Coagulation testing in atherosclerosis and liver disease

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Coagulation Testing in Atherosclerosis and Liver Disease

Marie-Claire Kleinegris

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PROEFSCHRIFT

ter verkrijging van de graad van doctor aan de Universiteit Maastricht,

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CHAPTER 1

General introduction and outline of the thesis

Interaction in haemostasis

Because of the constant interaction with various other control mechanisms regulating human homeostasis, haemostasis can and should not be seen as a separate and autonomous entity. Peripheral arterial disease and liver cirrhosis are two diseases that are often portrayed as 'self-inflicted' and therefore sadly come with a large social stigma. While these diseases may be *partly* caused by self-inflicted damage due to addiction the resulting diseases remain important public health issues, associated with great burdens of complications and related use of resources. Although of great interest, the etiologic and health impact consequences of both diseases are beyond the focus of this thesis. As haemostasis is a balanced system that can be disturbed by e.g. inflammation or bleeding both illnesses, peripheral arterial disease (PAD) and cirrhosis, affect and are possibly affected by the haemostatic system. This thesis will therefore focus on another important matter that deserves attention: the interaction between haemostasis on the one hand and these two chronic inflammatory diseases on the other hand. For a better understanding the different factors of haemostasis will be explained and summarized.

A quick take on haemostasis

Haemostasis is the systemic response that has developed in the course of evolution to protect man against lethal bleeding ensuing damage to the vascular wall. It also acts however to maintain the blood in a fluid state throughout the body when there is no damage to the vessel wall.¹ This response is a highly conserved mechanism that functions in most, if not all, mammals and is regulated through various processes. The current concept of haemostasis is that after damage of the vessel wall, there is 1. *Formation of a platelet plug* through activation, adhesion and aggregation of platelets (primary haemostasis). 2. This platelet plug is subsequently stabilized by the deposition of fibrin. Deposition of fibrin occurs upon *activation of the coagulation cascade* (secondary haemostasis). 3. Finally, to preclude (extensive) thrombus formation beyond the site of injury *fibrinolysis* takes place. These processes do not occur in separate, sequential phases, but are dynamic and highly intertwined.^{2,3} Furthermore, the quality of this haemostatic response ultimately depends on the delicate balance between the participating mechanisms.

Under normal conditions there is no adherence of platelets to the normal endothelial surface of the vascular wall. However, when the endothelium gets disrupted, platelets will start to adhere to the exposed subendothelial matrix or underlying layers of the vascular wall. Various receptors on the surface of the platelets (e.g. glycoprotein (GP) Ia/IIa and GP Ib/IX) interact with components present in the subendothelial matrix such

as adenosine diphosphate (ADP), collagen or von Willebrand Factor (vWF), thus mediating platelet adhesion. The interactions between the platelets and the vascular wall do not only result in platelet adhesion, but cause activation of the platelets as well. With the release of the platelet aggregation agents (e.g. ADP, fibrinogen, serotonin, vWF and collagen) the last step of the platelet plug formation has started: the platelets rapidly start to aggregate covering the damaged vascular wall.⁴

The coagulation cascade

There are two separate pathways that can be activated in order to start the coagulation cascade; these pathways merge halfway to activate factor (F) X, in the so-called common pathway (Figure 1.1). *The extrinsic pathway* starts when tissue factor (TF) is exposed at the site of injury. Cellular tissue factor activates the conversion of factor (F) VII into FVIIa. The TF-FVIIa complex thereupon activates FIX and FX leading to the formation of the initial amount of thrombin (known as the *initiation phase of thrombin formation*). Thrombin then activates FXI to FXIa via a feedback loop and subsequently FIX is activated which further amplifies the intrinsic pathway. Thrombin activates co-factors V and VIII as well, which leads to the bulk of thrombin generation, also known as the *propagation phase of thrombin formation*. This bulk of thrombin then cleaves the plasma protein fibrinogen into fibrin after which crosslinking of the fibrin strands occurs through FXIIIa (also activated by thrombin) promoting the formation of a stable clot. *The intrinsic pathway* becomes activated when there is a formation of a primary complex that comes into contact with specific negatively charged surfaces. Although the physiological source has been obscure for long, recent studies suggest that polyphosphates from platelets and other sources may play a role in activating the intrinsic route. Various proteins in the plasma, such as FXII, prekallikrein and high molecular weight kininogen are activated upon contact with such negatively charged molecules and potentially amplify the activation of FXI. This activation further activates FIX, which in complex with FVIIIa, forms the so-called intrinsic X-ase complex that leads to the formation of FXa. With the formation of Xa the common pathway has started.¹

Several mechanisms are in place to prevent excess fibrin formation. Tissue factor pathway inhibitor (TFPI) limits TF-FVIIa-mediated FXa-formation; thrombin itself serves as an anti-coagulant by binding thrombomodulin (TM); assembly of the thrombin-TM complex facilitates the conversion of protein C to activated protein C (APC) and as APC normally inactivates FVa and FVIIIa proteolytically which limits further thrombin generation. Furthermore, free thrombin is directly quenched by α 2-macroglobuline and as well by the serine protease inhibitor antithrombin (AT) forming a thrombin-antithrombin complex (TAT). To prevent excess clot formation the fibrinolytic system is in place as well.

Figure 1.1 a. The coagulation cascade.

b. Fibrinolysis.

Abbreviations: Prot, Protein; AT, antithrombin; TF, Tissue Factor; TFPI, Tissue Factor Pathway Inhibitor; α2M, alpha-2-macroglobulin; uPA, urokinase-like Plasminogen Activator; tPA, tissue Plasminogen Activator; PAI-1, Plasminogen Activator Inhibitor-1; TAFI, Thrombin-Activatable Fibrinolysis Inhibitor

Fibrinolysis

The overall physiologic effect of the fibrinolytic system is to facilitate lysis at the sites adjacent to the injury in order to prevent excess clot formation that would obstruct the blood flow. The fibrinolytic system is also responsible for clearance of the clot once the

endothelial damage is repaired. When endothelial cells are stimulated by either thrombin, fibrin, an altered flow or absence of the flow due to the thrombus, they start to secrete plasminogen activators. Tissue plasminogen activator (tPA) converts plasminogen, present in the thrombus, into plasmin that starts lysing the cross-linked fibrin. This forms fibrin breakdown products of which D-dimer is probably the best-known example (Figure 1.1). Fibrinolytic inhibitors in place to attenuate excess fibrinolysis are α_2 -antiplasmin and plasminogen activator inhibitor-1 (PAI-1). Cells able to produce PAI-1 are endothelial cells, platelets, smooth muscle cells, hepatocytes but also adipose tissue. Platelets for example release PAI-1 from their storage granules directly upon platelet aggregation, this to limit the fibrinolytic activity at the site of vascular damage and thereby allowing the stabilization of the thrombus.⁵

Based on the above-described haemostatic response, it can be concluded that three components are essential for thrombus formation: 1st an injured vessel wall 2nd a change in the constituents of the blood leading to hypercoagulability and 3rd alterations in the blood flow. These three components are also known as the triad of Virchow. Virchow's triad was initially proposed for venous thrombosis, however nowadays it seems that these same principles can be applied to other forms of thrombosis such as arterial thrombosis and disseminated intravascular coagulation (DIC).^{6,7} In this thesis we will, in the context of peripheral arterial disease and cirrhosis, examine two components of Virchow's triad: the injured vessel wall and the hypercoagulable state but also the interaction between these two elements. PAD and cirrhosis, both chronic inflammatory diseases, were chosen as being of interest as PAD has been well documented to strongly affect the vessel wall and the liver plays a major role in the synthesis of coagulation factors.

Peripheral arterial disease

Peripheral arterial disease (PAD) is one of the common manifestations of atherosclerotic vascular disease and results from progressive narrowing and/or occlusion of the major arteries to the lower limbs. It progresses locally, often without causing critical clinical manifestations, and can thus extend gradually over time.^{8,9} The earliest and most frequent presenting symptom of PAD is intermittent claudication, which is defined as pain in the legs with exertion that subsides with rest.¹⁰ Many patients however are asymptomatic, and although the diagnosis of symptomless PAD is of lesser significance with respect to the lower extremities, it still is a strong marker for future vascular events.⁹ The most widely used test to diagnose PAD is the ankle-brachial systolic pressure index (ABI) in which a resting ABI ≤ 0.9 is caused by a dynamically-significant arterial stenosis and therefore worldwide accepted as the cut-off value for PAD.^{11,12}

Hypoperfusion of the tissues progresses in the late stages of PAD to critical ischaemic ulceration, gangrene and in some cases therefore major amputation.¹⁰ Overshadowing the risk of limb loss however is the risk of cardio- and cerebro-vascular events and death.¹³ The survival of patients with PAD, both symptomatic and asymptomatic, is significantly reduced due to atherosclerotic complications in the cerebro- and cardiovascular beds. Within 5 years after the diagnosis of PAD approximately 20% of the patients experience a non-fatal cardiovascular event (myocardial infarction or stroke) and 10-15% of PAD-patients have died within that timeframe.¹¹

PAD is a growing clinical problem due to the aging population and the fact that the prevalence of PAD increases progressively with age.^{14,15} In 2007 it was estimated that in Europe and North America 27 million individuals were affected by PAD leading, yearly, to 413.000 hospital admissions.¹⁶

Various risk factors have been identified for PAD; these risk factors are nearly identical to the ones that have been established for atherosclerosis in general. Despite available data suggesting a moderate degree of heritability in PAD, a consistent genetic marker for PAD has yet to be identified.¹⁷ Risk factors as age, male sex, diabetes and smoking are however closely, and independently, linked to PAD.^{9,15,18,19} Additionally, hypertension, dyslipidaemia and non-white ethnicity have been described as well in increasing the PAD-risk.²⁰⁻²³

In the last years, in addition to the traditional risk factors, other possible mediators for PAD have been studied as well, including haemostasis related factors. In clinical research a lot of attention has gone out to “thrombophilia” such as activated protein C resistance (mostly related to Factor-V Leiden) and the prothrombin G20210A polymorphism.^{12,24-27} However, because of the small number of studies (and their small study populations on top of several methodological limitations) controversy regarding the role of these genetic thrombophilic factors in PAD remains. Despite the uncertain association between genetically determined hypercoagulability and PAD, other thrombogenic risk factors have been more consistently linked to the pathogenesis of PAD. Due to the advances in our knowledge of vascular biology a number of biomarkers that represent haemostasis and (since inflammation is closely linked to coagulation) activated inflammation have been proposed as novel risk factors for PAD.

The circulating markers of endothelial damage and/or dysfunction vWF and soluble tissue factor are both elevated in PAD and associated with the severity of the disease. For vWF it has even been established that it could be a marker for disease progression, for TF however this still needs to be elucidated.²⁸⁻³⁰ Soluble thrombomodulin has until now not been conclusively associated with PAD: various studies show that increased thrombomodulin levels correlate with the severity of PAD, illustrating that with increasing endothelial injury due to PAD probably more thrombomodulin is released. Other studies however fail to show this association.³¹⁻³⁴ The cellular adhesion molecules ICAM-1 and VCAM-1 (intracellular adhesion molecule 1 and vascular adhesion molecule-1, respectively), which facilitate platelet and leucocyte adhesion to the

endothelium are both elevated in PAD, but their prognostic value has so far not been established.^{35,36} Manifold blood constituents have been involved in the development, existence and progression of PAD. Platelet and soluble P-selectin levels for example independently predict disease severity in PAD.^{22,37} Fibrinogen is not only an important component of the coagulation system; it plays a major role in inflammation as well by promoting a chemotactic response via increased adherence of leukocytes to the endothelium.³⁸ Increased fibrinogen levels in PAD are partly due to e.g. smoking, diabetes and increased LDL cholesterol levels, however hyperfibrinogenaemia is also known as an independent risk factor in the prevalence, the disease severity and the prognosis of PAD. The mechanisms behind this association still have to be clarified though.^{22,38-40} The fibrinolytic system proved also to be affected as both tPA and PAI-1 have been associated with the prevalence and severity of PAD.^{40,41} Fibrin D-dimer, the breakdown product of cross-linked fibrin and thus a specific marker of fibrinolysis, is also a specific marker of the severity of a hypercoagulable state.⁴² High D-dimer levels have been associated with PAD-severity, concerning the progression of disease and occurrence of atherothrombotic events some ambiguity still exists.^{43,44}

Based on the aforementioned studies, there seems to be quite some evidence that abnormalities in the two selected components of Virchow's triad (the altered vessel wall and blood constituents) are related to the severity and prognosis of PAD. It remains uncertain if these haemostatic changes in PAD are a cause or a consequence of the vascular disease. It has been proposed that a hypercoagulable state in PAD-patients could predict future atherothrombotic events, even when this hypercoagulability is secondary to the atherosclerotic process. In order to provide more knowledge and thus answers for future management of this debilitating disease more research on this topic is desired.

Haemostasis in cirrhosis

Under physiological conditions the liver functions as a very inconspicuous organ with its many different functions interrelating with one another. When liver disease occurs these functions are often disturbed simultaneously, creating large problems. There are various causes of liver disease; common causes include alcoholic liver disease, chronic viral hepatitis, haemochromatosis and the increasingly prevalent non-alcoholic fatty liver disease (NAFLD). It has been suggested that, in most patients, already approximately 80-90% of the liver parenchyma is destroyed before liver disease manifests clinically.⁴⁵ Cirrhosis, complicated by portal hypertension, is the, generally considered, irreversible end stage of progressive hepatic fibrosis. The life expectancy of patients with cirrhosis is markedly reduced and in the last phase of the process, the only remaining treatment option may be liver transplantation.⁴⁶ The liver plays a major

role in haemostasis through various mechanisms: 1st The hepatic parenchymal cells synthesize the majority of the coagulation factors and proteins required for fibrinolysis. 2nd The reticuloendothelial system of the liver helps in regulating coagulation and fibrinolysis by clearing the excess products of haemostatic activation from the circulation. 3rd As the liver is a highly vascularized and expandable organ with the vital venous systems draining through the liver parenchyma, it has a major impact on systemic blood flow.

In the majority of patients with severe liver diseases, both quantitative as well as qualitative haemostatic abnormalities are detected (Table 1.1).⁴⁷ The magnitude of these abnormalities is mainly dependent on the severity of the liver failure and possibly due to the aetiology of the liver disease as well.⁴⁸ Furthermore, the haemostatic balance can be influenced by the existence of concomitant (coagulation) disorders or renal failure. Despite extensive research in the field of cirrhosis and coagulation, the pathogenesis of haemostatic disorders has not been fully elucidated. Due to the observation that patients with cirrhosis had a decrease in the majority of the levels of procoagulant factors combined with thrombocytopenia cirrhosis was long considered as “a prototype of the acquired coagulopathies” and thus precipitating to an increased bleeding risk.^{49,50} Bleeding episodes do occur in patients with cirrhosis and can be classified as *spontaneous* or *procedure-related*. Spontaneous bleeding includes variceal, non-variceal gastrointestinal bleeding and cerebral haemorrhage. Procedure related bleedings comprise bleeding associated with biopsies, paracentesis or even, albeit rare, central venous cannulation.^{51,52} Based on this bleeding risk, patients with cirrhosis were and still are today, treated with plasma or procoagulant agents to prevent haemorrhage by correcting the haemostatic abnormalities prior to invasive procedures or during surgery.⁴⁹

The well-accepted belief of a haemostasis related bleeding tendency in cirrhosis was challenged less than a decade ago when it was shown that liver disease patients were able to generate as much thrombin as healthy subjects and are thus not unquestionably “auto-anticoagulated”.^{50,53} More recently, it has been demonstrated that patients with cirrhosis even show a procoagulant imbalance in comparison to controls when the thrombin generation measurement is performed with the addition of thrombomodulin to fully activate the anticoagulant pathway.⁵⁴

Further supporting this was the fact that patients with cirrhosis indeed show an increased thrombosis risk on top of an increased bleeding tendency. Portal vein thrombosis has an incidence of 7.4-11% in cirrhotic patients and their relative risk of a venous thromboembolism is 1.65-1.74 when compared to controls.^{55,56} In a recent prospective study on decompensated cirrhotic patients it was found that 7% of patients presented with a deep venous thrombosis during a 6-month period.⁵¹ Based on laboratory data and clinical observations it was suggested that in patients with cirrhosis

compensatory mechanisms were at work causing a state where prothrombotic and bleeding enhancing factors are equally induced, compromising a delicate equilibrium.⁵⁷

Table 1.1 Qualitative and quantitative haemostatic changes occurring in patients with cirrhosis.

