative time forms a risk factor for venous thromboembolism [51]. Finally, hemodilution may also contribute to the decrease in coagulation factors following liver resection.

The results of chapter 5 on the hypercoagulable state following liver resection in combination with the discussed literature in the previous section, has important clinical implications in the management of patients following liver surgery. First, clinicians should be aware of the limitations of the use of conventional coagulation tests to guide hemostatic management. A prolonged PT or APTT following liver surgery should not be routinely corrected by transfusion of blood products. Second, patients following liver surgery should not be withheld from thrombosis prophylaxis. In fact, more aggressive anticoagulation may even be necessary in the post-operative period when certain risk factors for venous thromboembolism are present. Examples of such risk factors might be a history of venous thromboembolism, the presence of cancer, an extended operation time, or a prolonged length of stay.

Hemostasis in NAFLD

Nonalcoholic fatty liver disease (NAFLD) is, nowadays, considered to be the most frequent chronic liver disorder in Western countries [56,57]. It represents a histopathological spectrum ranging from simple hepatic steatosis to steatohepatitis (NASH) with increasing risk for progression to advanced fibrosis and cirrhosis [58-60]. NAFLD is considered the hepatic manifestation of the metabolic syndrome, and is associated with an increased risk of cardiovascular disease (CVD) [61]. Furthermore, rates of venous thromboembolism and portal vein thrombosis (PVT) appear also increased in patients with NAFLD [62,63]. Multiple processes probably contribute to this increased risk of thrombosis in NAFLD, which have been mentioned in chapter 6. Some recent studies have also suggested a role for a hypercoagulable state in the increased risk of thrombosis in patients with NAFLD. However, in chapter 7, we show that, except for some pro-thrombotic features, the overall hemostatic status is comparable between patients with NAFLD and controls. Therefore, our study suggests a limited role for hyperactive hemostasis in the increased thrombotic risk in NAFLD. In this section the results found in chapter 7 in combination with the current literature on the hemostatic alterations in NAFLD will be further discussed.

Alterations in primary hemostasis

Previous studies have suggested an increase in platelet activation in patients with the metabolic syndrome [64,65] and in patients with NAFLD [66-69], which might contribute to their increased risk of thrombosis. However, the results of these studies are inconsistent and most of these studies are limited by their relative small sample size. Furthermore, most of these studies use indirect markers of platelet activation, such as the mean platelet volume. In chapter 7, we show that NAFLD is not associated with an increase in platelet activation, which was measured using a direct flow cytometric assay of platelet activation status. Additionally, NAFLD was not associated with major changes in pivotal proteins in primary hemostasis. We, therefore, suggest a limited role for liver disease-induced platelet activation in the perceived increased risk of thrombosis in patients with NAFLD.

Alterations in coagulation & fibrinolysis

Levels of various individual pro-thrombotic factors appear to be increased in patients with NAFLD [70-76]. Although results of these studies on different factors have been inconsistent, an increase in PAI-1, fibrinogen, and factor VIII and a decrease in antithrombin are most frequently reported. In chapter 7, we also observed increased fibrinogen levels in patients with non-cirrhotic NAFLD, although the difference with controls did not reach statistical significance. However, levels of fibrinogen are mostly decreased in patients with cirrhosis [77], which we also observed in patients with alcoholic related cirrhosis. Nevertheless, patients with NASH-related cirrhosis had normal fibrinogen levels, which might be attributed to a relative increase in fibrinogen production due to the fatty liver disease.

Furthermore, we observed increased levels of factor VIII and decreased levels of antithrombin in patients with cirrhosis, which is in line with previous studies [7,28,30]. Finally, in chapter 7 we also observed increased levels of PAI-1 in patients with NAFLD. In fact, PAI-1 levels appeared to increase with increasing severity of the disease and increasing severity of steatosis. Also Verrijken et al. found significant elevated levels of PAI-1 in patients with NASH, which correlated with underlying histological parameters [72]. In fact, increased levels of PAI-1 and resultant reduction in fibrinolysis have been extensively reported as a risk factor for venous thrombosis [78,79]. However, levels of PAI-1 are not independently associated with risk of myocardial infarction, and PAI-1 levels appear to reflect other cardiovascular risk factors [80]. In chapter 7, we observed hypofibrinolysis using the clot lysis assay in patients with non-cirrhotic NAFLD. This hypofibrinolytic status might contribute to thrombosis risk in these patients since a hypofibrinolytic state as determined with the clot lysis assay has been demonstrated to form a risk factor for both venous and arterial thrombosis in the general population [81-83].

More important than to study individual hemostatic proteins, is to study if these changes lead to a hypercoagulability in patients with NAFLD which might explain their increased risk of thrombosis. We performed both TEG and thrombin generation testing in patients with NAFLD in chapter 7. TEG test results were comparable between non-cirrhotic NAFLD patients and controls. However, TEG revealed moderate hypocoagulability in cirrhotic patients. Furthermore, thrombin generation was also normal in patients with NAFLD. This contrasts with Tripodi et al., who concluded that NAFLD is characterized by a procoagulant-imbalance, as shown by an increase in ETP-ratio (with-to-without thrombomodulin) in these patients [73]. We also observed an increase in a similar ratio in patients with NASH-related cirrhosis. However, such ratios only represent the resistance of the plasma to the anticoagulant action of thrombomodulin, which is increased due to decreased levels of protein C and increased levels of FVIII in patients with liver disease. Furthermore, in patients with ASH-related cirrhosis in our study and in patients with alcoholic/viral cirrhosis in the study of Tripodi et al. the ratio was also increased. An increase in the ETP-ratio in patients with NAFLD, therefore, does not explain their increased risk of thrombosis compared to patient with alcoholic/viral cirrhosis. In chapter 7 we, therefore, argue against the use of this ratio to classify if patient samples are normo- or hypercoagulable, and instead believe that the thrombin generation performed in the presence of thrombomodulin is the most accurate laboratory measure of the coagulant potential of a patients' plasma.