Pro-haemostatic changes	Anti-haemostatic changes
Increased platelet aggregation due to ^{58,59} : - Increased levels of unconjugated bilirubin stimulating aggregation. - Ascitic fluid cause irreversible platelet aggregation.	Thrombocytopenia due to ⁶⁰⁻⁶¹ : - Decreased thrombopoietin production - Myelosuppression - Splenic platelet sequestration - Increased platelet consumption due to low grade DIC
Upregulated vWF on the endothelium due to chronic inflammatory state ⁶⁴ .	Qualitative platelet defects, such as ^{47,62,63} : - Acquired storage pool defects - Decreased thromboxane A ₂ synthesis - Altered transmembrane signal transduction
Decreased ADAMTS13 ⁶⁵	Decreased <u>pro</u> -coagulant factors ^{54,66} : - FII, FV, FVII, FIX, FX, FXI - Fibrinogen
Decreased <u>anti</u> -coagulant factors ⁵⁴ : - Antithrombin - Protein C and S	Vitamin K-deficiency due to poor nutrition or malabsorption caused by biliary obstruction or decreased bile production ⁶⁷ .
Increased production of procoagulant FVIII as “acute phase reactant” by the endothelium[54, 69].	
Increased endogenous heparinoids: heparin sulphate and dermatan sulphate	Decreased TAFI-levels ⁶⁸
Decreased plasminogen ⁶⁹	Decreased plasmin inhibitor ⁶⁹
Increased PAI-1 ⁶⁹	Increased tPA ⁶⁹

Abbreviations: DIC, Disseminated Intravascular Coagulation; vWF, von Willebrand factor; TAFI, Thrombin Activatable Fibrinolysis Inhibitor, PAI-1, Plasminogen Activator Inhibitor; tPA, tissue Plasminogen Activator.

To assess the haemostatic state in patients with cirrhosis, conventional laboratory tests prothrombin time (PT) and activated Partial Thromboplastin Time (aPTT) are still frequently used in the clinic. The PT and aPTT are only capable of measuring the amount of thrombin generated in plasma as a function of the procoagulant driver, but cannot take the anticoagulant forces into account. Therefore, these tests are unable to represent the coagulation balance in vivo and thus are inadequate to assess the haemorrhagic and thrombotic risk in cirrhosis.⁷⁰ With the introduction of the thrombin-generation tests it was possible to mimic the haemostatic conditions in plasma more closely to those in the vivo situation. Several studies so far have used this test, albeit with different test conditions, and found that indeed patients with cirrhosis show comparable thrombin generation potential as compared to controls.^{53,71,72} As the majority of the thrombin-generation tests still use platelet-free plasma and the amount of added thrombomodulin is chosen quite arbitrary they remain somewhat limited in probing the full coagulation process.[50] As the actual haemostatic and coagulation changes in cirrhosis are still not adequately reflected by the conventional tests the addition of whole blood testing to the haemostatic test panel would be of utmost importance.

Outline of this thesis

This thesis describes how two, globally prevalent, illnesses with major health care burden, PAD and cirrhosis of the liver, alter the haemostatic profile of affected patients. In **Part I** the emphasis of our research lies primarily on the interaction between the damaged vessel wall (atherosclerosis) and the coagulation system. **Chapter 2** starts with an overview on recent advances in research on the interaction between coagulation and the vessel wall in thrombosis and atherosclerosis. This narrative review discusses what the consequences of such interactions for both venous and arterial thrombosis are. **Chapter 3** gives a systemic evaluation of platelet reactivity in whole blood from patients with PAD using two high-throughput assays; flow cytometry and testing of thrombus formation on spotted microarrays. Furthermore, in **chapter 4**, we assess the differences in the haemostatic profile between patients with PAD and matched healthy controls by studying the coagulation-, clot formation- and fibrinolysis-status in a nested case control study. This is performed through the measurement of levels of various haemostatic markers and the plasmatic thrombin generation potential with the thrombin generation assay (TGA) combined with a modified tissue factor-triggered rotational thromboelastometry in whole blood. In **chapter 5** we further expatiate on the value of haemostatic changes in PAD with a systematic review and meta-analysis of the use of D-dimer as a predictive marker for cardiovascular and arterial thrombotic events in patients with PAD. In **Chapter 6** we prospectively evaluate the relationship between various coagulation parameters (D-dimer, vWF, fibrinogen and the thrombin generation assay) and the occurrence of cardiovascular events in 280 patients with PAD ourselves.

In the past two decades there have been tremendous advances in the understanding of the haemostatic abnormalities in patients with cirrhosis. However these data are not yet conclusive and it is becoming more apparent that the currently used coagulation tests are not able to assess the coagulation status of cirrhotics correctly. New tests that evaluate haemostatic alterations more accurately than now are needed to redefine the role of these alterations in the clinical bleeding- and thrombosis tendency of these patients. In Part II we therefore look deeper into the haemostatic problems that occur when the capacity of the liver to synthesize coagulation factors decreases due to end-stage liver disease with a set of newly developed, overall coagulation tests. It starts with **chapter 7** in which we analyse the problems caused by a deteriorating synthesis of coagulation factors by the liver in 73 patients with end-stage liver disease in an observational cohort study. Their coagulation profiles are assessed with the thrombin generation assay combined with assays for quantifying FVIIa and FIXa-AT, FXa-AT complexes and the modified tissue factor-triggered rotational thromboelastometry to which tPA was added in order to assess the fibrinolytic capacity in cirrhosis. In **chapter 8** we further investigate the prothrombin conversion and thrombin inactivation

curves from cirrhotics by an approach based on computational modelling of thrombin inactivation. **Chapter 9** describes our prospective study set up to investigate the value of classic haemostatic parameters including D-dimer and vWF, and overall coagulation assays such as plasma thrombin generation and whole blood clot formation in predicting coagulation related events and liver disease related mortality in patients with cirrhosis. In **chapter 10**, we discuss the key findings from the aforementioned studies as well as their possible implications for clinical practice and for possible future research in this area.

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PART I

**Interaction between the damaged vessel
wall and the coagulation system**

CHAPTER 2

Coagulation and the vessel wall in thrombosis and atherosclerosis

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Abstract

The blood coagulation system is a key survival mechanism that has developed to protect man against lethal bleeding. A second function of blood coagulation is its close interaction with immunity. The immune mediated coagulation responses may broadly be regarded as an element of response to injury. Pathological coagulation responses, including thromboembolism and disseminated intravascular coagulation (DIC), could therefore be regarded as excessive immune responses to a vessel wall injury. Virchow's triad, which comprises changes in the components of the blood, the state of the vessel wall and the blood flow, was originally proposed for venous thrombosis. However, lately it appears that the same principles can be applied to arterial thrombosis and even DIC. It has even been postulated that all forms of thrombosis may be part of a continuous spectrum of the same disease. Over the past few years an accumulation of evidence shows that the etiopathogenetic mechanisms behind venous and arterial thrombosis are quite similar. The traditional elements of Virchow's triad are found to apply to both arterial and venous thrombosis. Yet nowadays more emphasis is placed on the vessel wall and vascular bed specificity and the interaction with inflammation and hypercoagulability. This narrative review will discuss recent advances in research on the possible interactions between coagulation, the vascular endothelium, and atherosclerosis as well as the consequences of such interactions for venous and arterial thrombosis.

Introduction

The blood coagulation system is a key survival mechanism that has developed to protect man against lethal bleeding. A second function of blood coagulation, that has only in recent years received more attention and recognition, is its close interaction with immunity. The immune mediated coagulation responses may broadly be regarded as an element of response to injury. Along this line, pathological coagulation responses, including thromboembolism and disseminated intravascular coagulation (DIC), can be regarded as excessive immune responses to a vessel wall injury. Classically, three distinct forms of thrombosis are known, venous, arterial and the mixed, microvascular form (DIC). Thrombosis can be local in origin, such as in deep vein thrombosis (DVT) of the leg, or in cerebral venous sinus thrombosis but can likewise manifest itself systemically, such as with DIC affecting primarily the microvasculature. On top of this a focal occurrence may also cause a more distant pathology as manifested by pulmonary embolisms.

The triad predisposing to thrombus formation postulated by Virchow comprises changes in the components of the blood, the state of the vessel wall and the blood flow. It was originally proposed for venous thrombosis. However, lately it appears that the same principles can be applied to arterial thrombosis and even DIC. It has even been postulated that all forms of thrombosis may be part of a continuous spectrum of the same disease.¹ A more contemporary triad conserves the identical components, but these are much better characterized than in Virchow's days. Abnormal blood constituents are represented by abnormalities in platelet function, coagulation-, or fibrinolysis factors, and are influenced by metabolic, hormonal and inflammatory elements. Vessel wall changes are nowadays also better characterized with most arterial thrombosis developing on underlying atherosclerotic pathology. In the atherosclerotic artery, changes in hemorheology and/or turbulence at bifurcations and stenotic regions enable the formation of local arterial thrombosis.²⁻⁴ In contrast; in venous thrombosis one of the primary events is impaired oxygenation of the venous endothelium evoked by impaired venous flow, triggering inflammation.

In all forms of thrombosis, coagulation and inflammation are two principal pathways that act hand in hand in a "response to injury scenario". In spite of many differences in the contributing factors, the main interface appears to be the vascular endothelium, serving as the critical regulator of flow, hypercoagulability and thrombosis. The endothelial involvement depends in its responses on the dynamic interplay between the vessel wall and the blood compartment. The coagulation system plays an important role in maintaining endothelial integrity, as well as in initiating or accelerating pathophysiology leading to thrombosis. In addition, the endothelium is not a single entity but is highly heterogeneous in its phenotype and behaviour, which may be relevant to the localization of thrombosis. In atherosclerosis, coagulation proteases

play a major role, at least in experimental conditions, while the clinical relevance remains to be demonstrated.

Several pathophysiological conditions can alter one or more of the components of Virchow's triad leading to an activation of coagulation with in general, involvement of all three components being required to generate thrombosis. In venous and arterial thrombosis several risk factors are shared, including obesity, diabetes mellitus, age and hypertension, also suggesting a link between venous and arterial thrombosis.⁵⁻⁸ Indeed, recent studies show that patients with idiopathic venous thrombotic disorders are at a higher risk of developing arterial thrombotic complications than matched controls.⁹ Both with regard to atherosclerosis and thrombosis, the type and duration of anticoagulation may have clinical consequences that need to be addressed.

This narrative review will discuss recent advances in research on the possible interactions between coagulation, the vascular endothelium, and atherosclerosis as well as the consequences of such interactions for venous and arterial thrombosis.

The mechanisms leading to thrombin generation

Haemostasis is a highly conserved mechanism throughout species, aimed at protecting against lethal bleeding. In all mammals blood coagulation involves both a cellular and a protein component. The coagulation process is a dynamic, highly interwoven array of multiple processes. In the context of this review we will only address elements of the coagulation cascade that lead to generation of thrombin, along the lines discussed in our review paper on coagulation and atherosclerosis.¹⁰ Therefore we will not focus on the important role of platelets in complex processes like atherosclerosis, for this we refer the reader to other reviews on these topics.

Coagulation starts with the exposure of tissue factor (TF), followed by a stepwise activation of coagulation zymogens and culminating in the generation of thrombin. Thrombin, a key regulator of coagulation, then converts fibrinogen into fibrin.¹⁰ Cellular TF binds small amounts of FVII present in the circulation, but also activates the conversion of FVII into FVIIa. The TF-FVIIa complex in turn activates FIX and FX leading to the formation of a small amount of thrombin. The thrombin feedback loop results in the activation of FXI into XIa that additionally activates FIX leading to further amplification of the intrinsic cascade. In this process, thrombin activates the cofactors V, VIII and the FXIII zymogen, which leads to enhanced thrombin and fibrin formation (FXIIIa crosslinking fibrin monomers to form a polymerized clot). The end result of all these sequential reactions is the acceleration of fibrin formation at a specific time and place when this is required (e.g. bleeding).¹⁰

Excess fibrin formation is limited by several anticoagulant mechanisms. These include TF pathway inhibitor (TFPI), which limits TF-FVIIa mediated FXa formation, in a protein S dependent manner (at low TF concentrations).¹¹ In addition, thrombin serves an

anticoagulant role in binding the cell surface receptor thrombomodulin (TM), which complex converts protein C into activated protein C (APC). APC proteolytically inactivates the activated FV and FVIII, reducing the rate of thrombin generation. Additionally, free thrombin can be quenched by the serine protease inhibitor antithrombin (AT) into a thrombin-antithrombin complex (TAT).

Under physiological conditions a basal level of activated coagulation is maintained in a TF dependent manner.¹² This low level activity provides a flexible system that can rapidly respond to injury. Vascular endothelial disruption triggers the coagulation cascade, but also the fibrinolytic pathway. This pathway is initiated by endothelial cell derived tissue plasminogen activator (tPA), which mediates the conversion of plasminogen into plasmin. Plasmin will then degrade fibrinogen and fibrin, thereby limiting the size of the formed clot and furthermore clearing the clot once the endothelial damage has been repaired. Other proteins such as $\alpha 2$ -antiplasmin and plasminogen activator inhibitor-1 (PAI-1) inhibit the fibrinolytic pathway. Fibrinolysis is also controlled by thrombin-TM activatable fibrinolysis inhibitor (TAFI), which removes C-terminal residues from fibrin, which are important for the binding and activation of plasminogen. This way both coagulation and fibrinolysis are regulated in a fine-tuned, complex manner (Figure 2.1).

The vessel wall

The vascular endothelium can be regarded as a mediator between the components of Virchow's triad, for both anatomical and functional reasons.¹³ Recently, endothelial dysfunction has emerged as the most important constituent of Virchow's contemporary triad due to its ability to strongly influence the other constituents of haemostasis. Furthermore it also affects its natural sequels, inflammation and tissue repair.^{2,13}

Endothelial cells are located at the demarcation between tissue and blood and therefore are crucial for the protection against vascular injury and the maintenance of blood fluidity.¹⁴ Endothelium across the vascular bed does not consist of a collective of identical cells, but should be regarded as a heterogeneous conglomerate of cells with diverse structure and function.¹⁵ Adding to this, endothelium possesses a structure and function that can alter in space and time, under the influence of sickness and health. It is therefore important to distinguish normal endothelium from disturbed endothelium.^{16,17} The endothelium, under normal circumstances, supports vasodilatation, inhibits adhesion and activation of blood platelets, quenches the coagulation cascade while amplifying fibrinolysis and antagonizing inflammatory processes and is consequently considered to be antithrombotic and anti-inflammatory. The endothelium can be regarded to be as important for haemostasis as the liver. However, while the liver constantly synthesizes coagulation zymogens, the pro-and anticoagulants produced by the endothelium are both *regulated* and *site-specific*.¹⁵

Figure 2.1 Coagulation cascade and the fibrinolytic process: Tissue factor pathway inhibitor (TFPI), Antithrombin (AT), Heparin cofactor II (HCII).

Four mechanisms have been described that contribute to the inhibition of fibrin deposition on a normal endothelium¹³: 1. The glycocalyx produces heparan- and dermatansulfate molecules that activate heparin cofactor II and antithrombin. 2. The expression of TFPI limits the activity of the extrinsic pathway. 3. The activation of the

protein C/protein S system down regulates the intrinsic coagulation route; the endothelial protein C receptor (EPCR) mediates APC's effects on endothelium and other vessel wall cells. 4. The expression of tissue plasminogen activator (tPA) and urokinase stimulates fibrinolysis.

These four mechanisms are not regulated evenly distributed over the vascular tree, but are probably site-specific. Based on the limited evidence available, it seems that large vessels mainly express EPCR, micro vessels express TFPI and the pulmonary and cerebral arteries primarily tPA. Thrombomodulin is present in all endothelial cells, being prominent in the microvessels of the lung, yet only minimally present in the blood-brain barrier (Figure 2.2).^{14,18} In response to systemic inflammation an alteration of this site-specificity has been observed.¹⁵ This alteration can be caused by signals from the microenvironment around the endothelial cells (through e.g. cytokines, mechanical forces, components of the extracellular matrix and surrounding cells), which are converted through endothelial cell-signalling networks and can lead to alterations in the mRNA expression of haemostatic proteins and their function.¹⁹⁻²¹ If problems occur in the synthesis of coagulation factors by the liver (for example due to impaired liver function in cirrhosis) a haemostatic imbalance can arise within the vascular system. This systemic imbalance can however be counteracted to some extent via site-specific responses of the endothelium.^{15,21} Clinically, it is therefore possible that with the loss of function of one of the liver generated natural anticoagulants, like protein C, while one would expect a *diffuse* hypercoagulability, only *local* thrombotic lesions in *discrete* vascular segments are seen.²¹ This supports a model of the so-called 'vascular bed-specific haemostasis' and it seems credible that this model may be applicable to both venous and arterial vasculature.

Interplay between coagulation and the endothelium of the vessel wall

When perturbation of the endothelium occurs, either in the course of haemostasis (arresting bleeding) or in prothrombotic conditions, the overall response of the endothelium will be a local prothrombotic, procoagulant and proinflammatory state. Concurrent functional endothelial alterations include the enhancement of permeability, the production of cytokines and growth factors as well as the increase in the expression of chemokines, leukocyte and platelet adhesion molecules (e.g. V-CAM, I-CAM).¹³

vascular-bed-specific hemostasis

Figure 2.2 A. Haemostasis is mediated by a balance of procoagulants versus anticoagulants. Procoagulant activities of the endothelium are the expression of receptors for cell-surface tissue factor (TF) and the release of von Willebrand factor (vWf), plasminogen-activator inhibitor type 1 (PAI-1). Anticoagulant forces of the endothelium are, besides providing the nonthrombogenic cell-surface membrane, the release of heparin sulphate and prostacyclin, the expression of thrombomodulin (TM), tissue-type plasminogen activator (tPA) and endothelial nitric oxide (NO) synthase. B. The components for the balance between procoagulant forces and anticoagulant forces vary per vascular bed.

Inflammation triggers a down-regulation of TM synthesis leading to a decrease in protein C activation and an increase in PAI-1 production. Synergistically the endothelial cells start to express TF and procoagulant FV in response to inflammatory mediators (e.g. TNF- α and IL-1).^{22,23} These collective alterations then in turn enhance local fibrin deposition to the vessel wall. The changes in permeability of the vessel wall further promote the passage of inflammatory cells and coagulation proteins into the vessel wall stimulating local production of thrombin, a prothrombotic, but also pro-inflammatory enzyme. It is therefore clear that endothelial dysfunction is an important constituent of Virchow's triad leading to a site-specific up regulation of coagulation and thereby increasing the risk of thrombosis.

In addition to these inflammation dependent mechanisms, serine proteases like FVIIa, FXa and thrombin can directly act on vessel wall cells, through the activation of the so-called protease-activated receptors (PARs).²⁴ As with many coagulation proteases, the net effects of thrombin activation of PAR-1 may act in two, apparently opposite ways. For instance, thrombin activation can on the one hand lead to endothelium-dependent vasodilatation (through PAR-1), but on the other hand also induce vasoconstriction.^{25-28,29-31} Various studies have furthermore proposed a role for thrombin in causing an increase in the permeability of the endothelium, thus disrupting the endothelial barrier-function.³²⁻³⁴ Theoretically, it is difficult to classify the resulting phenotypical endothelial changes as either "positive" or "negative", as a balance between those is probably essential for adequate immune responses, wound healing and tissue repair. We have to consider the interaction between coagulation and vascular endothelium in a time, location and situation dependent manner. In addition, age may be a crucial factor. While in a young individuals endothelial cells will mostly be unperturbed, the cumulative exposure to cardiovascular risk factors, driving inflammatory mediators, provides an increasing challenge to the protective properties of endothelial cells at a more advanced age. This may be associated by a progressive decay in protective molecules like TM and EPCR that have been shown to vanish from the endothelium upon increasing atherosclerosis.³⁵ Thus, effects of essentially protective proteases like thrombin, may become more prothrombotic and pro-inflammatory in time, due to reduced reserve in anticoagulant and anti-inflammatory mechanisms. Therefore, one of the consequences of ageing may be a shift from "positive" to "negative" functions of activated coagulation, in those at risk of CVD, while protective functions may be maintained for a long time in those that maintain a healthy vascular system.³⁶

The role of coagulation in atherosclerosis

One of recently discovered effects of thrombin that is considered "negative" is related to the pathophysiological process causing atherosclerosis. There is abundant

(experimental) data showing that thrombin is a crucial mediator in the cross talk between coagulation, inflammation and the vessel wall. Although the impact that thrombin has on atherosclerotic development is a relatively novel topic of investigation, it has been observed that both in the early stages of atherosclerotic plaque formation and in the advanced stages of atherosclerosis with plaque progression and destabilization, thrombin is involved.

By generating pro-inflammatory mediators, thrombin stimulates the recruitment of monocytes and T-cells into the vessel wall encouraging early plaque formation. The signalling mechanisms that have a pro-atherogenic impact on the vessel wall are mainly mediated through PARs. When activated, the endothelium in turn expresses certain surface markers such as V-CAM and I-CAM allowing leucocyte adhesion and rolling. These actions are essential for further development and progression of atherosclerosis.³⁷

Thrombin is also a potent mediator for the expression of selectins on the endothelium; it causes the expression of E-selectin on the endothelial surface and has the potential to release P-selectin from endothelial Weibel-Palade bodies.³⁸⁻⁴⁰ Besides its effects on the endothelium, thrombin also induces effects on other parts of the haemostatic process stimulating atherogenesis. By targeting PAR-1 and PAR-4 receptors on the surface of human platelets, thrombin induces atherogenic signals, boosting the synthesis and release of pro-inflammatory mediators. Furthermore, it enhances the interaction between platelets and leukocytes to increase chemotaxis, adhesion and the migration of leukocyte subsets (neutrophils) into the vessel wall. Thrombin activation of platelets leads to expression of CD40 ligand on the platelet surface, resulting in downstream atherogenic signals to other cells including, once more, endothelial cells and smooth muscle cells. Several additional pathways implicating thrombin in the development of atherosclerosis including vascular smooth muscle proliferation and proangiogenic responses.²⁴

Numerous experimental studies have indicated that hypercoagulability aggravates atherosclerosis in ApoE^{-/-} mice, while mice with a defect in coagulation (e.g haemophilia A) tend to be protected or may show phenotypic effects on atherosclerosis (fibrinogen deficiency),¹⁰ The protective effect of haemophilia A is however dependent on the genetic mouse background; while ApoE^{-/-} mice were protected the effect was absent in LDLR^{-/-} mice. Since in humans the protective effect of haemophilia on atherosclerosis is also disputed it remains uncertain whether any atherosclerosis modifying effect of coagulation proteases is confined to a genetically susceptible background (both in humans and other mammals).⁴¹ In addition, it is generally inferred from experimental studies that the effector mechanisms in atherosclerosis are mediated through thrombin generation and PAR activation, but protease specific effects of fibrinogen, FXa, FVIIa-TF and TFPI may also be relevant. As a proof of principle of the important role of thrombin several studies showed that direct inhibition of thrombin (either by Melagatran or

Dabigatran) protects against atherosclerosis development in ApoE^{-/-} mice, suggesting that at least thrombin is a key enzyme in this context.^{42,43}

Despite the experimental evidence suggesting that there is an association between inherited hypercoagulability and the development of atherosclerosis, clinical studies have so far shown conflicting results.^{41,44-46} Early studies indicated that certain hypercoagulable states (e.g. prothrombin mutation 20210A and FV Leiden) were associated with arterial occlusive disease^{47,48} with later studies however casting doubt on these observations by showing no such associations.⁴⁹ Based on these conflicting results a large meta-analysis was performed in 2006 by Ye et al. to investigate the association between certain haemostatic gene polymorphisms and coronary artery disease (CAD).⁵⁰ It was found that FV Leiden and the prothrombin G20210A were moderately associated with the risk of CAD with the per-allele relative risks for FV Leiden and of prothrombin G20210A being 1.17 (95% CI 1.08–1.28) and 1.31 (95% CI 1.12-1.52), respectively. Although these relative risks are small and make one wonder as to what the direct clinical implications should be, the results are biologically relevant and highlight the importance to gather more knowledge about the mechanisms behind hypercoagulability and (arterial) thrombotic events. When for example the two genetic defects are combined the hazard ratio for ischemic heart disease increases from 1.5 (95% CI 1.1-2.1) for the prothrombin G20210A mutation alone to 6.0 (95% CI 2.0-19) in combination with the FV Leiden mutation, possibly explained by a common prothrombotic pathway.⁵¹

Effects of hypercoagulability on endothelium, thrombosis risk and thrombosis location

Now that it has been established that thrombin itself can cause endothelial disruptions and even promote atherosclerosis, one of the remaining questions is whether the thrombosis risk associated with 'hypercoagulability' acts through upregulation of inflammation and perturbation of vascular endothelium. For this review we consider 'hypercoagulability' as a condition of the blood, arising from an imbalance between pro- and anticoagulant forces and which can be driven by inherited and/or acquired factors. Over the past years extensive research has further developed our understanding of the interplay between hypercoagulability and inflammation, also propagating venous thrombosis.⁵²

Hypercoagulability can be congenital or acquired in origin. Although the effect of congenital deficiencies in any of the known anticoagulant proteins including antithrombin, protein C, protein S, as well as the FV-Leiden and the prothrombin 20210A mutations on risk of venous thrombosis has been firmly established, their actual contribution to inflammation and response to injury effects are poorly known in the clinical setting.^{52,53} Interestingly, in experimental models of sepsis, defects in any of

the TM=protein C, antithrombin or TFPI pathways appear to be of major influence on the defence against sepsis and the development of DIC. Animals with an inborn or acquired defect in any of these natural anticoagulant proteins were more vulnerable to die of sepsis and had more aggravated DIC. Several studies by the Taylor group established that intact levels and function of each of the natural anticoagulants AT, PC or TFPI were relevant in baboons in order to survive severe sepsis.⁵⁴⁻⁵⁶ These studies also provided the basis for the subsequent clinical intervention studies like PROWESS that appeared to herald a way of reducing mortality due to severe sepsis by infusing recombinant APC.⁵⁷ Unfortunately, subsequent studies failed to corroborate this protective effect and the drug has now been withdrawn.⁵⁸

The overall lesson from the experimental studies may be that lack of or defect in any of the natural anticoagulant proteins may impair the immune defence in conditions of sepsis, resulting in an aggravated DIC response, mostly confined to the microvascular endothelium.

Whether the susceptibility to venous thrombosis in anticoagulant defective humans is also based on an increased inflammatory tendency remains to be proven.

Several acquired hypercoagulable conditions have been associated with an increased risk for both VTE and arterial thrombosis. These acquired hypercoagulable conditions include a variety of syndromes including cancer, myeloproliferative syndromes, antiphospholipid syndrome, hyperhomocysteinemia and heparin induced thrombocytopenia.

Although thrombi related to these acquired hypercoagulable states may present as VTE, or even as arterial thrombosis, thrombosis can be seen in atypical vascular locations as well. Patients with the antiphospholipid-antibody syndrome have widespread thrombus formation in segments of both the venous and arterial vascular tree, including for example thrombosis in the retinal artery or vein.⁵⁹ Vascular bed specificity is applicable to the acquired hypercoagulable states caused by paroxysmal nocturnal hemoglobinuria (PNH) and myeloproliferative disorders. PNH has a predilection for intra-abdominal and cerebral vessels. Myeloproliferative diseases are characterized by a high incidence of thrombosis in the hepatic, portal and mesenteric veins.⁶⁰ Recent research indicates for example that in a subpopulation of patients with the myeloproliferative disorder polycythaemia vera, which is associated with an increased rate of intra-abdominal thrombi, hepatic venule endothelial cells are affected by the malignant process.⁶¹ Falanga and coworkers have shown that such endothelial cells become procoagulant (by upregulation of TF and downregulation of TM) and that this phenotype can be corrected by the application of All-trans retinoic acid (ATRA).⁶² The mechanism of hyperhomocysteinemia related thrombosis is not yet established, however it is known that human umbilical vein endothelial cells (HUVECs) treated with homocysteine increase their externalization of procoagulant phosphatidylserine and shedding of procoagulant endothelial microparticles. Consequently these changes lead to an enhanced clot-promoting activity of the endothelial cells.⁶³ With regards to the

antiphospholipid syndrome it has now been generally accepted that the endothelium is a predominant target of antiphospholipid antibodies. Pathogenic antibodies bind to the β_2 glycoprotein 1 causing a prothrombotic endothelial cell phenotype with an upregulated expression of TF and E-selectin, but an increased release of microparticles as well.⁶⁴ These diverse observations provide strong support for the existence of distinct interactions between the blood components and the local vessel wall, possibly depending on the type of endothelial cell present, in both the venous and arterial vascular bed.

Association between venous thrombosis and atherosclerosis

An association between venous thromboembolism and atherosclerosis has been postulated. In separate studies patients with an unprovoked VTE were shown to have a higher prevalence of carotid artery plaques, coronary artery calcium and arterial thrombosis in comparison to healthy controls.⁶⁵⁻⁶⁷ A recent review by Ageno et al. showed that important risk factors associated with VTE, were concordantly associated with atherosclerosis (e.g. obesity, hypertension, diabetes mellitus and hypertriglyceridemia).⁶⁸ The nature of this association has not been clarified, but the risk factors all share effects on vascular endothelium. Inflammation-coagulation crosstalk may be at the basis of this observed risk interaction. As recently reviewed by Reitsma and colleagues, venous thrombosis is characterized by several proinflammatory reactions involving expression of leukocyte adhesion molecules by the endothelium, activation of platelets secreting chemokines, activation of neutrophils releasing NETs that act in different prothrombotic ways.⁵² Since inflammation is at the basis of atherosclerosis as well, it may be inferred that particularly in patients with a protracted course of idiopathic thrombosis ongoing inflammation may aggravate atherosclerosis. Since an increased risk of AMI was observed after VTE, it is also likely that plaque-destabilizing effects are involved.

Suggested implications for research and medical practice that have been stated are a modification of (atherosclerotic) life style counselling in VTE-patients and a potential role for prophylaxis with antiplatelet therapy and statins. Also, it becomes of interest to determine the effects of anticoagulant treatment beyond preventing (recurrent) thrombosis, on the inflammatory effects on the vessel wall.

Effects of anticoagulation on the vessel wall

If we assume that hypercoagulability interacts with and affects the vessel wall through mediation via the endothelium, it is tempting to speculate on the possible effects that anticoagulation may have. Several researchers have postulated that the use of

anticoagulant medication, through the inhibition of thrombin, could have beneficial effects on slowing down atherosclerosis. Anticoagulant medication is used for a variety of arterial and venous thromboembolic disorders, for the prevention as well as treatment of thrombosis. The commonly used vitamin K-antagonists (VKA), act directly on the inhibition of carboxylation by the liver of the so-called vitamin K-dependent proteins (VKDP). When there is a deficiency of vitamin K or vitamin K is “blocked” with the use of VKA, the VKDP’s will be non-functional and are called PIVKA’s (Proteins Induced by Vitamin K deficiency or Antagonists). However, the desired effects VKA have on inhibiting coagulation, may be overshadowed by the effect of VKA on the *extra-hepatic* VKDPs.⁶⁹ One of these VKDPs is the Matrix Gla Protein (MGP) that is involved in the inhibition of calcification of the arterial vessel wall. MGP is synthesized locally in the vessel wall by smooth muscle cells and, likewise to the coagulation-VKDPs, it needs vitamin K-dependent carboxylation for its function. It has been shown that MGP knockout mice (MGP^{-/-}) die within a few weeks after birth due to a complete calcification of the medium and large arterial vessels.⁶⁹⁻⁷¹ Limited experimental data suggest a protective effect of VKA treatment in ApoE^{-/-} mice by large calcifications that stabilize the atherosclerotic plaque.⁶⁹ On the other hand it has been stated that with large agglomerations of calcification present, micro-calcifications are present as well, making the plaque vulnerable and prone to rupture. The research regarding the effect of VKA on vascular calcification in humans is relatively new, only since the beginning of this century studies concerning this topic have been published.⁷²⁻⁷⁵ These studies strongly suggested that the use of VKA accelerates the process of calcification of the cardiac valves, the vessel wall and in the atherosclerosis plaque, and therefore, in certain patient populations the use of VKA could potentially be harmful.⁶⁹

In the past few years novel anticoagulants (NOACs), such as the direct thrombin- and factor Xa-inhibitors, have been introduced clinically. Quite clearly, it should be investigated what the effects of these NOACs are on the venous and arterial vascular bed. Unlike the VKA’s, these novel anticoagulants do not seem to interfere with the VKDP’s and therefore not with MGP. However they probably do have pleiotropic effects that could also interfere with atherosclerosis or vascular calcification. So far only a handful of studies concerning this topic have been published. The direct thrombin inhibitor Melagatran showed beneficial effects on both size and composition of advanced atherosclerotic lesions in mice, possibly due to a reduced activation of pro-inflammatory transcription factors (NFκB and AP-1) and a decreased synthesis of MMP-9.⁴³ Dabigatran, a direct thrombin inhibitor as well, showed in ApoE^{-/-} a reduced atherosclerotic lesion size and an improved endothelial function due to a decrease of oxidative stress and ROS production in (hypercholesterolemic) atherosclerosis.^{42,76} Administration of Rivaroxaban, a direct FXa-inhibitor, in ApoE-deficient mice showed no effect on lesion progression however did result in the downregulation of inflammatory mediator expression (of e.g. IL-6, TNF-α, MCP-1, and Egr-1) and promoted stability of advanced atherosclerotic lesions.⁷⁷ These data appear to confirm the idea that

thrombin (generation) is a relevant element in the process of atherosclerosis, at least in experimental animal models.