Besides hypofibrinolysis, we did find another pro-thrombotic feature of patients with NAFLD, namely a pro-thrombotic structure of the fibrin clot. This was observed by decreased fibrin clot permeability and increased clot density in patients with NAFLD. Indeed, previous studies have shown decreased fibrin clot permeability to be associated with thrombotic diseases [84-86].

Finally, it should be mentioned that we found a higher variability of several hemostatic test results in patients compared to controls. This might suggest that individual patients have a more thrombogenic hemostatic profile. In chapter 7, we could, however, not identify any characteristics of individual patients to explain the more extreme values in individual patients.

Conclusion: hemostasis in NAFLD

Despite a slight hypofibrinolysis and pro-thrombotic structure of fibrin clots in patients with NAFLD, in chapter 7 we show that the overall hemostatic status is comparable between patients with NAFLD and healthy individuals. This suggests that the role for hemostasis in the increased risk of thrombosis in patients with NAFLD and NASH-related cirrhosis is probably limited.

Thrombotic complications in patients with liver disease

Various thrombotic complications do occur frequently in patients with liver disease. For instance, PVT is a common complication of chronic liver disease and is perceived to be associated with clinical deterioration [87]. However, recent studies question whether PVT truly affects cirrhosis outcome [88,89]. A recent systematic review and meta-analysis of multiple studies on anticoagulation for treatment of PVT in cirrhosis concluded that anticoagulant therapy could lead to a relatively high rate of portal vein recanalization [90]. However, further randomized controlled trials are warranted to confirm the risk-to-benefit ratio of anticoagulation in such patients, especially concerning anticoagulant-related bleeding. Recent case studies and one retrospective study have also reported both the efficacy and safety of new oral anticoagulants as a treatment of PVT [91-94]. Currently, there are no strategies available to prevent the development of PVT in cirrhosis. Nevertheless, one recent randomized trial reported that enoxaparin prevents PVT in patients with cirrhosis and, in addition, appears to delay hepatic decompensation [95].

Another frequent occurring thrombotic complication in patients with chronic liver disease is venous thrombosis (which includes deep vein thrombosis (DVT) and pulmonary embolism (PE)). In fact, the estimated incidence of venous thrombosis in cirrhotic patients is 0.5% to 6.3%, compared with 0.5% to 0.9% in non-cirrhotic patients, and the incidence appears to increase with increasing severity of cirrhosis [96]. Current guidelines on the treatment of venous thrombosis in the general population recommend the use of new oral anticoagulants (e.g. Dabigatran, Rivaroxaban, Apixaban, or Edoxaban) over vitamin K antagonist (VKA) therapy [97]. Furthermore, initial parenteral anticoagulation (typically with low molecular weight heparin (LMWH)) is given before Dabigatran and Edoxaban, is not given before Rivaroxaban or Apixaban, and is overlapped with vitamin K antagonist therapy. Except in cancer-associated thrombosis the guidelines suggest LMWH therapy over VKAs or new oral anticoagulant drugs. Primary prevention of venous thrombosis in the general population

is achieved by LMWH, fondaparinux, low-dose unfractionated heparin, or by new oral anticoagulant drugs [98]. There is mounting evidence that thromboprophylactic treatment with LMWH is safe in patients with chronic liver disease [96].

Finally, arterial thrombotic events also occur in patients with liver disease and, in fact, patients with NAFLD have been repeatedly shown to have an increased risk for arterial disease (see chapter 6). For primary prevention of cardiovascular disease in the general population with an age of >50 years, low dose aspirin therapy is suggested. For secondary prevention of cardiovascular events in patients with established coronary artery disease, monotherapy with aspirin or clopidogrel is recommended. Finally, following acute coronary syndromes with percutaneous coronary intervention and stent placement, dual antiplatelet therapy with aspirin in combination with ticagrelor, clopidogrel, or prasugrel is recommended [99]. Secondary prevention of arterial events should probably not be withheld from patients with liver disease, but the risk of bleeding complications may be increased [100]. This thesis further focuses on the use of anticoagulant drugs in patients with liver disease, the use of anti-platelet agents in patients with cirrhosis has been extensively reviewed elsewhere [100].

Different anticoagulant drugs in patients with liver disease

Table 1 shows the different anticoagulant drugs with their mechanisms of action, advantages or disadvantages, and in vitro potency (as studied in chapter 8) in patients with liver disease.

Heparins

Heparins act by binding to antithrombin and enhancing its effect to inhibit factor Xa and/or thrombin. They can be used for the prevention or treatment of both venous thrombosis and PVT. Currently, three classes of heparins are available: unfractionated heparin, LMWH, and fondaparinux. Several studies reported that heparins are efficacious and safe in patients with cirrhosis and PVT or venous thrombosis [95,101-105]. Most studies used LMWH, rather than unfractionated heparin or fondaparinux. Furthermore, studies suggest that the use of LMWH may be more favorable in terms of bleeding complications than unfractionated heparin for the indication of thromboprophylaxis in cirrhotic patients [105,106]. Nevertheless, there are important drawbacks in using heparins in both the general population and, specifically, in patients with liver disease. For instance, the long-term use of these drugs may be limited by the mode of administration (i.v. for unfractionated heparin or s.c. for LMWH and fondaparinux) as well as the concern for heparin-induced thrombocytopenia (HIT). The mode of administration may be associated with both poor compliance and skin reactions. In patients with renal failure LMWH accumulation is known to occur, which may require dose adjustments in patients with cirrhosis and decreased renal function. Furthermore, heparin requires antithrombin to exert its anticoagulant effect and antithrombin levels are frequently decreased in patients with liver disease, which theoretically leads to an unpredictable anticoagulant effect. Additionally, monitoring issues of heparins occur in patients with liver disease, which will be discussed further on. Recently, Senzolo et al. showed an increased anticoagulant response to LMWH in plasma from patients with cirrhosis [107]. In chapter 8, we also show a modestly increased anticoagulant response to both unfractionated heparin and LMWH in plasma from patients with cirrhosis, which was, however, only significant in the absence of thrombomodulin. In contrast, we observed a reduced response to fondaparinux in patients with, especially, advanced cirrhosis (e.g. Child-Pugh C). Dose adjustments based on both altered pharmacokinetics and altered drug potency might, thus, be required for

these drugs in patients with cirrhosis. However, the limited data showing that LMWH is safe to use in patients with cirrhosis contradicts that dose adjustments for this drug are needed.