Conclusion

Traditionally, the origin of thrombosis has been viewed separately for arterial and venous thrombosis, and the pathophysiology has been explained by distinct mechanisms and influenced by different risk factors. Over the past few years however there has been a paradigm shift due to the accumulation of evidence showing that the etiopathogenetic mechanisms behind venous and arterial thrombosis are quite similar. The traditional elements of Virchow's triad are found to apply to both arterial and venous thrombosis. More emphasis is placed on the vessel wall and vascular bed specificity and the interaction with inflammation and hypercoagulability. The increased understanding of the interactions between endothelial dysfunction, vascular inflammation, thrombosis and atherosclerosis opens possibilities for novel diagnostic and therapeutic approaches for both venous and arterial cardiovascular disease, including atherosclerosis.

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CHAPTER 3

Normal platelet activation profile in patients with peripheral arterial disease on aspirin

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Abstract

Background

Peripheral arterial disease (PAD) is a progressive vascular disease associated with a high risk of cardiovascular morbidity and death. Antithrombotic prevention is usually applied by prescribing the antiplatelet agent aspirin. However, in patients with PAD aspirin fails to provide protection against myocardial infarction and death, only reducing the risk of ischemic stroke. Platelets may play a role in disease development, but this has not been tested by proper mechanistic studies. In the present study, we performed a systematic evaluation of platelet reactivity in whole blood from patients with PAD using two high-throughput assays, i.e. multi-agonist testing of platelet activation by flow cytometry and multi-parameter testing of thrombus formation on spotted microarrays.

Methods

Blood was obtained from 40 patients (38 on aspirin) with PAD in majority class IIa/IIb and from 40 age-matched control subjects. Whole-blood flow cytometry and multiparameter thrombus formation under high-shear flow conditions were determined using recently developed and validated assays.

Results

Flow cytometry of whole blood samples from aspirin-treated patients demonstrated unchanged high platelet responsiveness towards ADP, slightly elevated responsiveness after glycoprotein VI stimulation, and decreased responsiveness after PAR1 thrombin receptor stimulation, compared to the control subjects. Most parameters of thrombus formation under flow were similarly high for the patient and control groups. However, in vitro aspirin treatment caused a marked reduction in thrombus formation, especially on collagen surfaces. When compared per subject, markers of ADP- and collagen-induced integrin activation (flow cytometry) strongly correlated with parameters of collagen-dependent thrombus formation under flow, indicative of a common, subject-dependent regulation of both processes.

Conclusion

Despite of the use of aspirin, most platelet activation properties were in the normal range in whole-blood from class II PAD patients. These data underline the need for more effective antithrombotic pharmacoprotection in PAD.

Introduction

Peripheral arterial disease (PAD) is a systemic vascular disorder with manifestations of atherosclerosis in particular in the lower extremities, resulting in obstruction of the arterial blood flow, and strongly associated with cardiovascular events.¹⁻³ In the Western world, a considerable part of the elderly population appears to have characteristics of PAD, albeit the affected subjects often remain asymptomatic.^{4,5} Symptomatic patients, already at an early stage (Fontaine class IIa/b), suffer from intermittent claudication and cannot walk a long distance without feeling pain.² At later stages (Fontaine classes III/IV), PAD patients progressively suffer from chronic and critical leg ischemia.²

While it is recognized that systemic atherosclerosis is one of the underlying diseases of PAD, the pathophysiology of this disease is still not well understood. One of the early concepts was that aberrant coagulation, inflammation and platelet activity in these patients lead to ongoing thrombogenesis in the lower extremities.⁶ In agreement with this, markers of systemic coagulation such as levels of D-dimer are increased in patients with PAD, but it is unclear whether this also reflects causality.⁷⁻¹⁰ Consistently, also plasma levels of the inflammation marker C-reactive protein are elevated.^{10,11} There is also ample evidence in the literature for increased platelet activation and aggregation in PAD,¹²⁻¹⁵ although this is not an undisputed finding.^{8,16,17} The role of platelets in PAD may indeed be complex. On the one hand, antiplatelet treatment with aspirin, inhibiting thromboxane formation, is a standard therapy for PAD patients, with inhibitors of the platelet P2Y₁₂ receptors (clopidogrel, prasugrel) as effective alternatives.¹⁸ On the other hand, there is evidence that in these patients, even after aspirin treatment, platelet functions remain high,¹⁹ platelet-monocyte aggregates are still formed,²⁰ and platelet cleavage products such as soluble CD40L accumulate in the plasma.²¹ Since PAD in many patients is a progressive disease with high cardiovascular risk, the question is whether residual high on-treatment platelet activity may contribute to the disease progression.

In recent years, a number of whole-blood based tests for detailed and overall platelet phenotyping in bleeding and thrombosis have been developed and validated. Platelets based flow cytometric tests allow simultaneous determination of key platelet responses, *i.e.* fibrinogen binding (integrin $\alpha_{IIb}\beta_3$ activation) and secretion (P-selectin exposure) in response to a panel of receptor agonists.^{22,23} Such flow cytometric tests are advantageous in detecting changed platelet activation in thrombotic patients.²⁴ Furthermore, flow chamber devices for whole-blood perfusion over a thrombogenic surface like collagen have appeared to be highly valuable in detecting a loss or gain of platelet function, supporting their use as *ex vivo* assays reflecting arterial thrombus formation.²⁵⁻²⁷ Recently, we have extended this method into a multi-parameter platelet function test to assess thrombus formation on arrays of microspotted platelet-adhesive surfaces.²⁸

In the present paper, we used both high-throughput whole-blood assays, *i.e.* multi-agonist testing of platelet activation by flow cytometry and multi-parameter testing of thrombus formation on microarray spots, for detailed evaluation of the platelets in patients with PAD receiving aspirin. The data show that platelet function in these patients is in general high despite aspirin treatment, thus not pointing to a decreased prothrombotic propensity.

Methods

Patients and controls

Included were 40 patients with established PAD. Patients were selected based on a decrease in the ankle brachial index <0.9 ^{29,30} and most of them suffered from a stage IIa/b disorder according to the Fontaine classification.² As a control group, 40 healthy control subjects were included with similar age and gender. Fitness (*i.e.*, non-disease state) of the control subjects was assessed with the Edinburgh claudication questionnaire.³¹ Medication use was checked in all patient and control subjects by personal interview. Exclusion criteria for patients and controls were: proved coagulation or chronic inflammatory disorders, active infection, malignancy, anti-phospholipid syndrome, pregnancy and/or prescribed antiplatelet or anticoagulant medicines other than aspirin. The study was approved by the Medical Ethics Committee of the Maastricht University Medical Center. All patients and healthy subjects gave written informed consent for participation according to the declaration of Helsinki.

Blood collection

Blood was drawn from subjects in resting condition, and collected into 10 ml tubes containing 3.2% trisodium citrate. Portions of the whole-blood were directly used for flow cytometric and flow chamber assays. Separate blood samples were collected into 4 ml EDTA tubes (BD Vacutainer, Breda, the Netherlands) for determination of haemoglobin and blood cell counts with a Sysmex XN-9000 analyzer (Kobe, Japan).

Whole-blood flow cytometry

Agonist-induced platelet activation was assessed by an optimized and validated test using diluted whole-blood samples without centrifugation steps.²² In brief, a 96-well plate was thawed, containing phycoerythrin (PE)-conjugated anti-P-selectin mAb (BD 555524, Pharmingen, Franklin Lakes, NJ) and FITC-conjugated anti-fibrinogen Ab (F011102, Dako, Denmark) together with varying concentrations of ADP (01897-1G, Sigma-Aldrich, Zwijndrecht, the Netherlands), collagen peptide (CRP-xl, Cambridge University, UK, a generous gift)²² or TRAP-6 (H2936.0025, Bachem, Weil am Rhein,

Germany); volume was 50 μ l in buffer A (10 mM Hepes, 150 mM NaCl, 5 mM KCl 1 mM $MgCl_2$). Activations were started by adding to each well 5 μ l of blood, while mixing. Final agonist concentrations were: 0.1-2.7 μ M ADP, 5-135 ng/ml collagen peptide or 0.4-3.2 μ M TRAP-6. A separate row did not contain platelet agonists and served as vehicle. After 20 minutes of activation, 500 μ l fixation solution (0.9% NaCl, 0.2% formaldehyde) was added to each well. Integrin activation and P-selectin expression in platelets were analyzed on an Accuri C6 flow cytometer (Becton and Dickinson, CA; 10,000 events), as described.²⁴ Data are represented as percentage positive cells.

Whole-blood thrombus formation on microspots

Whole-blood thrombus formation under flow was investigated with the Maastricht flow chamber (depth 50 μ m, width 3 mm, length 30 mm), employing a newly developed and validated multi-parameter assay in combination with three microspots.²⁸ (Where indicated, control blood samples were pre-incubated with lysine acetylsalicylate for 10 minutes at 37°C (100 μ M, Aspegic, Sanofi, Gouda, the Netherlands). Microspots on glass coverslips were prepared by applying 0.5 μ l collagen type I Horm (100 μ g/ml), von Willebrand factor (vWF, 50 μ g/ml) combined with CLEC-2 agonist rhodocytin (250 μ g/ml), and vWF plus fibrinogen (250 μ g/ml). Sources of microspots were as described before.³² Citrate whole-blood perfusion was at arterial wall-shear rate of 1600 s^{-1} for 3.5 minutes. Brightfield images were taken from all three microspots, while chambers were stained with Alexa Fluor (AF) 647-labeled annexin A5 (1:200, A23204, Life Technology, Eugene, OR) and FITC-labelled anti-P-selectin mAb (1:40, A07790, Beckman Coulter, Woerden, the Netherlands) in buffer B (10 mM Hepes, 136 mM NaCl, 2.7 mM KCl, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 0.1% glucose, 0.1% bovine serum albumin, 5 U/ml fragmin (low-molecular weight heparin) (RVG20607, Pfizer, Capelle a/d IJssel) and 1 U/ml unfractionated heparin (1760 U/ml, 125K1336, Sigma), pH 7.45). The anticoagulants were added to all Ca^{2+} -containing perfusion buffers to prevent thrombin and fibrin generation. Images of FITC and AF647 fluorescence were captured after a short rinse with buffer B. For microscopic imaging, an inverted EVOS fluorescence microscope (Life Technology) was used, basically as described elsewhere.²⁸

Whole-blood thrombus formation under flow was also assayed in real-time using a microfluidics polydimethyl siloxane (PDMS) flow chamber with a straight 1 mm wide x 50 μ m height channel, principally as described,³³ but without stenosis. The disposable chamber was prepared with a Sylgard 184 kit, by mixing 43 g of silicone elastomer with 7 g curing agent (Dow Corning, Wiesbaden, Germany). The degassed mixture was poured into a round mold containing water to harden at room temperature. The flow channel inlet and outlet were made using a 3 mm biopsy punch and a blunt syringe needle, respectively. The flow chip was mounted onto a glass coverslip, coated with collagen type I Horm (100 μ g/ml),³⁴ fixed with a pressure plate, and pre-rinsed. Whole-blood perfusion, using a pulse-free syringe pump (pull mode), was performed as described.³⁵ Blood samples (300 μ l) were pre-labelled with 0.5 μ g/ml DiOC₆ (6975,

AnaSpec, San Jose, CA), and flowed at a wall-shear rate of 1600 s^{-1} for 4 minutes. Microscopic images of deposition of fluorescently labelled platelets were taken as previously described,²⁸ and analyzed for increases in fluorescence.

Microscopic imaging and analysis

Phase-contrast images were analyzed for surface-area-coverage and integrated feature size, using a standardized journal in Metamorph software.³⁶ Images were also assessed for morphological score from 0 (no platelet adhesion) to 5 (large platelet thrombi).²⁸ Fluorescence images of antibody staining were analyzed for surface-area-coverage above background with the program Image J (open access).

Statistics

Group comparisons were performed using the Mann-Whitney U-test for continuous variables. Assay parameters were compared using the Spearman correlation test. The statistical package for the Social Sciences version 22 was used (SPSS, Chicago, IL). *P* values <0.05 were considered to be significant.

Results

Undiminished platelet activation in whole-blood from PAD patients on aspirin

Baseline characteristics of the 40 control subjects and 40 PAD patients were comparable for age, gender and body mass index (Table 3.1). With respect to the medical history, the patients (vs. controls) more frequently suffered from diabetes mellitus (5 vs. 1) and hypertension (29 vs. 13), and in vast majority used prescribed aspirin (38 vs. 0). All patients, and none of the control subjects, were diagnosed as PAD with confirmed Fontaine classification IIa/IIb (37 out of 40). Most hemostatic variables of the PAD patients were in the normal range, but leukocyte counts and plasma levels of the coagulation product, D-dimers, were significantly increased in the patient group (Table 3.1).

Platelet activation tendency was measured in diluted whole blood samples, using a validated, 96-plate-well-based flow cytometric test, in which both integrin $\alpha_{IIb}\beta_3$ activation and P-selectin exposure are determined in response to dose ranges of the key agonists, ADP (stimulating $P2Y_{12}/P2Y_1$ receptors), CRP-xI (stimulating the collagen receptor glycoprotein VI) and TRAP-6 (stimulating the thrombin receptor PAR1).²² For various agonists, intra- and inter-individual variation coefficients of the test were <10% and <15%, respectively, for the area under the curve.³⁷

Table 3.1 Baseline characteristics of control subjects and PAD patients. Data are represented as medians (ranges).

Subject characteristics	Controls	Patients	P
General			
Age, years	67 (60 - 71)	67 (65 - 72)	
Male gender, n	22/40	24/40	
Body mass index, kg/m ²	26 (23.4 - 27.8)	26.1 (23.7 - 29.3)	
Diagnosis PAD (Fontaine), n			
I, IIa/IIb, III	0, 0, 0	1, 37, 2	
Medical history, n			
Diabetes mellitus	1	5	
Hypertension	13	29	
Aspirin intake	0	38	
Anticoagulant intake	0	0	
Hemostatic variables			
Platelet count, x10 ⁹ /l	255 (205 - 291)	249 (213 - 284)	n.s.
Mean platelet volume, fl	10.8 (10.2 - 11.4)	11.1 (10.4 - 11.9)	n.s.
Hematocrit, L/l	0.44 (0.42 - 0.47)	0.43 (0.39 - 0.45)	n.s.
Leukocyte count, x10 ⁹ /l	6.15 (5.60 - 7.45)	7.50 (6.03 - 9.13)	0.003
D-dimers, ng/ml	366 (259 - 520)	528 (363 - 835)	0.005

In response to the weak agonist ADP, platelets from patients and control subjects showed a dose-dependent increase in integrin activation (fibrinogen binding) and limited P-selectin expression (Figure 3.1A, B). Dose-response curves varied per subject but, overall, did not markedly differ between the patient and control groups. Platelet stimulation with CRP-xI (Figure 3.1C-D) or TRAP-6 (Figure 3.1E-F) resulted in dose-dependent increases in integrin activation and P-selectin expression that, with these stronger agonists, reached maximal levels at high doses. At lower CRP-xI concentrations, both integrin activation and P-selectin expression were significantly higher in the patient group than in the control group ($P \leq 0.037$), but ranges were overlapping. In contrast, in response to higher doses of TRAP-6, integrin activation ($P = 0.021$) and P-selectin expression ($P \leq 0.015$) were significantly lower in the patient group compared to the controls. Two of the patients did not use aspirin. In blood samples from these patients, agonist-induced integrin activation and P-selectin expression were mostly but not always in the higher ranges (see Figure 3.1). Upon stimulation with ADP, no significant effect of aspirin treatments was observed (not shown). This is in agreement with the known sensitivity for aspirin of glycoprotein VI-induced platelet responses.³⁸ Taken together, these data indicate that, in spite of the use of aspirin, the platelets from PAD patients were in general similarly high in response as the platelets from control subjects, with only reduced activity at high TRAP-6 doses.

Figure 3.1 Activation tendency of platelets from PAD patients and control subjects. Diluted whole-blood from control subjects (c) or PAD patients (p) was stimulated in plate wells with indicated doses of 0.1–2.7 μ M ADP (A, B), 5–135 ng/ml CRP-xI (C, D) or 0.4–3.2 μ M TRAP-6 (E, F). Binding of fibrinogen to the platelets (left panels) and P-selectin expression of platelets (right panels) were determined by flow cytometry, using a FITC-anti fibrinogen and a PE-anti-P-selectin mAb, respectively. Indicated are per subject the percentages of positively stained platelets. Patients using aspirin are represented by open squares. Bars indicate mean and S.D. values (n=40), *P<0.05.

Essentially unchanged thrombus formation in patient whole blood

Whole-blood thrombus formation was tested on three microspots, representing adhesive ligands for major platelet receptors, *i.e.* collagen type I (activating via glycoprotein VI and integrin $\alpha_2\beta_1$), vWF/rhodocytin (acting via GPIb and CLEC-2 receptors), and vWF/fibrinogen (acting via GPIb and integrin $\alpha_{IIb}\beta_3$). The presence of vWF (also binding to collagen) allowed thrombus formation to be assessed at high, arterial shear rates.²⁸ On collagen, large platelet aggregates were formed, staining positively for P-selectin, and individual platelets in the thrombus were present, staining positively with annexin A5 for phosphatidylserine exposure (Figure 3.2A), as established before.³⁹ On vWF/rhodocytin smaller aggregates formed, while staining for P-selectin and phosphatidylserine exposure was still high (Figure 3.2B). On vWF/fibrinogen mostly single platelets adhered with P-selectin but no phosphatidylserine exposure (Figure 3.2C). Principal assay parameters per surface were obtained following quantitative analysis of the recorded images, according to standard protocols.³⁶ These were: morphological score, platelet deposition (surface-area-coverage of brightfield images), integrated feature size (weighted cumulative size of platelet aggregates), and extent of P-selectin and phosphatidylserine exposure (Figure 3.3A-D). In agreement with earlier assessment of high inter-individual variation of most of these parameters,²⁸ values obtained per subject (controls and patients) showed large differences. Markedly, however, only few differences in these parameters were detected between the patient and control groups. With the patient blood samples, thrombi on collagen were lower in morphological score, integrated feature size and phosphatidylserine exposure ($P \leq 0.02$). None of the parameters differed for thrombi formed on vWF/rhodocytin or vWF/fibrinogen.

Furthermore, real-time imaging of platelet adhesion to collagen using DiOC₆-labeled blood samples did not show differences between the control group and patient group (Figure 3.4A-B). Similarly, as reported before,²⁸ markers of thrombus formation – per surface and per patient/control sample – were correlated (data not shown). Together, this indicated that for the patient group platelet adhesion, activation and aggregation under flow was mostly unaltered compared to the healthy, control group.

In vitro studies using blood samples from healthy controls were carried out to specifically determine the effect of aspirin on the various assay parameters. As indicated in Figure 3.5, on collagen and to a lesser extent on vWF/rhodocytin (morphological score and phosphatidylserine exposure), but not on vWF/fibrinogen, aspirin treatment reduced aspects of thrombus formation. Especially on collagen, thrombus parameters were reduced by more than half.

Figure 3.2 Thrombus formation with blood from patients and controls at microspot surfaces. Whole blood from control subjects or patients was perfused for 3.5 minutes at wall shear rate of 1600 s^{-1} over microspots containing collagen type I (A), vWF/rhodocytin (B), and vWF/fibrinogen (C). Shown are typical brightfield images (left) and fluorescence images of P-selectin expression (middle) or phosphatidylserine exposure (right) from a representative control subject and patient. Bars = $20 \mu\text{m}$ (for all pictures equal).

Figure 3.3 Parameters of thrombus formation with blood from patients and controls. Whole blood from control subjects (c) and PAD patients (p) was perfused over coated microspots, as described for Figure 2. Shown are analyzed data per subject of brightfield images of morphological score (A) and platelet deposition (B); and of staining for P-selectin (C) and exposed phosphatidylserine (D). Patients using aspirin are represented by open squares. Bars indicate mean and S.D. values (n=40), * $P<0.05$.

Comparing the various parameters of collagen-dependent thrombus formation per subject (patients and controls), gave high correlations ($P<0.001$, $r^2=0.25-0.56$, Spearman). This compares well with a previous systematic analysis of the thrombus-forming process with blood from healthy subjects,²⁸ and confirms that – in the absence of platelet aberrations – the various platelet activation responses are highly linked. Furthermore, compared per subject, markers of ADP- and collagen-induced integrin activation (flow cytometry) strongly correlated with parameters of collagen-dependent

platelet aggregation under flow (Table 3.2), pointing to a subject-dependent activation factor commonly regulating both types of platelet responses.

Figure 3.4 Flow-dependent adhesion of platelets with blood from patients and controls. Whole blood pre-treated with DiOC₆ from control subjects (c) and PAD patients (p) was perfused through a collagen-coated PDMS chamber for 3.5 minutes at wall shear rate of 1600 s⁻¹. Analyzed data per subject of brightfield images of platelet deposition (A) and integrative adhesion of DiOC₆-labeled platelets (B). Patients using aspirin are represented by open squares. Bars indicated mean and S.D. values (n=40).

Figure 3.5 Effect of aspirin on whole-blood thrombus formation at microspot surfaces. Whole blood from control subjects was perfused over coated microspots, as in Figure 2. Blood samples were incubated with vehicle or lysine acetylsalicylate (100 μ M) for 10 minutes at 37 $^{\circ}$ C prior to perfusion. Images were analyzed for morphological score, platelet deposition, staining for P-selectin and phosphatidylserine (PS), as described for Figure 3.3. Data are expressed as percentages relative to vehicle control. Means $\pm$ S.E.M. (n = 4), * P <0.05.

Table 3.2 Correlations of agonist-induced fibrinogen binding and P-selectin expression with parameters of thrombus formation on collagen microspots. Compared are analyses of platelet deposition (brightfield % surface area coverage, SAC), aggregate size (integrated feature size, IFS), and DiOC₆ staining. Spearman correlation analysis of data from all subjects (n = 80); given are two-sized P values per flow-cytometric data set. Colour scale from green to white: low to high P values.

Thrombus parameter	Fibrinogen binding			P-selectin expression		
	SAC	IFS	DiOC ₆	SAC	IFS	DiOC ₆
ADP (0.1)	0.056	0.014	0.323	0.417	0.929	0.394
ADP (0.3)	0.039	0.014	0.125	0.226	0.424	0.599
ADP (0.9)	0.016	0.007	0.058	0.096	0.232	0.239
ADP (2.7)	0.041	0.064	0.164	0.321	0.665	0.558
ADP (AUC)	0.033	0.008	0.202	0.243	0.361	0.664
CRP-xI (5)	0.007	0.011	0.058	0.346	0.336	0.466
CRP (15)	0.012	0.020	0.012	0.104	0.198	0.882
CRP-xI (45)	0.083	0.050	0.066	0.393	0.219	0.310
CRP-xI (135)	0.072	0.083	0.107	0.579	0.805	0.627
CRP-xI (AUC)	0.048	0.033	0.048	0.188	0.161	0.176
TRAP-6 (0.4)	0.023	0.001	0.077	0.805	0.781	0.775
TRAP-6 (0.8)	0.280	0.032	0.011	0.649	0.198	0.083
TRAP-6 (1.6)	0.152	0.090	0.073	0.836	0.844	0.436
TRAP-6 (3.2)	0.096	0.340	0.015	0.608	0.904	0.149
TRAP-6 (AUC)	0.162	0.019	0.008	0.565	0.484	0.104
All agonists (ΣAUC)	0.070	0.048	0.064	0.017	0.012	0.012

Discussion

A recent meta-analysis of randomized trials indicates that in patients with PAD, treatment with aspirin alone led to a statistically insignificant decrease in cardiovascular events, but a significant reduction in non-fatal stroke.⁴⁰ Several small-size studies have pointed to high activity of platelets in PAD patients taking aspirin,^{20,21} which can be reversed by the antiplatelet agent iloprost, a prostacyclin analogue.¹⁹ Here, we used high-throughput whole-blood assays, *i.e.* multi-agonist testing of platelet activation by flow cytometry and multi-parameter testing of thrombus formation on microarray spots, to determine the platelet activation tendency in 40 patients with PAD receiving aspirin compared to healthy controls not on antiplatelet medication. This systematic analysis points to a general high on-treatment function of the patient platelets in thrombus formation, and thus supports the hypothesis that the prothrombotic propensity known for PAD patients is not accompanied by an increased platelet activation profile after intake of aspirin.

In collagen-induced thrombus formation, platelet stimulation via glycoprotein VI and ADP increases the affinity state of integrins $\alpha_2\beta_1$ and $\alpha_{IIb}\beta_3$,^{41,42} and leads to stable platelet adhesion and aggregate formation.³⁹ Given that ADP is a primary agonist in establishing stable platelet-platelet interactions under flow,⁴³ the similarity in thrombus

formation between the patient and control groups matches the comparable ADP-induced integrin activation (fibrinogen binding) in both groups determined by flow cytometry. This notion is confirmed by the observation that markers of ADP- and collagen-receptor dependent $\alpha_{IIb}\beta_3$ activation (flow cytometry) strongly correlated with markers of the thrombus-forming process on collagen, which is indicative of a common, subject-dependent regulation of both processes.

Although platelet activation and thrombus parameters in the patient group were generally comparable with the control group, there were some typical differences versus the healthy control group. The indication for increased platelet activity in PAD patients came from flow cytometric analysis of platelet activation with low but not high CRP-xI concentrations (stimulating glycoprotein VI). In contrast, decreased activity in the PAD group was observed for TRAP-6-induced platelet activation (stimulating thrombin receptors), which is an aspirin-sensitive response. Furthermore, we found moderate reductions in some (morphological score, phosphatidylserine exposure), but not all (P-selectin and platelet deposition) parameters of thrombus formation on collagen in the patient group. Although these can be explained by the use of aspirin, the effect size appears to be smaller than observed by direct and complete inhibition of control platelets with aspirin *in vitro*. In agreement with the present results, others have also shown that aspirin markedly affects thrombus formation on collagen under flow.^{44,45}

The seeming contrasting differences between increased stimulation of patients' platelets with low doses of CPR-xI and reduced parameters of collagen-dependent thrombus formation, can be explained by the fact that, in thrombus formation under flow, not only glycoprotein VI, but also other platelet receptors like integrin $\alpha_2\beta_1$ and GPIb-V-IX are determinative for this process.⁴⁶ This suggests a partly diminished role of these other receptors in the patient group, concerning morphological score and phosphatidylserine exposure on collagen. The effect on phosphatidylserine exposure yet is relevant, because this platelet response is a major trigger of coagulation factor binding and thrombin formation on the platelet surface.⁴⁷

Strengths of this study are the relatively large size of the PAD patients (class IIa/IIb) and control groups; the use of well standardized tests using whole blood samples with no platelet activation due to centrifugation steps; and the ability to evaluate multiple platelet activation processes and markers in the same tests. Given the overall consistency of the various assay results, this study may help in improving the management and treatment of PAD, *e.g.* by considering more effective antithrombotic pharmacoprotection.

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CHAPTER 4

Increased clot formation in the absence of increased thrombin generation in patients with peripheral arterial disease

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Abstract

Objective

In peripheral arterial disease (PAD) activation of the haemostatic system may contribute to atherosclerosis progression and atherothrombotic events. This case control study assesses the overall coagulation status in PAD-patients by evaluating coagulation markers in combination with thrombin generation potential, whole blood clot formation and fibrinolysis.

Methods

In blood from 40 PAD-patients (n=20 with cardiovascular event within one year after initial diagnosis and n=20 without) and 40 healthy controls thrombin generation was determined in whole blood (WB) and platelet poor plasma. WB-ROTEM-measurements were triggered with tissue factor with/without tissue Plasminogen Activator.

Results

We observed increased levels of erythrocyte sedimentation rate, leucocytes, eosinophil granulocytes, vWF antigen, fibrinogen and D-dimer in PAD-patients ($p < 0.05$). Markers of thrombin generation potential showed no difference between patients and healthy controls. In PAD-patients with event compared to patients without, WB-thrombin generation showed a lower thrombin potential when triggered with 0 pM and 2.5 pM tissue factor. The ROTEM clotting assay showed significantly faster clot formation and increased clot firmness in PAD-patients compared to controls. No significant differences were found for parameters of clot degradation.

Conclusions

There are no significant differences between the thrombin generation profiles of PAD-patients and healthy controls. Between PAD-patients with and without cardiovascular event the whole blood thrombin generation appears to differ. Mechanistically, PAD-patients show an increased ability to form a stable clot in whole blood in comparison to healthy controls. This is most likely due to the increased fibrinogen levels related to the inflammation in atherosclerosis, confirming the importance of the inflammation-coagulation axis.

Introduction

Peripheral arterial disease (PAD) results from atherosclerosis in the major arteries of the lower extremities, causing an imbalance between the demand and supply of oxygenated blood flow.¹ This decreased blood flow in the legs can cause 'intermittent claudication', characterized by a cramp-like pain or aching in the calves or thighs typically brought on by ambulation and relieved with rest.² The total disease prevalence of PAD is estimated to be 2-7% in the general population aged 50 to 70 years, and believed to increase to 15-20% in people over 70 years.³ Due to this high prevalence, PAD should be considered an important global healthcare problem, in particular since patients with PAD have a high risk of cardiovascular complications and death.² After initial diagnosis, morbidity and mortality rates after 5, 10 and 15 years are approximately 30%, 50% and 70%, respectively. Several studies suggest that ongoing activation of the haemostatic system may contribute both to atherosclerotic progression and to the occurrence of atherothrombotic events.⁶ Indeed, in a recent meta-analysis we showed that an increased D-dimer was independently associated with a 2-fold increased risk of cardiovascular events in the near-term future (within 4 years of the assessment of D-dimer).⁷ Previously it was demonstrated that PAD-patients have a systemic increase in concentrations of various rheological and haemostatic factors. These factors include a significant increase of fibrinogen, thrombin-antithrombin (TAT) complexes, prothrombin fragment 1.2 (F1.2) and D-dimer.⁸⁻¹⁴ In the present study we wanted to gain insight into the overall haemostatic profile (i.e. coagulation-, clot formation- and clot lysis capacity) of patients with occlusive PAD versus that of healthy controls. Furthermore, we wanted to assess whether PAD-patients that experienced a cardiovascular event within one year of follow-up after the initial diagnosis of PAD differed in their haemostatic profile from PAD-patients that did not experience a cardiovascular event.

Therefore, we performed a nested case control study in which we assessed the levels of various coagulation markers, both the plasmatic thrombin generation potential with the Calibrated Automated Thrombogram (CAT) and the recent whole blood thrombin generation analysis. In addition, we studied the fibrin formation and lysis effects by a modified tissue factor-triggered rotational thromboelastometry (ROTEM) test in whole blood.