Vitamin K antagonists

VKAs act by decreasing vitamin K-dependent procoagulant factors II, VII, IX, X, and anticoagulant proteins C and S, with a reduction of hemostatic potential as a net result. A major advantage of VKA therapy is the oral mode of administration. However, the major concern with the use of VKAs in patients with liver disease is the monitoring of the drug by INR levels, which are already abnormal in patients with liver disease. The target INR for VKAs in patients with cirrhosis is, therefore, unclear. In addition, the use of VKAs in patients with cirrhosis has been associated with an increased risk of bleeding complications [108,109]. A recent retrospective study demonstrated significantly better recanalization rates of PVT in patients treated with warfarin therapy (with a target INR range of 2.0-3.0) compared to untreated patients. However, the study did report on bleeding events [110]. Given the reported increased bleeding risk and unclear target INR, the use of VKAs in patients with cirrhosis is likely associated with an unfavorable risk-to-benefit ratio.

New-generation oral anticoagulant drugs

The new-generation oral anticoagulant drugs include the direct factor Xa inhibitors Rivaroxaban, Apixaban, and Edoxaban and the direct thrombin inhibitor Dabigatran. Major advantages of these drugs include the oral mode of administration, the action independent of antithrombin, and the lack of need for monitoring. Although, the lack of need for monitoring can also be interpreted as a disadvantage of the drug as it may increase the risk for non-compliance. Previously, one of the major concerns for these drugs was the lack of specific antidotes to reverse the anticoagulant effect in emergency situations. However, Idarucizumab is currently being used in the clinics as an antidote against Dabigatran, and Andexanet alfa is currently being studied in a phase IV trial as a reversal agent against factor Xa inhibitors [111]. Due to favorable efficacy and safety, new guidelines currently recommend the use of these newer drugs over VKAs and LMWH as long-term anticoagulant therapy in venous thrombosis in the general population (in the absence of cancer) [97]. The direct factor Xa and thrombin inhibitors may also be applicable in the prevention and treatment of thrombotic complications in patients with liver disease. Reports of success of these new-generation oral anticoagulant drugs in patients with cirrhosis are emerging [91,93,94], however larger clinical trials on efficacy and safety of these drugs in cirrhotic patients are lacking. We studied the efficacy of three of the new oral anticoagulant drugs in plasma from patients with cirrhosis in chapter 8 and the appendix to chapter 8. Addition of the direct thrombin inhibitor Dabigatran resulted in an increased response in plasma from patients with cirrhosis. The enhanced effect of Dabigatran was proportional to the severity of the disease. In contrast, the in vitro anticoagulant potency of both factor Xa inhibitors Rivaroxaban and Apixaban was substantially reduced in patients with moderate and advanced cirrhosis. Thus, the new oral anticoagulants may work differently in liver disease patients compared to patients with intact liver function, which is a potential caveat. Drug-specific dose adjustments may be required for these drugs in patients with liver disease. Since these drugs are cleared by the liver and kidneys, the pharmacokinetics may also potentially be altered in patients with liver disease. Dose adjustments should, thus, ideally take both the altered drug potency and the altered pharmacokinetics into account. Furthermore, since the in vitro anticoagulant potency of Dabigatran is substantially increased in patients with cirrhosis, clinicians may be cautious in

using this drug in patients with liver disease. The results of chapter 8 (and appendix) suggest that anticoagulant treatment with direct factor Xa inhibitors will likely not result in over-anticoagulation, with a potentially increased bleeding risk, provided drug levels remain in the target range. Future clinical trials should, therefore, focus on the efficacy and safety of the direct factor Xa inhibitors in patients with liver disease. Finally, monitoring of these new drugs in patients with liver disease may be advisable, which will be discussed later on.

Drug	Mechanism	Pros	Cons	*In vitro* potency
Unfractionated heparin	AT-dependent inhibition of factor Xa and thrombin	- Costs - Fully reversible with protamine	- I.v. administration - Risk of HIT - Dependent on AT - Monitoring issues with APTT and anti-Xa assay	Modestly increased
LMWH	AT-dependent inhibition of factor Xa and (to a lesser extent) thrombin	- Reduced risk for HIT - Route of administration (s.c. *vs.* i.v. for UFH) - Excellent safety profile in cirrhosis perceived by limited studies	- S.c. administration - Dependent on AT - Monitoring issues with anti-Xa assay - Accumulation in renal failure	Modestly increased
Fondaparinux	AT-dependent inhibition of factor Xa	- Reduced risk for HIT compared to other heparins - Synthetic drug	- S.c. administration - Dependent on AT - Monitoring issues with anti-Xa assay - Accumulation in renal failure	Decreased, especially in advanced cirrhosis
Vitamin K antagonists	Reduce functional levels of vitamin K-dependent proteins	- Costs - Oral mode of administration	- Monitoring issues in patients with already elevated INR - Bleeding risk	
Rivaroxaban, Apixaban, Edoxaban	Direct factor Xa inhibitor	- Lack of AT dependence - Mode of administration - Wider therapeutic window than VKAs	- Lack of experience - GI bleeding risk - Accumulation in renal and liver disease	Rivaroxaban and Apixaban: decreased in moderate and advanced cirrhosis
Dabigatran	Direct thrombin inhibitor	- Lack of AT dependence - Mode of administration - Wider therapeutic window than VKAs	- Lack of experience - GI bleeding risk - Accumulation in renal failure	Substantially increased, proportional to severity of disease

Table 1. Anticoagulant drugs in the prevention or treatment of venous thrombosis and portal vein thrombosis in patients with liver disease. Shown are their mechanisms of action, advantages or disadvantages, and in vitro potency (as studied in chapter 8) in patients with liver disease.
AT: antithrombin, HIT: heparin-induced thrombocytopenia, APTT: activated partial thromboplastin time, LMWH: low molecular weight heparin, VKA: vitamin K antagonist, GI: gastro-intestinal.