Methods

Patients

From 2009 to 2013, 301 consecutive patients from the vascular surgery departments of three hospitals in South-Limburg, the Netherlands, with newly diagnosed occlusive PAD

were enrolled in a prospective cohort study with a follow-up duration of 2 years. The diagnosis of occlusive PAD was based on an ankle-brachial index <0.9 . After 1-year follow-up in this study, patients with one or more cardiovascular events during this period were selected for this nested case control study as 'cases'. From this cohort a total number of 20 cases were thus identified. Patients, from the same cohort, with PAD and of comparable age and sex but without CV-event during this follow up were selected as 'controls' ($n=20$). The study was set up as an explorative, hypothesis generating study to obtain insights into the prothrombotic mechanism in PAD-patients, therefore a formal sample size calculation was not included. Cardiovascular events were defined as ischaemic cerebrovascular accident (CVA), transient ischaemic attack (TIA), new unstable angina pectoris (UAP), myocardial infarction (MI), coronary revascularisation, and/or revascularisation for PAD. Exclusion criteria were the use of medication known to affect coagulation (e.g. cumadins, direct factor Xa-or thrombin inhibitors, or heparin), documented congenital coagulation disorders, documented chronic inflammatory disease (however COPD Gold I and II were accepted), active malignancy, current pregnancy, age <18 years. Patients were included after written informed consent was obtained. The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki (Seoul 2008) and was approved by the local medical ethics committee (METC azM / UM).

Healthy subjects

Forty apparently healthy individuals of comparable sex- and age (mean age of 67 years, 55% males) were also enrolled in the study, after informed consent was obtained. The Edinburgh Claudication Questionnaire was used to select controls in which the presence of PAD was deemed unlikely. Exclusion criteria were: the use of medication known to affect coagulation (e.g. cumadins, direct factor Xa-or thrombin inhibitors, or heparin), known congenital coagulation disorders, chronic inflammatory disease, active malignancy, current pregnancy, and age <18 years.

Normal pooled plasma

Normal pooled plasma (stored frozen at -80°C) used for the normalization of thrombin generation samples was prepared by pooling plasma from 80-90 apparently healthy volunteers.

Blood collection and plasma preparation

Venous blood was collected in a 4.0 ml EDTA-tube (Vacutainer plastic, K2E (EDTA) 7.2 mg, Becton and Dickinson) and 4.5 ml 3.2% citrate tubes (Vacutainer glass, 0.105 M, Becton and Dickinson). Blood was drawn by clean venipuncture in the antecubital fossa and within one hour processed to obtain platelet-poor plasma (PPP). PPP was obtained

by initial centrifugation at 2500 g for 5 minutes (18°C) followed by centrifugation at 10.000 g for 10 minutes (18°C) and stored at -80°C until use. All samples were thawed for 15 minutes at 37°C before testing. One 4.5 ml 3.2% citrate tube (Vacutainer glass, 0.105 M, Becton and Dickinson) was used to collect whole blood for the 'tPA-ROTEM'.

Measurements

Conventional coagulation tests and haematologic parameters

Haemoglobin, haematocrit, erythrocyte sedimentation rate (ESR), thrombocyte count, leucocyte count and leucocyte differentiation, red blood cell distribution width (RDW) and mean platelet volume (MPV) measurements were performed on a Sysmex XN-9000 (Sysmex Corporation, Kobe, Japan) using blood from an EDTA-tube. The measurements of aPTT (Dade[®] Actin[®] FSL Activated PTT Reagent, Calcium Chloride Solution), PT (Dade[®] Innovin[®]), Fibrinogen (Dade[®] Thrombin Reagent), D-dimer (INNOVANCE D-Dimer), and vWF-antigen (vWF Ag), were performed on the Sysmex CS-2100i analyzer (Sysmex Corporation, Kobe, Japan) according to the manufacturer's instructions (all reagents by Siemens Healthcare Diagnostics Products, Marburg, Germany).

Plasma thrombin generation

Thrombin generation in tissue factor-triggered platelet-poor plasma was measured by means of the Calibrated Automated Thrombogram method (Thrombinoscope BV, Maastricht, The Netherlands); according to our in-house standardized procedure.¹⁵ In brief, measurements were conducted on 80 µl platelet-poor plasma with a final concentration of 1 or 5 pM tissue factor (PPP Reagent, PPP Reagent Low; Thrombinoscope BV) and in the presence of 4 µM phospholipids. The measurements were conducted in the absence or presence of 0.56 nM recombinant soluble thrombomodulin (Thrombinoscope).

Whole blood thrombin generation

Whole blood thrombin generation was performed as previously described with minor modifications.¹⁶ Briefly, whole blood (50%) was mixed with a rhodamine based thrombin substrate (300 µM; P2Rho, Diagnostica Stago Gennevilliers, France) and activated with a mixture of tissue factor (0, 0.5 or 2.5 pM) and CaCl₂ (16.7 mM). For calibration, 2-macroglobulin-thrombin complex (100 nM thrombin activity; α2M-T) was added to the mixture of whole blood and rhodamine based thrombin substrate (all concentrations are final concentrations). After activation, the mixture was transferred onto 6 mm paper disks (Whatman 589/1, Whatman GmbH) and covered with mineral oil (USB Corporation). Samples were run in triplicate for 50 minutes and fluorescence was recorded every 6 seconds with a Fluoreskan Ascent multiplate fluorometer with λ_{ex} = 485 nm and λ_{em} = 538 nm (Thermolabsystems, Helsinki, Finland). All procedures were performed at 37°C.

Rotational thromboelastometry

The tissue factor triggered ROTEM (EXTEM based) and the 'tPA-ROTEM' give a graphic representation of the clot formation process, and due to the addition of tPA also include the subsequent fibrinolysis process. Both the ROTEM (EXTEM based) and the 'tPA-ROTEM' were carried out by means of the 4 channel ROTEM[®] Gamma device operated according to manufacturer instructions using tissue factor (Innovin) and tPA (Actilyse). For the tPA modified ROTEM procedure recalcified citrated blood (300 μ l) was incubated, within one hour after venipuncture, at 37°C in a preheated cup. For the purpose of this study the tPA-ROTEM was determined in recalcified citrated whole blood incubated with two different triggers: 35 pM tissue factor alone in the presence or absence of 175 ng/ml tissue plasminogen activator (tPA) (Kuiper et al, unpublished data). The following numerical parameters investigated on whole blood were (1) α -Angle (α) defined as the speed (sec/min) at which the clot is formed; (2) Clot Formation Time (CFT), defined as the time (sec) from initiation of clotting until a clot firmness of 20 mm is detected; (3) Maximum Clot Firmness (MCF) defined as the maximal amplitude (mm) of the clot; (4) Lysis Onset Time (LOT) defined as the time (sec) until 15% reduction of the MCF; (5) Lysis Time (LT) defined as the time (sec) until 90% reduction of the MCF and (6) Fibrinolysis velocity (FV) defined as the decline in % per minute between the LOT and the LT.

Fibrinolysis capacity was assessed with the addition of 175 ng/ml recombinant tPA. This concentration was chosen after our lab determined that this was the optimal concentration to distinguish between a hypo- or normo-fibrinolytical potential in ROTEM-measurements with an acceptable runtime.

Statistical analyses

Statistical analysis was performed using the GraphPad Prism version 6.00 software (GraphPad Software, Inc. For Mac OS X, San Diego, CA, USA) and IBM SPSS Statistics 22 for Windows (Armonk, New York: IBM Corp.). Normality of the data was tested with the D'Agostino & Pearson omnibus normality test. According to the distribution of the variables, data are expressed as median $\pm$ interquartile ranges (IQR) or mean $\pm$ standard deviation (SD). Differences between the results of the PAD-patients with an event, PAD-patients without an event and the healthy controls groups were analysed with the Kruskal-Wallis ANOVA with Dunn's post-test or with the Oneway ANOVA with Boferroni's multiple comparison test. The Mann-Whitney U test was used to test for differences between median values of two separate groups; all PAD-patients and the healthy controls. A p-value of <0.05 was considered to be statistically significant.

The associations between the haemostatic markers, thrombin generation measurements and the (tPA-)ROTEM were assessed using Spearman correlation coefficient. Statistical significance was considered as a two-tailed probability <0.05.

Results

The main characteristics of the study population (n=80) are reported in Table 4.1.

Table 4.1 Demographic and clinical characteristics of the study population (median with IQR).

Characteristics	PAD patients (n=40)	PAD with event during 1 st year FU (n=20)	PAD without event during 1 st year FU (n=20)	Healthy controls (n=40)	p-value
Demographics					
Age, years	67 (65-72)	67 (61-73)	68 (65-72)	67 (60-71)	0.2
Male sex (%)	24 (60)	9 (45%)	15 (75)	22 (55)	0.8
BMI, kg/m ²	26.1 (23.7-29.3)	26.2 (23.5-29.3)	25.9 (24.0-29.8)	26.0 (23.4-27.8)	0.4
Medical history					
Smoking (%)					
Never smoked	1 (2.5)	1 (5)		16 (40.0)	<0.0001
Active smokers	15 (37.5)	8 (40)	7 (35)	5 (12.5)	0.02
Stopped smoking >1yr ago	2 (5.0)	11 (55)	11 (55)	18 (45.0)	<0.0001
Stopped smoking <1yr ago	22 (55.0)		2 (10)	1 (2.5)	<0.0001
Self-reported PV, years	40 (30-45)	37 (30-43)	44 (27-52)	22 (6-37)	0.0032
Non Insulin-Dependent Diabetes Mellitus (%)	5 (12.5)	3 (15)	2 (10)	1 (2.5)	0.2
Hypertension (%)	29 (72.5)	13 (65)	16 (80)	13 (32.5)	0.0007
Previous ischemic coronary vascular disease (%)	5 (12.5)	1 (5)	4 (20)	1 (2.5)	0.2
Previous ischemic cerebral vascular disease (%)	2 (5)	0 (0)	2 (10)	1 (2.5)	0.4
Using thrombocyte 2 inhibitors (%)	38 (95)	20 (100)	18 (90)	2 (5)	<0.0001
Using cholesterol lowering medication (%)	30 (75)	15 (75)	15 (75)	7 (17.5)	<0.0001
Fontaine Classification at diagnosis (%)				NA	
I	1 (2.5)	1 (5)			
Ila	28 (70)	11 (55)	17 (85)		
IIlb	9 (22.5)	6 (30)	3 (15)		
III	1 (2.5)	1 (5)			
IV	1 (2.5)	1 (5)			
Time since initial PAD- diagnosis, years	2.2 (1.9-4.3)	4 (2-4.4)	2.1 (1.9-3.5)	NA	

BMI, Body Mass Index; NIDDM, Non Insulin-Dependent Diabetes Mellitus; NA, Not Applicable. PAD, Peripheral Arterial Disease. Continuous data are expressed as median with interquartile ranges. Dichotomous data are shown as n (%). The P-values were calculated using either the unpaired t-test with Welch's correction, when data were normally distributed or Mann Whitney test when the data were not normally distributed.

Main differences between PAD-patients and healthy controls are the history of tobacco use, the use of platelet aggregation inhibitors, the use of cholesterol lowering medication and the prevalence of hypertension (all significantly higher in the PAD-patients). Inspection of the individual clinical records showed that 17 PAD-patients underwent a revascularisation procedure for PAD, 3 patients had another ischaemic cardiovascular event (one patient experienced a retinal infarction during the revascularisation procedure with two stents for renal artery stenosis, one patient experienced a TIA, one patient had unstable angina with ischaemia in multiple areas on thallium scintigraphy). These 20 events made up the cardiovascular events that classified PAD patients as “cases” ($n=20$), the other 20 PAD-patients (“controls”) had remained free of cardiovascular events during the follow-up after the diagnosis of PAD. Table 4.2 shows the haematological and haemostatic parameters of the study population. In line with previous findings, median levels of the coagulation markers vWF, fibrinogen and D-dimer, were increased in the combined group of PAD-patients, as compared to the healthy controls. Contrasting with previous literature PAD-patients had a lower haematocrit level as compared to healthy controls. Both markers of inflammation, ESR and leucocyte count, were significantly increased in PAD-patients versus healthy controls; besides a significant increase in the eosinophil granulocytes, there were no significant differences in the leukocyte subpopulations. There were no significant differences in the haematologic and haemostatic markers between PAD-patients with and PAD-patients without an event.

Thrombin generation in platelet poor plasma

Global coagulation potential was measured in platelet poor plasma triggered with either 1 or 5 pM tissue factor. In Figure 4.1 parameters derived from the thrombin generation curve performed in platelet poor plasma triggered with 1 pM tissue factor (lag time (LT), endogenous thrombin potential (ETP), peak height (PH), time to peak (TTP)) are depicted for both PAD-patients (with and without event) and healthy controls. There are no significant differences between PAD-patients and the healthy controls. Furthermore no significant differences were found between PAD patients with and patients without an event. Measurements performed in platelet poor plasma triggered with 5 pM tissue factor showed comparable results (data not shown).

To assess possible differences in the activity of the anticoagulant protein C pathway between PAD-patients and healthy controls, the same experiments were performed in the presence of 0.56 nM thrombomodulin (a concentration that was chosen such that it inhibits thrombin generation in pooled normal plasma by 50%¹⁷). The addition of thrombomodulin caused a similar reduction of the endogenous thrombin potential in both healthy controls and PAD-patients and no significant difference between both groups was observed (the median ETP-levels in healthy controls decreased from 773 nM.min to 375 nM.min, in PAD-patients from 801 nM.min to 410 nM.min).

Table 4.2 Hematological and hemostatic parameters of the study population (median with IQR).

Hemostatic parameters with reference values	PAD (n=40)	PAD with event during 1 st year FU (n=20)	PAD without event during 1 st year FU (n=20)	Healthy controls (n=40)	p-value
Hemoglobin (7.3-9.7 mmol/l)	8.7 (8.2-9.3)	8.6 (7.9-9.3)	8.7 (8.4-9.4)	9.0 (8.6-9.5)	0.06
Hematocrit (0.36-0.48 L/l)	0.43 (0.39-0.45)	0.44 (0.38-0.45)	0.43 (0.40-0.45)	0.44 (0.42-0.47)	0.008*
ESR (0-14mm)	8 (6-13)	8 (7.5-13.8)	8 (5-13)	6 (3.5-7)	0.004*
Trombocytes (130-150x10E9/l)	247 (214-282)	258 (222-281)	247 (205-286)	255 (205-291)	0.62
vWF-antigen (50-150%)	149 (119-174)	146 (113-159)	157 (125-203)	133 (107-154)	0.044*
RDW (11.4-14.5%)	13.0 (12.4-13.5)	13.1 (12.3-13.5)	12.8 (12.5-13.7)	12.9 (12.6-13.2)	0.66
MPV (9.2-12.7fL)	11.1 (10.4-11.9)	11.0 (10.6-11.6)	11.2 (10.3-12.0)	10.8 (10.2-11.4)	0.31
Leukocytes (3.5-11.0x10E9/l)	7.5 (6.0-9.1)	7.7 (6.3-8.9)	7.4 (6.0-9.5)	6.2 (5.6-7.5)	0.003*
Lymphocytes (15-48%)	28 (24-33)	29 (24-33)	26 (23-34)	31 (26-35)	0.21
Monocytes (4-11%)	9 (7-10)	8 (6-10)	10 (8-11)	7 (6-10)	0.06
Neutrophil granulocytes (40-70%)	60 (55-64)	60 (55-64)	60 (54-65)	58 (51-63)	0.37
Eosinophil granulocytes (0-10%)	3 (2-4)	3 (2-5)	3 (2-4)	2 (1-3)	0.012*
aPTT (23-32 sec)	26 (25-27)	26 (25-27)	26 (24-28)	26 (26-27)	0.36
PT (9.9-11.5 sec)	10.0 (9.8-10.2)	10.0 (9.8-10.1)	10.0 (9.8-10.3)	10.1 (9.8-10.3)	0.36
Fibrinogen (1.7-4.0 g/l)	3.6 (3.1-3.9)	3.5 (3.1-3.9)	3.6 (3.0-4.1)	3.1 (2.8-3.5)	0.015*
D-Dimer (0-500 ng/ml)	528 (363-835)	497 (358-816)	564 (389-959)	366 (259-520)	0.005*

Abbreviations: ESR, erythrocyte sedimentation rate; vWF, von Willebrand factor; RDW, Red blood cell distribution width; MPV, Mean Platelet Volume; aPTT, activated partial thromboplastin time; PT, Prothrombin time.

Values expressed as median with interquartile ranges. The P-values between the total PAD-group and the healthy control group were calculated using either the unpaired t-test with Welch's correction, when normal Gaussian distribution) or Mann Whitney test when data were not normally distributed. * indicates significant difference ($p < 0.05$). There were no significant differences between the two PAD-groups.