Prevention or treatment of thrombosis in patients with liver disease

There are multiple indications for antithrombotic treatment in patients with liver disease. However, due to the lack of clinical data, the anticoagulant drug of choice and dosing regimens are in most instances unclear. In this section, some guidance on how to approach prevention or treatment of thrombosis in patients with liver disease will be provided. In addition, it is critical to evaluate each patient individually to best determine which benefits and burdens are most prominent in the setting of prevention or treatment of thrombosis. Finally, before starting thrombosis prophylaxis or treatment in patients with liver disease, pharmacologic (i.e. non-selective beta-blockers) or endoscopic variceal bleeding prophylaxis should be considered in case of (known) esophageal varices.

Portal vein thrombosis

To date, only one study reported the efficacy and safety of prevention of PVT with LMWH in patients with cirrhosis [95]. Since insufficient data on this topic exists, the prevention of PVT with anticoagulant therapy should, at present, only be considered in the context of clinical trials.

In general, evidence indicates that anticoagulant therapy for PVT in carefully selected patients with cirrhosis is safe and effective. However, conservative dosing, in particular in patients with advanced disease, and in those patients with risk factors for bleeding, such as renal failure and severe thrombocytopenia, may be suggested. Furthermore, certain patients may benefit more from anticoagulant treatment of PVT, such as liver transplantation candidates compared to patients not suitable for liver transplantation and with an otherwise poor prognosis [88]. In addition, patients being considered for anticoagulation should undergo evaluation for esophageal varices before initiation of anticoagulation. At present, LMWH may be the anticoagulant drug of choice, since the available clinical data suggest LMWH to be safe and effective in patients with liver disease. VKAs are discouraged given the high rates of bleeding and the lack of suitable target INR. In the future, the new oral anticoagulants may be applicable in the treatment of PVT, however clinical data on this are currently lacking.

Venous thrombosis

Thrombosis prophylaxis should not be withheld in patients with a liver disease when risk factors for thrombotic complications are present, even in the presence of abnormal routine tests of hemostasis. Risk factors include hospitalization and immobilization, surgery, and the presence of (hepatocellular) cancer. Dosing regimens may be altered in patients with risk factors for bleeding, such as renal failure or profound thrombocytopenia. Mechanical thromboprophylaxis should be considered in those patients with clear contraindications for anticoagulant treatment. For the same reasons as for treatment of PVT, in prevention of venous thrombosis LMWH is also currently the drug of choice.

While studies on prevention of venous thrombosis and prevention or treatment of PVT exist, no current study examined the efficacy or safety of anticoagulants for the treatment of venous thrombosis in cirrhotic patients. Clinicians are, therefore, left to extrapolate therapeutic choice and regimen for venous thrombosis in cirrhosis patients from trials and guidelines in medical patients [97]. This would imply that treatment of venous thrombosis should be initiated with LMWH, and may be continued with VKAs or new-generation oral anticoagulant drugs. However, the use of VKAs should be performed with great care, since dosing regimens are unclear. Furthermore, the use of new oral anticoagulant drugs has not been studied in clinical trials in patients with cirrhosis. As mentioned before, due to the unpredictable effect of Dabigatran observed in chapter 8, the use of direct factor Xa inhibitors may be more promising in patients with cirrhosis. It may, therefore, be suggested to use either prolonged LMWH administration or direct factor Xa inhibitors (with initial LMWH for Edoxaban) in the treatment of venous thrombosis in patients with liver disease. Future studies on the efficacy and safety of these anticoagulant drugs for treatment of venous thrombosis in patients with cirrhosis are urgently needed.

Antithrombotic therapy to reduce progression of liver disease

Increasing evidence from animal models of liver disease suggests that antithrombotic therapy reduces progression of liver disease [112-119]. Furthermore, a single randomized clinical trial showed that LMWH therapy delays decompensation in patients with cirrhosis [95]. There may be two hypotheses that could explain the involvement of the coagulation cascade in the rate of liver fibrogenesis: the formation of thrombi resulting in tissue ischemia (also referred to as parenchymal extinction) and the activation of disease-promoting cells by coagulation proteases [120]. If antithrombotic therapy truly prevents progression of liver disease and substantially delays decompensation, this will profoundly impact the clinical management of patients with (early) cirrhosis. However, future clinical studies on the benefits and safety of antithrombotic treatment in different patient groups are required before this may be considered in clinical practice.

Monitoring of anticoagulant drugs

Due to the extensive hemostatic changes in patients with cirrhosis, careful monitoring of anticoagulant therapy may be required. For monitoring of the different heparins in the general population the anti-Xa assay and/or the APTT (for unfractionated heparin) can be used. However, recent data suggest that plasma levels of LMWH are substantially underestimated by the anti-Xa assay in patients with cirrhosis [104,107,121]. This is most probably related to the reduced antithrombin levels in these patients. Indeed, in chapter 9 we show that the anti-Xa assay not only underestimates the LMWH mass, but the test also underestimates the plasma levels of other antithrombin-dependent drugs (i.e. unfractionated heparin and fondaparinux) in plasma from patients with cirrhosis. Furthermore, when exogenous antithrombin was added to the anti-Xa assay, the reduced recovery of LMWH in patients compared to controls was fully blunted.Based on these data, we strongly suggest that anti-Xa levels should not be relied upon for monitoring heparins (that exert their effect through antithrombin) in patients with cirrhosis, unless exogenous antithrombin is added to the test. However, this modification of the test is not readily available in many routine diagnostic laboratories. Since the use of LMWH appears safe in patients with cirrhosis, monitoring of LMWH should maybe not be performed at all, except in certain situations, for example in patients with severe renal failure in which the anti-Xa with the addition of exogenous antithrombin may be used. In contrast to the underestimation of levels of heparins with the anti-Xa assay, we show in chapter 9 that the APTT overestimates levels of unfractionated heparin in plasma from patients with cirrhosisThis test, therefore, also seems unsuitable for heparin monitoring in patients with cirrhosis. Since the different anticoagulant drugs may also have an altered anticoagulant potency in patients with cirrhosis (as outlined before), the ideal monitoring test for patients with cirrhosis should take both drug levels and drug effect into account.