Figure 4.1 Comparison of plasma thrombin generation potential values in PAD-patients and healthy controls

Parameters of thrombin generation in PAD-patients and healthy controls. Thrombin generation was measured following a 1pM tissue factor trigger in the presence of 4μM phospholipids in platelet-poor plasma of PAD-patients with (PAD+) and without (PAD-) a cardiovascular event during the first year after initial diagnosis of PAD and in platelet poor plasma of healthy controls (A.). Thrombin generation measurements of PAD-patients both with and without event were also grouped and compared to healthy controls (B.). Shown are the thrombin generation parameters lag time in minutes, endogenous thrombin potential (ETP), peak height and time to peak in minutes. Data of the groups are presented as median with interquartile ranges and were analyzed statistically as described.

Thrombin generation in whole blood

Furthermore, we determined the thrombin generation capacity in a thin layer of whole blood. In these measurements thrombin generation in blood was triggered with 0, 0.5 or 2.5 pM tissue factor and 16.7 mM CaCl_2 . In Figure 4.2 the parameters (LT, ETP, PH and TTP) derived from the thrombin generation curve performed in whole blood triggered with 2.5 pM tissue factor are depicted for PAD-patients and healthy controls. We observed that the ETP-values were lower in PAD-patients with an event compared to PAD-patients without an event (800 nM.min (SD=105) compared to 935 nM.min (SD=163), $p=0.013$). We observed comparable results at 0 pM tissue factor, however, at 0.5 pM tissue factor no significant differences were observed. There were no significant differences in the other thrombin generation parameters, and there were no differences between PAD-patients and healthy controls.

Rotational thromboelastometry

To assess global clot formation, we performed ROTEM measurements on whole blood samples of PAD-patients and healthy controls following stimulation with 35 pM tissue factor, and analyzed the different parameters (clot formation time, α -angle and maximum clot firmness, Figure 4.3). The clot formation time was significantly diminished in PAD-patients (median CFT: PAD with event 55 sec, PAD without event 50 sec and healthy controls 59 sec). Despite a difference of the median α -angle (denoting the speed at which a clot forms in whole blood) of only 1 degree, this difference was significant and increased in PAD-patients in comparison to healthy controls (79° versus 78°, Figure 4.2B). This increase is, comparable to the CFT, mainly caused by the increase in PAD-patients that did not experience a cardiovascular event as the median α -angle in this group was 80°. Between patients with and without event no differences were observed.

Additionally, with stable blood clot formation, the maximum clot firmness, reflecting the absolute strength of the fibrin and platelet clot, was significantly increased in PAD-patients versus healthy controls (median MCF: PAD 69 mm and healthy controls 65 mm); no differences between PAD-patients with and without event were found.

In order to assess determinants of this increased clot formation capacity in PAD-patients we calculated the correlation coefficients of various haematological and haemostatic parameters and the ROTEM parameters of clot formation. The ESR, fibrinogen levels and thrombocyte count were positively correlated with parameters of clot formation. The haematocrit and haemoglobin levels on the other hand showed an inverse correlation with clot formation parameters α -angle and MCF (Table 4.3).

Figure 4.2 Comparison of whole blood thrombin generation potential values in PAD-patients and healthy controls .

Parameters of whole blood thrombin generation in PAD-patients and healthy controls. Thrombin generation was measured following a 2.5 pM tissue factor trigger in the presence of 16.7 mM CaCl₂ in whole blood of PAD-patients with (PAD+) and without (PAD-) a cardiovascular event during the first year after initial diagnosis of PAD and in whole blood of healthy controls (A.). Thrombin generation measurements of PAD-patients both with and without event were also grouped and compared to healthy controls (B.). Shown are the thrombin generation parameters lag time in minutes, endogenous thrombin potential (ETP) in nM.min, peak height in nM and the time to peak in minutes. Data of the groups are presented as median with interquartile ranges and were analyzed statistically as described, with * p<0.05.

Figure 4.3 Rotational thromboelastometry parameters of clot formation in PAD-patients and healthy controls.

Rotational thromboelastometry (EXTEM-based ROTEM) was assessed for clot formation in whole blood of PAD-patients with (PAD+) and without (PAD-) a cardiovascular event during the first year after initial diagnosis of PAD and in whole blood of healthy controls (**A.**). Parameters of clot formation of PAD-patients both with and without event were also grouped and compared to healthy controls (**B.**). Shown are the α -angle, maximum clot firmness (MCF) and clot formation time (CFT). Data of the groups are presented as median with interquartile ranges and were analyzed statistically as described, with * $p < 0.05$.

tPA-induced fibrinolysis by rotational thromboelastometry

In Figure 4.4 the thromboelastometry parameters representing fibrinolysis of a blood clot in PAD-patients and healthy controls are depicted. These parameters are set as the lysis onset time (LOT; 15% lysis of the clot) and lysis time (LT; when 90% clot lysis has occurred). We also calculated the fibrinolysis velocity (FV), which is the decline of the clot between the LOT and LT in % per minute. To measure the various parameters of clot lysis ROTEM was performed with the addition of 175 ng/ml tPA, this in order to achieve full clot lysis within two hours. There were no significant differences between the parameters of clot lysis between PAD-patients and healthy controls, nor between the two PAD-groups.

Table 4.3 Correlation coefficients between various hematological and hemostatic parameters and the ROTEM parameters of clot formation.

	ESR	Hemoglobin	Hematocrit	Thrombocytes	Fibrinogen
CFT	$r=-0.5$ ($p=3.27 \times 10^{-7}$)	$r=0.5$ ($p=3.48 \times 10^{-6}$)	$r=0.5$ ($p=1.54 \times 10^{-5}$)	$r=-0.5$ ($p=6.84 \times 10^{-7}$)	$r=-0.6$ ($p=1.42 \times 10^{-7}$)
α-angle	$R=0.6$ ($p=2.69 \times 10^{-8}$)	$r=-0.5$ ($p=6.36 \times 10^{-6}$)	$r=-0.5$ ($p=2.61 \times 10^{-5}$)	$r=0.5$ ($p=7.63 \times 10^{-7}$)	$r=0.6$ ($p=2.06 \times 10^{-8}$)
MCF	$r=0.6$ ($p=5.89 \times 10^{-10}$)	$r=-0.5$ ($p=1.42 \times 10^{-5}$)	$r=-0.4$ ($p=0.000365$)	$r=0.4$ ($p=0.000159$)	$r=0.7$ ($p=4.75 \times 10^{-13}$)

Abbreviations: ESR, erythrocyte sedimentation rate; CFT, clot formation time; MCF, maximal clot firmness. Values calculated with the Spearman correlation coefficient.

Figure 4.4 Rotational thromboelastometry parameters of clot lysis in PAD-patients and healthy controls. Rotational thromboelastometry ('tPA-ROTEM') was assessed for clot lysis in whole blood of PAD-patients with (PAD+) and without (PAD-) a cardiovascular event during the first year after initial diagnosis of PAD and in whole blood of healthy controls (A.). Parameters of clot lysis of PAD-patients both with and without event were also grouped and compared to healthy controls (B.). Shown are the lysis onset time (LOT), lysis time (LT) and the fibrinolysis velocity (FV). Data of the groups are presented as median with interquartile ranges and were analyzed statistically as described.

Discussion

In order to explore the coagulation status in patients with PAD we assessed the coagulation-, clot formation- and clot lysis-status with a concise and state of the art panel of assays. This case-control study demonstrates that patients with PAD have a hypercoagulable state as indicated by increased markers of coagulation activity. However, the potential to generate thrombin upon additional stimulation with TF is not different between PAD and controls, or even somewhat lower in those patients with PAD with an ischaemic complication on short-term follow up. The unaltered capacity to generate thrombin in spite of increased levels of formed thrombin suggests a hypercoagulability characterized by consumption of coagulation proteins diminishing the capacity to generate more thrombin. Such a phenomenon (lower levels of thrombin generation capacity in plasma) has recently also been documented for patients PAD in another study¹⁸ as well as in a controlled study of cardiovascular complications in elderly subjects.¹⁹ Our study is the first to also show such an inverse association between thrombin generation, in whole blood, and thrombotic outcomes.

One confounding factor in these patients may be the use of statins. Several studies have indicated that the plasma thrombin generation potential significantly decreased upon the use of statins, already detectable within two months of starting statin therapy.^{16,20-25} Such an attenuating effect on thrombin generation may play a role in the lack of difference between PAD patients and controls as 75% of our PAD-patients versus only 17.5% of the healthy controls used statins to lower cholesterol levels.

The difference in whole blood thrombin generation must be related to the presence of cells in the whole blood assay. While a more pronounced level of depletion of coagulation proteins may be present in whole blood as compared to plasma, direct effects of erythrocytes or leukocytes on thrombin generation cannot be excluded.

The hypercoagulability in PAD patients is further characterized by an increased ability to form a stable clot at an increased rate, without evidence of clot resistance to lysis, as measured by ROTEM. In PAD-patients the α -angle and maximal clot firmness were significantly increased, whereas the clot formation time was significantly shortened, thus indicating that a clot is formed faster and has more firmness than clots formed by healthy controls. However, there were no differences in clot formation between the PAD-patients who experienced a cardiovascular event and the patients who did not.

The increased ability to form a stable clot in PAD-patients is most likely explained by the increased levels of fibrinogen and the increased erythrocyte sedimentation rate, as both were positively correlated with the parameters of clot formation.²⁶⁻²⁹ In a ROTEM-study performed by Lang et al. it was observed that the strength of a clot increased in a fibrinogen concentration-dependent manner independent of platelet count. At normal fibrinogen levels however, the clot strength increased when platelet count increased from $<10 \times 10^3 \text{ mm}^{-3}$ to $50\text{-}100 \times 10^3 \text{ mm}^{-3}$. This increase in clot firmness tended to reach

a plateau phase at normal platelet counts.³⁰ The potential contribution of erythrocytes to the clot formation process requires further investigation. Previously, others observed denser and faster formed fibrin clots in patients with PAD in comparison to healthy controls.^{29,31,32} However, fibrinogen levels are not always implicated unequivocally.³¹ Previous research has demonstrated an increase in Factor XIII, the transglutaminase that is responsible for cross-linking fibrin and thus strengthening the clot, in patients with PAD, albeit only significantly increased in women.³³ This illustrates that clot formation is a complex and most likely multi-factorial process in which fibrinogen cannot be assumed to be the sole player.

Increased fibrinogen levels are known to interfere with plasminogen and therefore reduce the fibrinolytic capacity.²⁹ We therefore expected that PAD-patients would have reduced fibrinolysis. However, we did not observe any differences in fibrinolysis between PAD-patients and healthy controls. These findings contrast with previous findings by Undas et al. who observed that the fibrin clots of patients with PAD had a reduced susceptibility to clot lysis compared to the clots made from plasma from healthy controls. Because the latter measurements were performed in plasma, the effect of platelets and other blood cells on fibrin clot formation and lysis were not included.³¹

The patients in our study are also characterized, as expected, by a chronic inflammatory state, indicated by elevations in the plasma levels of von Willebrand factor, erythrocyte sedimentation rate, leucocyte count, fibrinogen and D-dimer levels. It is commonly assumed that the systemic atherosclerosis in this condition is responsible for these effects, although a direct contribution of an activated coagulation system in blood, to the generation of d-dimer fragments due to combined fibrin formation and increased cleavage, is likely present.^{26,34,35}

Despite the fact that the thrombin generation potential appeared comparable between PAD-patients and healthy controls we did find a significant difference in the ability to build a stable clot between those two groups. Based on our results, the observed increase in D-dimer levels in PAD-patients is not caused by increased fibrinolysis, but more likely is the consequence of the increased (inflammation related) fibrinogen levels and subsequent fibrin turnover and clot formation. Previous studies have shown a correlation between the plasma fibrinogen levels and the fibrin D-dimer levels.³⁶⁻³⁸ This was principally thought to be due to fibrin degradation products (including D-dimer) stimulating the hepatocytes to synthesize fibrinogen through an increased release of interleukin-6.³⁹ However the reverse mechanism, in which increased fibrinogen contributes to hypercoagulability and increased D-dimer levels has also been proposed.^{40,41}

One of the remaining questions of our study is why we observed hardly any differences in the coagulation status between the two PAD-groups: the patients with a

cardiovascular event within one year after PAD-diagnosis and the patients without. The fact that we did not find differences brings us to, possibly, one of the limitations of this study. The defined criteria for 'cardiovascular events' (being CVA, TIA, unstable AP, MI, coronary revascularisation and revascularisation for PAD) may not have been set rigorously enough; a revascularisation procedure for PAD for example is not necessarily a (direct) consequence of a hypercoagulable state. In hindsight we probably should have focused more on *atherothrombotic events* leading to acute ischaemia than on *cardiovascular events*. As 17 out of 20 included patients with events in our study underwent a peripheral revascularization procedure and only 3 patients experienced an atherothrombotic event, this could have influenced our outcomes. The patients that experienced an atherothrombotic event, albeit a small sample size, however did not differ in outcomes of the various coagulation assays from the patients with a revascularization procedure. Another limitation of this study is the fact that our healthy controls did not have their ankle-brachial index measured to exclude PAD. We did however ask the healthy control group to fill in the Edinburgh Claudication Questionnaire (ECQ). This questionnaire has a high specificity (99.3%, 95% CI 98.9-100%) in excluding intermittent claudication. Eligible healthy controls that scored positive on the ECQ therefore were excluded from study participation, thus limiting the chances of including healthy controls with PAD.⁴²

In conclusion, our data show clear evidence of hypercoagulability in whole blood as well as plasma from patients with PAD, up to the level of fibrin clot formation, without obvious effects on clot lysis. This prothrombotic state, linked to a phenotype of systemic atherosclerosis, may play an important role in the high risk of cardiovascular complications and vascular death that is typical for PAD. These findings provide additional basis for new studies on the use of anticoagulant agents, possibly in combination with antiplatelet therapy (the current cornerstone of antithrombotic management in PAD).

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CHAPTER 5

D-dimer as a marker for cardiovascular and arterial thrombotic events in patients with peripheral arterial disease; a systematic review

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Abstract

Background

Peripheral Artery Disease (PAD) is associated with an increased risk for cardiovascular events. D-dimers are a marker for hypercoagulability and are linked with thrombotic events in patients with venous as well as arterial thrombosis. The predictive value of plasma D-dimer levels in relation to cardiovascular events in patients with PAD is not unambiguously established.

Objective

To synthesize evidence evaluating the value of D-dimer as a predictor of arterial thrombotic events patients with PAD.

Design

The Pubmed, Embase, and Cochrane databases were searched (January 1980-November 2012) and 65 abstracts were found. The strategy was supplemented with manual review of reference lists. Case-control, cohort or prospective cohort studies that measured fibrin D-dimer in patients with PAD, were included. Studies were excluded if there was no follow-up for arterial thrombotic events or when no specific information on D-dimer was available.

Results

The search yielded 10 studies for our analysis, comprising 2420 patients with PAD, with a total of 1036 cardiovascular events in 10599 patient years.

Two studies with a follow-up of 1 year show that fibrin D-dimer predicts both deterioration of PAD and subsequent thrombotic events. Five out of six studies with a median follow-up of 2-4 years reveal that an increased D-dimer is predictive of various arterial thrombotic events including mortality. Two studies with a longer follow-up (over 6 years) do not show an independent association between increased D-dimer levels, arterial thrombotic events and CVD mortality.

Conclusion

An increased D-dimer appeared to be independently associated with a two times increased risk of near-term cardiovascular events (RR 2.30 (95% confidence interval 1.43-3.68)). However formal meta-analysis was only feasible for four out of ten included studies. Due to the extended heterogeneity of the included studies cautious interpretation of these data is warranted.

Introduction

Peripheral arterial disease (PAD) is a manifestation of generalized atherosclerosis and is associated with coronary artery disease (CAD) and cerebrovascular disease (CBVD).¹ The mortality rate of patients with claudication is approximately 2.5-times higher than that of patients without claudication.² Literature shows an annual overall major cardiovascular event rate (comprising myocardial infarction, ischemic stroke and vascular death) of approximately 5-7%.² This high risk of death is only partially explained by the coexistence of ischaemic heart disease.³⁻⁵ Within 15 years after the initial diagnosis the all-cause morbidity and mortality rates increase up to 70% and only 20-30% of these patients die of non-cardiovascular causes. Despite extensive research, there still is poor understanding of the natural history of PAD.