Monitoring of the new oral anticoagulant drugs may be required in patients with liver disease, since these drugs are cleared by the liver and kidneys and the anticoagulant potency of these drugs may be altered. The direct factor Xa and thrombin inhibitors may be monitored through the respective anti-Xa and anti-IIa assays, as in chapter 9 comparable anti-Xa and anti-IIa levels were observed after the addition of Rivaroxaban and Dabigatran, respectively, in plasma from patients and controls. For monitoring of Dabigatran the thrombin time (TT), ecarin clotting time (ECT), or APTT may also be used [122]. However, the TT and ECT are not widely available, and the APTT is mostly already prolonged in patients with cirrhosis.

Future perspectives

The results of this thesis have contributed to our understanding of the hemostatic system and the prevention or treatment of thrombosis in patients with liver disease. However, as mentioned throughout this last chapter, there is still a lot of research to be done in order to provide better strategies to deal with hemostatic abnormalities in patients with liver disease. Therefore, future studies should include (but should not be restricted to) the following topics:

- Prospective studies to assess the clinical value of promising laboratory tests (thrombin generation testing, TEG, or Thromboelastography) in the prediction of bleeding or thrombosis risk in patients with liver disease.

- Randomized controlled studies assessing efficacy and safety of restrictive transfusion strategies during invasive procedures in patients with liver disease.

- Randomized controlled studies on reduction of bleeding complications during invasive procedures in patients with small volume prohemostatics, such as prothrombin complex concentrate.

- Randomized studies on efficacy and safety of prophylaxis or treatment of venous thrombosis and portal vein thrombosis in patients with liver disease, specifically with the use of LMWH or new-generation oral anticoagulants.

- Prospective studies on anticoagulation in patients with NAFLD with thrombotic disease.

- Studies on the use of TEG or thrombin generation to monitor anticoagulant therapy in patients with chronic liver disease.

- Finally, prospective clinical studies on the benefits and safety of antithrombotic therapy to slow down progression of liver disease.

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Nederlandse samenvatting

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Hemostase

Hemostase is het proces wat ervoor zorgt dat een beschadiging aan een bloedvat wordt hersteld om zo overtollig bloedverlies te voorkomen. In normale omstandigheden is het hemostatische proces strikt gereguleerd. Een verstoring in de hemostatische balans kan aanleiding geven tot een bloeding of trombose.

Wanneer een bloedvaatwand beschadigd raakt, ontstaat er vasoconstrictie: samentrekking van het bloedvat om de bloeddoorstroming te verminderen. Vervolgens volgen er een aantal opeenvolgende processen die resulteren in afsluiting van het bloedvat. De volgende processen kunnen theoretisch worden onderscheiden: primaire hemostase, secundaire hemostase en fibrinolyse. In de primaire hemostase hechten bloedplaatjes aan blootgestelde eiwitten vlak onder de vaatwand, zoals collageen, en raken geactiveerd. Deze bloedplaatjes kunnen weer andere bloedplaatjes binden en vormen zo uiteindelijk een prop om het bloedvat af te sluiten. Echter voor permanente afsluiting van het bloedvat dient de plaatjesprop te worden versterkt door een fibrine netwerk. In de secundaire hemostase wordt fibrine gevormd met hulp van de stollingsfactoren. De stolling wordt in gang gezet door het vrijkomen van weefselfactor (tissue factor) na de weefselbeschadiging. Interactie met verschillende stollingsfactoren leidt uiteindelijk tot de vorming van fibrine. Daarnaast is er een actief antistollingsmechanisme, dat voorkomt dat teveel stolling optreedt. Ten slotte kan enige tijd nadat de vaatwand is hersteld, het bloedstolsel worden opgeruimd. Dit is het proces van fibrinolyse.

Hemostatische veranderingen bij leverziekte

De lever speelt een grote rol in het hemostatische systeem doordat het de meeste stollingsfactoren, antistollingsfactoren en eiwitten betrokken bij de fibrinolyse synthetiseert. Daarnaast is de lever ook betrokken bij de primaire hemostase. Acute en chronische leverziekten leiden daardoor tot grote veranderingen in het hemostatische systeem. In **hoofdstuk 2** is een literatuuroverzicht van de hemostatische afwijkingen in patiënten met leverziekte gegeven. De belangrijkste veranderingen in de primaire hemostase omvatten een verminderd aantal bloedplaatjes (trombocytopenie), verhoogde waarden van von Willebrand factor (VWF) (betrokken bij de adhesie van bloedplaatjes) en verlaagde waarden van ADAMTS13 (enzym wat VWF grootte reguleert). In de secundaire hemostase zijn de plasma spiegels van de belangrijkste stollings- en antistollingseiwitten verlaagd, behalve van het stollingseiwit VIII wat verhoogd is. Tot slot zijn de plasma spiegels van eiwitten betrokken bij de fibrinolyse veranderd.

De lever speelt een grote rol bij de synthese van stollingsfactoren. Daardoor zijn de spiegels van de stollingsfactoren V, VII, IX, X, XI en protrombine verlaagd. De plasma spiegel van factor VIII is echter vaak verhoogd. De verminderde productie van stollingsfactoren wordt echter gecompenseerd door een verminderde productie van de antistollingsfactoren, zoals proteïne C en antitrombine. Proteïne S plasma spiegels zijn ook verlaagd in patiënten met een chronische leverziekte (cirrose). Recent onderzoek heeft aangetoond dat proteïne S naast een cofactor voor geactiveerd proteine C ook een cofactor voor tissue factor pathway inhibitor (TFPI) in de remming van de stolling is. Door een directe interactie tussen proteïne S en TFPI zijn zowel erfelijke als verworven proteïne S deficiënties geassocieerd met een gelijktijdige TFPI deficiëntie. In **hoofdstuk 3** laten we echter zien dat patiënten met cirrose

een verworven proteïne S deficiëntie hebben dat niet gepaard gaat met een verlaging in TFPI plasma spiegels. Patiënten met cirrose hadden vergelijkbare TFPI spiegels als gezonde controles. Een verklaring voor de normale TFPI spiegels kan zijn dat in patiënten met cirrose de TFPI afgifte is verhoogd door continue activatie van het endotheel, maar dat dit (deels) is gemaskeerd door de proteïne S deficiëntie. TFPI wordt inderdaad primair geproduceerd in het endotheel. Ondanks normale TFPI plasma spiegels, laten we in hoofdstuk 3 zien dat de activiteit van het TFPI/proteïne S systeem is verminderd in patiënten met cirrose in vergelijking met gezonde controles. De verminderde activiteit van het TFPI/proteïne S systeem in de remming van de stolling kan mogelijk bijdragen aan het verhoogde risico op trombotische complicaties in patiënten met cirrose.

Chronische leverziekten werden vroeger beschouwd als het prototype van een verworven coagulopathie met een verhoogde bloedingsneiging. Conventionele stollingstesten, zoals de protrombine tijd (PT, of afgeleide international normalised ratio (INR)) of geactiveerde partiële tromboplastinetijd (APTT), zijn verlengd in patiënten met een leveraandoening. Deze testen meten echter alleen de afname van pro-stollingsfactoren en niet de vermindering van de antistollingsfactoren. Recente klinische en laboratorium studies pleitten voor een gebalanceerde hemostase in patiënten met een chronische leverziekte door de gelijktijdige daling in zowel pro- als antihemostatische eiwitten. Studies die gebruik maken van trombine generatie testen, een meer geavanceerde test van de secundaire hemostase, laten inderdaad zien dat in patiënten met leverziekte de trombine generatie gelijk is (of zelfs verhoogd is) aan die van gezonde controles. De 'hemostatische herbalans' in patiënten met chronische leverziekte kan echter wel snel verstoord raken en aanleiding geven tot bloedingen of trombotische complicaties. In **hoofdstuk 4** laten we zien dat de nieuwe 'Thrombodynamics assay', een globale test van de secundaire hemostase die niet trombine generatie maar vorming van een stolsel als eindpunt heeft, een vergelijkbare stolselvorming in plasma van patiënten met cirrose in vergelijking met plasma van gezonde controles meet. Toekomstig onderzoek moet uitwijzen of deze nieuwe hemostatische test ook kan worden gebruikt om een verhoogde bloedingsneiging of een verhoogd risico op trombo-embolische complicaties in patiënten te voorspellen. Momenteel is de test echter alleen geschikt voor onderzoeksdoeleinden en kan nog niet worden toegepast in de kliniek.

Hemostase na lever resectie

Men dacht vroeger dat patiënten na een leverresectie ook erg gevoelig waren voor bloedingscomplicaties, doordat de PT of INR ook verlengd is in deze patiënten. Echter, we weten nu dat deze conventionele stollingstesten niet betrouwbaar zijn in de beoordeling van de hemostatische status in deze patiënten. In **hoofdstuk 5** is daarom gekeken naar de stollingsstatus van patiënten na leverresectie. Door gebruik te maken van de trombine generatie test laten we zien dat het plasma van patiënten na leverresectie juist een verhoogde stollingsneiging heeft. Ook klinische studies laten zien dat het ontstaan van veneuze trombo-embolieën na leverresectie niet ongewoon is, het risico op trombose neemt zelfs toe met de omvang van de leverresectie.

De resultaten van hoofdstuk 5 in combinatie met de beschreven literatuur hebben belangrijke klinische implicaties in de behandeling van patiënten na leveroperaties. Allereerst dienen artsen zich bewust te zijn van de beperkingen van het gebruik van conventionele stollingstesten als leidraad voor hemostatische behandelingen. Een verlenging van de

PT/INR na leverresectie dient niet routine matig te worden gecorrigeerd door transfusie van bloedproducten. Daarnaast is trombose profylaxe geïndiceerd in patiënten na een leverresectie, ondanks dat de PT/INR suggereert dat patiënten 'auto-ontstold' zijn.

Hemostatische veranderingen in patiënten met niet-alcoholische leververvetting

In de westerse wereld is niet-alcoholische leververvetting (non-alcoholic fatty liver disease; NAFLD) de meest voorkomende oorzaak van chronische leverziekten. Het omvat een spectrum van steatose (leververvetting) tot niet-alcoholische steatohepatitis (NASH, een combinatie van vervetting en ontsteking in de lever) en uiteindelijk cirrose. Obesitas, voornamelijk abdominale obesitas, vormt de grootste risicofactor voor de ontwikkeling van NAFLD. Door de toenemende prevalentie van obesitas is er een sterke stijging in het aantal patiënten met NAFLD.

Uit recent onderzoek blijkt dat NAFLD is geassocieerd met een verhoogd risico op hart- en vaatziekten. Daarnaast lijkt er ook een verhoogd risico te zijn op veneuze trombo-embolische complicaties en vena porta trombose in patiënten met NAFLD. In **hoofdstuk 6** gaan we in op de mogelijke processen die bijdragen aan een verhoogd risico op trombose in patiënten met NAFLD. Een aantal studies suggereert een verhoogde stollingsneiging als oorzaak van het verhoogd risico op trombose in patiënten met NAFLD. In **hoofdstuk 7** onderzoeken we daarom het hemostatische systeem in patiënten met NAFLD. In dit onderzoek laten we zien dat de algehele hemostatische status vergelijkbaar is tussen patiënten met NAFLD en gezonde controles. Het plasma van patiënten met NAFLD toont echter wel enige pro-trombotische eigenschappen, zoals een geringe hypofibrinolyse en pro-trombotische structuur van fibrine stolsels. Uit de resultaten van hoofdstuk 7 kunnen we concluderen dat er waarschijnlijk een beperkte rol is van het hemostatische systeem in het verhoogd risico op trombose in patiënten met NAFLD.

Behandeling van trombose in patiënten met een chronische leverziekte

Er kunnen diverse trombotische complicaties ontstaan in patiënten met een chronische leverziekte. Vena porta trombose is bijvoorbeeld een bekende complicatie van patiënten met cirrose. Andere frequent voorkomende trombotische complicaties zijn diep veneuze trombose en longembolieën. Behandeling en preventie van trombotische complicaties is dus vaak nodig. Echter, door de beperkte klinische ervaring in patiënten met cirrose, is nog steeds niet bekend welk antistollingsmiddel het beste is voor de verschillende indicaties.

Veel gebruikte antistollingsmiddelen in de algemene populatie zijn vitamine K antagonisten, heparines en de nieuwe orale antistollingsmiddelen met een directe remming van factor X of trombine. Deze middelen hebben allemaal verschillende voor- en nadelen in patiënten met een chronische leverziekte. Heparines werken door binding aan antitrombine en versterken daarmee de remming van factor X en trombine. Drie klassen worden onderscheiden: ongefractioneerde heparine, laag moleculair gewicht heparine (LMWH) en fondaparinux. LMWHs lijken veilig en effectief in patiënten met cirrose en vena porta trombose of veneuze trombose. Echter, heparines zijn antitrombine-afhankelijke antistollingsmiddelen en antitrombine spiegels zijn vaak verlaagd in patiënten met een leverziekte. Een ander nadeel van heparines is de wijze van toediening (intraveneus voor ongefractioneerde heparine en subcutaan voor LMWH en fondaparinux), wat het langdurig gebruik kan beperken. Vitamine

K antagonisten verlagen de vitamine K afhankelijke stollingsfactoren II, VII, IX, X en de antistollingseiwitten proteïne C en S. Een groot voordeel van vitamine K antagonisten is hun orale toediening. Echter, een groot nadeel in patiënten met een leverziekte is het monitoren van het medicijn met de INR, want INR levels zijn al afwijkend in patiënten met een leverziekte. Nieuwe antistollingsmiddelen zoals de directe factor X remmers (rivaroxaban, apixaban en endoxaban) en de directe trombine remmer (dabigatran) worden steeds meer toegepast door de orale toediening en het ontbreken van de noodzaak tot monitoren van medicijn spiegels. Deze middelen worden, echter, (ten dele) door de lever of nieren geëlimineerd en worden daardoor nauwelijks gebruikt in patiënten met een leverziekte. Enkele kleine onderzoeken suggereren dat deze nieuwe middelen mogelijk effectief en veilig zijn in patiënten met cirrose, echter grote klinische trials naar de effectiviteit en veiligheid van deze middelen in patiënten met cirrose ontbreken.

Doordat er weinig onderzoek is gedaan naar de effectiviteit en veiligheid van de verschillende antistollingsmiddelen in patiënten met cirrose, is het preferente antistollingsmiddel voor de verschillende indicaties onbekend. In **hoofdstuk 8** hebben we daarom de in vitro effectiviteit van verschillende antistollingsmiddelen in patiënten met een chronische leverziekte onderzocht. In dit hoofdstuk tonen we aan dat het in vitro effect van de verschillende antistollingsmiddelen significant verschilt tussen patiënten met cirrose en gezonde controles. Dabigatran, en in mindere mate heparine en LMWH, toonden een versterkt antistollingseffect in plasma van patiënten met cirrose in vergelijking met gezonde controles. Aan de andere kant was er een verminderd effect van fondaparinux en rivaroxaban in patiënten met cirrose. Naast een veranderd effect van de middelen, kan ook de farmacokinetiek van de middelen anders zijn. De dosering van de verschillende antistollingsmiddelen in patiënten met cirrose zal dus moeten worden aangepast op basis van een veranderde farmacokinetiek en een veranderd effect. De resultaten van hoofdstuk 8 en appendix suggereren dat antistollingsbehandeling met directe factor X remmers waarschijnlijk veilig zullen zijn met het oog op risico op bloeding (op voorwaarde dat medicijn spiegels binnen de streefwaarden blijven). Toekomstige klinische trials kunnen zich daarom het beste richten op de effectiviteit en veiligheid van de directe factor X remmers in patiënten met een leverziekte.

Gezien de uitgebreide hemostatische veranderingen in patiënten met cirrose, kan monitoring van antistollingsmiddelen noodzakelijk zijn. Monitoring van heparines kan middels anti-Xa testen of voor ongefractioneerde heparine middels de APTT. In **hoofdstuk 9** laten we echter zien dat de anti-Xa testen niet betrouwbaar zijn in de monitoring van heparines in patiënten met cirrose. Er werd een substantiële afname gevonden in anti-Xa waardes gemeten na in vitro toevoeging van LMWH, ongefractioneerde heparine of fondaparinux aan plasma van patiënten met cirrose in vergelijking met gezonde controles. Deze test onderschat waarschijnlijk de plasma spiegels van heparines in patiënten met cirrose, omdat heparines antitrombine afhankelijk zijn en antitrombine spiegels verlaagd zijn in patiënten met cirrose. Wanneer we exogeen antitrombine toevoegen aan de test, zijn de anti-Xa waardes na toevoeging van de verschillende heparines inderdaad gelijk tussen patiënten en controles. Aangezien de anti-Xa test plasma spiegels van antitrombine-afhankelijke antistollingsmiddelen onderschat in patiënten met cirrose, adviseren we deze test niet te gebruiken in deze patiënten, tenzij exogeen antitrombine aan de test wordt toegevoegd. In tegenstelling tot de onderschatting van plasma spiegels met de anti-Xa test, laten we in hoofdstuk 9 zien dat de APTT plasma spiegels van ongefractioneerde heparine in plasma van

patiënten met cirrose overschat. Deze test is daarom ook niet geschikt voor de monitoring van ongefractioneerde heparine in patiënten met cirrose.

Monitoring van de nieuwe orale antistollingsmiddelen is mogelijk ook noodzakelijk in patiënten met cirrose, omdat deze middelen worden geëlimineerd door de lever en nieren en de antistollingspotentie van deze middelen veranderd kan zijn. De directe factor X remmers en directe trombine remmers kunnen echter wel betrouwbaar worden gemonitord met de respectievelijke anti-Xa en anti-IIa testen, aangezien we in hoofdstuk 9 vergelijkbare anti-Xa en anti-IIa waardes laten zien na de respectievelijke toevoeging van Rivaroxaban en Dabigatran in plasma van patiënten en controles. De ideale monitor test voor antistollingsmiddelen in patiënten met cirrose zou echter rekening moeten houden met zowel verandering in geneesmiddel spiegels als een veranderd effect.

Conclusie

Resultaten van dit proefschrift hebben bijgedragen aan de kennis omtrent de functionering van het hemostatische systeem in patiënten met een leverziekte. Daarnaast hebben we de effectiviteit en monitoring van antistollingsmiddelen in plasma van patiënten met cirrose onderzocht. De resultaten van dit proefschrift dragen bij aan een beter inzicht in strategieën om trombotische complicaties in patiënten met een leverziekte te voorkomen of te behandelen. Er is echter nog veel toekomstig onderzoek nodig om inzicht te krijgen in de beste preventie en behandeling van hemostatische veranderingen in patiënten met een leverziekte.

List of publications

1. **Potze W**, Arshad F, Adelmeijer J, Blokzijl H, van den Berg AP, Meijers JC, Porte RJ, Lisman T. Decreased tissue factor pathway inhibitor (TFPI)-dependent anticoagulant capacity in patients with cirrhosis who have decreased protein S but normal TFPI plasma levels. Br J Haematol 2013; 162(6):819-826.

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Curriculum Vitae

Nederlands

Wilma Potze werd geboren op 22 januari 1990 te Hardenberg. Zij volgde haar lagere school opleiding aan de Kardoen te Ommen en de middelbare schoolopleiding aan het Vechtdal College te Hardenberg, waar zij in 2008 haar VWO diploma behaalde. In datzelfde jaar begon zij aan de opleiding Farmacie aan de Rijksuniversiteit Groningen waar zij haar propedeuse cum laude heeft behaald. In 2009 begon Wilma aan haar opleiding Geneeskunde aan de Rijksuniversiteit Groningen. Tijdens haar opleiding werkte ze als student assistent op verscheidene afdelingen in het Universitair Medisch Centrum Groningen (UMCG), was ze actief binnen de studentenschaatsvereniging TJAS en deed ze vrijwilligerswerk bij het Rode Kruis in Groningen. Daarnaast volgde zij tijdens haar bachelor het Honours College programma waarmee zij in aanraking kwam met het doen van onderzoek. In het tweede jaar van haar opleiding deed zij een pilot project bij de afdeling Dermatologie in het UMCG. In het derde jaar van de opleiding geneeskunde begon Wilma met een pilot project bij de onderzoeksgroep van Prof. dr. Ton Lisman en Prof. dr. Robert J. Porte waar ze gefascineerd raakte van het onderzoek naar de hemostase in patiënten met een leverziekte. Na het behalen van haar bachelor diploma in 2012 (cum laude) begon zij met haar wetenschappelijke stage van de master geneeskunde. Daarna werd Wilma aangenomen voor het MD/PhD traject, waarbij de masteropleiding gecombineerd wordt met een promotieonderzoek. Na haar eerste jaar coschappen in het UMCG deed zij een fulltime jaar onderzoek, waarvan een deel in Richmond, Virginia werd uitgevoerd. Daarna volgde Wilma haar senior coschappen in Isala in Zwolle, waar ze ondertussen ook heeft mee geholpen aan de organisatie van het J.F. van Gils symposium in 2015. In datzelfde jaar won zij de GUF-100 award, een prijs toegekend aan de meest excellente student van elke faculteit aan de Rijksuniversiteit Groningen. Na haar senior coschappen deed Wilma haar semi-arts stage bij de interne geneeskunde en de spoedeisende hulp in het Wilhelmina ziekenhuis. In november 2016 behaalde zij haar master geneeskunde diploma. Een paar maanden later ronde zij haar promotieonderzoek vervroegd af, waarvan de resultaten in dit proefschrift zijn beschreven. Sinds januari 2017 is Wilma werkzaam als ANIOS op de interne geneeskunde in de Isala in Zwolle.

English

Wilma Potze was born on the 22nd of January 1990 in Hardenberg, the Netherlands. She attended the 'Kardoen' primary school in Ommen and thereafter went to high school ('Vechtdal College') where she graduated in 2008. Before she enrolled in the study of Medicine at the University of Groningen in 2009, she studied one year of Pharmaceutics at the University of Groningen (first year diploma cum laude). During her medical studies, Wilma worked as an assistant at different departments at the University Medical Center Groningen, was a member of the Promotion and Competition Committee at the student speed skating association TJAS in Groningen, and worked as a volunteer at the Rode Kruis in Groningen. Furthermore, she successfully attended the Honours College program at the University of Groningen during her Bachelor of Medicine. As part of this program she performed a part-time research project at the Department of Dermatology. In the third year of medical school Wilma started a pilot project in the research group of Prof. dr. Ton Lisman and Prof. dr. Robert J. Porte, where she developed an enthusiasm for medical research on the hemostasis in liver diseases. After finishing her Bachelor of Medicine (cum laude), she continued with her research internship of the Master of Medicine, entitled: 'Efficacy of anti-hemostatic drugs in patients with cirrhosis'. Based on this work, Wilma successfully applied for the MD/PhD program to combine her Master of Medicine with a PhD project. After a year of clinical rotations at the University Medical Center Groningen, she performed one year of fulltime research on hemostasis in (fatty) liver diseases, part of which was performed in Richmond, Virginia. During her second year of clinical rotations in Isala in Zwolle, Wilma organized the yearly J.F. van Gils symposium in 2015. Wilma graduated from medical school in November 2016. Officially she had one year of research of the MD/PhD program left, but she had enough publications to shorten the program and defend her PhD thesis in April 2017. Currently Wilma is working as a resident in Internal Medicine at the Isala in Zwolle.