Conventional risk factors: e.g. age, smoking, male sex, and coronary artery disease have been associated with increased morbidity and mortality rates.^{3,5,6} More recently thrombogenic and inflammatory risk factors have also been implicated in the pathogenesis of PAD, as well as the acute cardiovascular complications.⁷⁻¹¹ In selected populations, an association between plasma D-dimer level and an increased risk of venous and arterial thrombosis has been proposed.¹⁰ In cardiovascular disease (CVD), an increased fibrin turnover may represent not only a prothrombotic state, but also be a marker for the severity of atherosclerosis.^{10,12,13} Based on a variety of experimental data, it now seems evident that activated coagulation and atherogenesis are closely linked processes.¹⁴

It is known that D-dimer is associated with an increased risk of arterial thrombotic events, irrespective of baseline vascular disease, even after adjusting for confounders such as age, smoking and diabetes, as has been shown in various large prospective studies published over the last 20 years.¹⁵⁻²⁹ In most of these studies a relative risk of around 2 was found for D-dimer in relation with future myocardial infarction, ischemic heart disease or stroke. In a number of the studies, the cohorts were screened for concordant PAD. However, as these PAD-patients were part of the total study-population and the associations were solely calculated for this *total* population, it was impossible to transpose the results to PAD-patients only.^{30,31}

Although the risk factor profile for cardiovascular events is comparable for PAD and CAD/CBVD, the incidence of future events is higher in PAD than the latter two groups.³² Despite clear guidelines on risk factor management in the PAD-population, recent studies show that risk factor control in patients with PAD is reached less frequently in comparison with patients with CAD or CBVD.^{33,34} This stresses the need for further optimization of risk factor management in PAD-patients, as it is known that with good risk factor control the rate of major CV-events in PAD-patients is lower. Given the high risk of cardiovascular death in patients with PAD and the uncertainty regarding the individual risk profile, there is a need for simple markers that can help identify patients at risk. D-dimer is such a simple test, which is widely available and adds no extra burden

as part of routine cardiovascular risk assessment. High-risk patients could thus be monitored and treated more intensely. Our objective was therefore to assess the utility of D-dimer as a marker of morbidity and death due to arterial thrombotic events in the PAD-population.

Methods

A systematic literature search was performed including the Pubmed, Medbase and Cochrane databases to locate all prospective clinical studies on D-dimer as a marker for arterial thrombotic events. We searched the three databases for publications from January 1980-November 2012 using the subject headings (“fibrin degradation products OR D-dimer”) AND (“peripheral arterial disease OR intermittent claudication OR claudicants”) AND (“coronary artery disease OR myocardial infarction OR stroke OR amputation OR thrombotic events OR mortality”). The following search filters were applied: English language, studies using human adults of 19+ years of age.

In an effort to prevent a possible publication bias the authors searched a clinical trials registry (clinicaltrials.gov) for possible (unpublished) studies concerning this topic. Since no studies were found with this search a publication bias cannot be completely excluded however is less apparent.

Study selection

Studies were eligible for this review if: 1. The study contained data on patients with either baseline stated intermittent claudication (IC), symptomatic or asymptomatic PAD of the lower extremities. PAD had to be confirmed with either an ABI<0.9, other diagnostic (imaging) tests such as Doppler ultrasound or angiography showing arterial stenosis or patients had to be treated with stenting or revascularization for flow limiting stenosis, 2. Information on baseline levels of D-dimer could be extracted, 3. Information on cardiovascular events or (cardiovascular) mortality in the follow-up could be extracted.

Studies were excluded if: 1. Patients had no baseline IC or PAD, 2. Fibrin D-dimer measurement was not included as a possible predictor of CVD, 3. There was no follow-up for arterial thrombotic events or (cardiovascular) mortality.

Manual search of the references of all studies meeting the inclusion criteria and abstracts was carried out. Two of the authors (MCK, AtC) reviewed all identified titles and abstracts and independently assessed eligibility (Figure 5.1). Both authors reviewed all data and consensus was reached for all data described. In addition, the quality of all studies included in the review was assessed, using a validated generic checklist designed for quantitative studies³⁵ (Table 5.1).

Figure 5.1 Study selection.

Data extraction

Data were extracted making use of a standard data extraction form, including:

1. information on the study including author, journal, and year of publication
2. total number of patients per included study
3. patient characteristics (patients with PAD alone or in conjunction with other atherosclerotic burden (coronary artery disease etc)
4. definition of PAD
5. type of D-dimer assay
6. Outcome measures (total number of arterial thrombotic events and D-dimer values)
7. Statistical analyses, results and adjustments.

Table 5.1 Methodological quality assessment of the included studies.

	Fowkes 1993 (40)	Smith 1997 (29)	Cortellaro 1994 (37)	Boneu 1998 (42)	Komaov 2002 (41)	Bosevski 2005 (39)	Muscant 2006 (45)	Vidula 2008 (9)	Vidula 2010 (31)	Criqui 2010 (44)
1. Clearly defined objective	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
2. Appropriate study design	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
3. Patient selection appropriate	Y	Y	Y	Y	Y	Y	PA	Y	Y	PA
4. Subject characteristics sufficiently described	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
5. Interventional and random allocation described	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
6. Interventional and blinding investigators reported	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
7. Interventional and blinding subjects reported	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
8. Outcome well defined	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
9. Sample size appropriate	Y	Y	N	Y	Y	PA	PA	PA	PA	PA
10. Analytic measures described appropriately	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
11. Estimate of variance reported	Y	Y	Y	Y	Y	PA	Y	Y	Y	Y
12. Controlled for confounding	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
13. Results in sufficient detail	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
14. Results support conclusions	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y

Y; Yes, N/A; Not Applicable, PA; Partially, N; No

Patients and event calculation

Calculation of the number of included patients and cardiovascular events during follow up was performed by creating a summary table. In this table the number of included patients, the mean follow-up time and the number and the type of events were registered. Per study, the number of included patients was multiplied with the mean follow-up time in years to calculate the total number of patient-years. The number and type of events (if stated in the article) were registered to assess whether they were due to cardiovascular causes or non-cardiovascular causes. Only the cardiovascular events were taken into account in the event calculation. Because we have included two publications that reported on early findings of the same study populations in two later reported studies with increased follow-up duration, we excluded these early papers from the calculation (with the exception of 10 patients in the Fowkes-study with one year follow-up that were not included in the Smith-study with 6 years follow-up).

Study quality assessment

Assessment of the methodological quality of the included studies was performed using a checklist for quality assessment for evaluating primary research papers from a variety of fields.³⁵ This checklist, with a scoring system based on fourteen standardized questions, allows appraising the methodological quality of quantitative studies. This checklist includes criteria which are consistent with the recommendations from the Centre for Reviews and Dissemination (CRD).³⁶ The quality of the included studies was appraised independently by two authors (MK and AtC); inter-rater agreement was reached on all criteria (Table 5.1).

Analysis

The ten selected studies presented different effect size estimates. Some studies indicated the mean difference of levels of D-dimer between patients who experienced cardiovascular events and patients who did not.^{37,38} Other studies used relative risks (RR)^{3,39-42} or hazard ratios (HR) as estimates of relative risk for cardiovascular events. To determine sources of heterogeneity, sensitivity analyses were performed in case of considerable heterogeneity (defined as $I^2 \geq 50\%$). Fixed-effect models or random-effects models were incorporated in the generic variance method of data pooling in Review Manager 5.1 (The Cochrane Collaboration).

Results

Study identification

Our search identified 65 papers. Of these, 24 were excluded based on the title not meeting the inclusion criteria. Of the 41 potentially eligible studies using D-dimer on

patients with either claudication, or diagnosed PAD, 19 had to be excluded based on the abstract, since the patients had no PAD (or claudication) at baseline, or because there was no follow-up for arterial thrombotic events. Hence, of 22 studies the full text was analyzed. One article appeared to be published under two different titles, but the content of the full article was identical for the two publications.^{39,43} After manual screening of the reference lists two extra studies that met the primary inclusion criteria based on title and abstract were found.^{6,42} After assessment of the full text, 14 studies were excluded because they did not meet the inclusion criteria. Finally, ten prospective, observational studies combined measurements of D-dimer at baseline with follow-up for arterial thrombotic events in patients with either diagnosed peripheral arterial disease or claudication (Figure 5.1).

After a second full text assessment, two publications that reported on early findings of the same study population as reported in later studies, were identified^{9,40}; we decided to report the findings of both studies in the narrative part of this systematic review. Therefore in total ten studies were included (Figure 5.1). The ten identified studies represented a total number of 2420 patients (corrected for the overlap of the two extra studies) with either diagnosed PAD (ABI ≤ 0.9) or (intermittent) claudication with a total of 1036 cardiovascular events in 10599 patient years, which translates to 9.77 cardiovascular events per 100 patient years.

The majority of the studies were prospective, observational cohort-studies where D-dimer was used as a laboratory parameter to predict arterial thrombotic events. We created a summary table of study characteristics. The primary outcome was either short-term (1-2 years), or long-term (3-6 years) incidence of all arterial thrombotic events in patients with either claudication or objectively confirmed PAD in relation to a D-dimer measurement at baseline (Table 5.2).

General characteristics of the included studies

Studies identified for this review were published between 1993 and 2011. Three studies contained a patient population where PAD was confirmed with an ABI < 0.9 ,^{9,38,42} in one other study an ABI of either < 1.00 , or > 1.40 was deemed indicative of PAD.⁴⁴ Two studies included a target population of subjects with symptoms of intermittent claudication and in most of these patients an index ABI confirmed the diagnosis of PAD.^{29,40} In two studies PAD had to be confirmed with either angiography, or ultrasound Doppler and duplex scanning of the lower limb arteries.^{37,41} One study included only patients with coexisting PAD and coronary artery disease³⁹ and one other study only included patients with symptomatic disease and at least one lower extremity vascular surgical procedure.⁴⁵

Table 5.2 Characteristics of the included studies.

Source	Type of study	Study population	Outcomes of interest	Confounders	Results	Conclusion
1993 Edinburgh	Longitudinal study Mean FU: 1yr	n=617 Consecutive pts. with intermittent claudication. Mean ABI: 0.58±0.2	- Deterioration of PAD - Risk of coronary events: death, coronary heart disease	- Age - Sex - Smoking - Other coagulation, fibrinolytic and rheological factors	N=36 CHD-events Coronary events RR: 4.4 (1.3-19.0) between top and bottom quintiles (DD<65 ng/ml vs. DD>179 ng/ml)	Increased concentration XLFDP Predict coronary events and progression of PAD
Fowkes, Housley et al. ³⁸						
1994 Milan	Case-control study Mean FU: 1yr	n=50 Pts. with ath. lesions in zone of 10 leg arteries Doppler confirmed or a documented history of revascularization. Mean ABI: 0.74±0.2	- (non)fatal MI - Sudden cardiac death - (non)fatal stroke - TIA - Acute peripheral ischaemia - Peripheral bypass occlusion	- Age - Fibrinogen - tPA antigen - delta tPA antigen - fibrinol. activity - delta fib. activity - PAI-1 - total cholesterol - HDL cholesterol - triglycerides	N= 18 events DD cases: 182.55 ng/ml (±21.89) DD controls: 136.44 ng/ml (±16.41)	D-dimer was significantly higher in subgroup with subsequent thrombotic events.
Cortellaro, Turri et al. ⁴²						
1997 Edinburgh	Cohort study Mean FU: 6yrs	Consecutive pts. with IC Mean ABI: Not stated	Combined: - (non)fatal stroke - MI - AP - coronary events - death - coronary and vascular procedures	- Age - Sex - Packyears - Syst. Bloodpress. - Glucose - Baseline evidence IHD (angina and/or MI)	N= 160 IHD and stroke Total RR: 1.26 (0.97-1.65) S RR: 1.27 (0.87-1.85) NF IHD RR: 1.37 (0.97-1.92)	Haemostatic factors may contribute to prediction of CVD risk, but no longer sign. when adjusted for confounders
Smith, Lowe et al. ³						
1998 Royat	Prospective study Mean FU: 2yrs	n=324 Patients with moderately severe forms of PAD. Mean ABI: 0.64 ± 0.17	- MI - angina - cerebral ischaemic event - clinical progression of PAD: - amputation, - surgery	- History of smoking - Arterial hypertension - Hypercholesterolaemia - Other haemostatic factors	N=51 events D-dimer R: 1.7 (95% CI 0.86-3.47) P=0.12	Levels of Ddimer Not predictive of vascular event when adjusted for confounders
Boneu, Leger et al. ³⁷						

Table 5.2 (continued)

Source	Type of study	Study population	Outcomes of interest	Confounders	Results	Conclusion
2002 Moscow	Longitudinal study Mean FU: 4.2yrs	n=121 Pts. with PAOD and stable claudication (FS II-III), confirmed by angiography/Doppler and duplex scanning of the lower limb arteries	Fatal and non-fatal thrombotic events in any main arterial area: - sudden cardiac death - definite MI - ischaemic stroke - deaths from non- vascular causes	- Conventional cardiovascular risk factors - Baseline clinical manifestations of cardiac, cerebral, and peripheral atherosclerosis.	N=38 thrombotic events DD > 861ng/ml: RR 5.99 (1.3-27.2)	Increased DD is independent Haemostatic predictor of thrombotic events in PADpts
Komarov, Markova et al. ⁴¹		Mean ABI: Not stated Fontaine stage: IIA 7.5% IIB 85% III 7.5%				
2005 Skopje	Observational study Mean FU: 3yrs	n=90 Pts. with PAD and CAD (previous MI/ myocardial revascularization/ clinical symptoms) Mean ABI: 0.58±0.23	- Mortality	- Smoking - Age - Dislipidemia - Arterial hypertension	N=126 events (acute MI, heart failure, peripheral thromboembolism. Hazard ratio: 2.55 for onset cardiovascular events	Pts. Stratified for elevated DD >300ng/ml) Higher mortality than other patients.
Boševski, Borožanov et al. ³⁵		n=332 Pts. with symp. PAD (and/or cerebral vascular disease) in at least one lower extremity and only when ≥1 leg had undergone surgery. Mean ABI: 0.75±0.24	Composite of: - ABI progression - stroke - MI - amputation - death from CV disease - death from any cause	- Age - Gender - Diabetes mellitus - Hypertension - Log-cholesterol - Loghomocysteine level - Smoking	N=19 CVD-death N=161 primary composite Hazard ratio: 2.31 (1.12-4.76)	D-dimer significantly associated with time to subsequent MI (not with stroke, amputation, CV- death)
2006 Oregon	Prospective trial Mean FU 3.2yrs					
Muscant, Moneta et al. ⁴³						

Table 5.2 (continued)

Source	Type of study	Study population	Outcomes of interest	Confounders	Results	Conclusion
2008 Chicago San Diego, Boston	Prospective cohort study Mean FU: 3.4yrs	n=377 Consecutive pts. with lower extremity testing with ABI <0.9. Mean ABI: 0.659 ±0.008.	- (Cardiovascular) death including deaths due to: - coronary heart disease - stroke - PAD - other CV disease	- Age - Sex - Race - Diabetes - Number of CV diseases - Smoking - ABI	N=76 all cause death, not specified for CVD. Hazard ratio 1yr: 1.2 (1.08-1.33) Hazard ratio 2yrs: 1.14 (1.02-1.27)	Increased DD and inflammatory biomarkers independently associated with higher mortality
Vidula, McDermott et al.9	Observational prospective study Mean FU: 3.7yrs	n=569 Consecutive pts. with PAD (ABI ≤ 0.9). Mean ABI: 0.64±0.15	- (Cardiovascular) death	- Age, gender, race - ABI and BMI, PY - Stroke - Angina, MI and heart failure - Diabetes - Hypertension - Pulmonary disease - Cancer - TC and HDL	N=43 CV-death	Not stated in the text: Ddimer is associated with CVD-events
2010 Chicago Boston Chapel Hill	Cohort study Average FU: 6.6yrs	n=379 Pts. seen in pr. 10 years for non-invasive arterial testing with ABI <1.00 or >1.40, or PPTF- velocity of <11cm/sec, or previous leg vascularization Mean ABI: Not stated	- All cause mortality - CVD mortality - non-CVD mortality	- Age - Gender	After 6.6 years: N=150 CVD-death. Comparison survivors vs. CVDmortality: DD: 920 ng/ml vs. DD 1020 ng/ml resp. P-value of 0.4	D-dimer did not meet P<0.1 inclusion criterion for the multivariable Survival models: DD did not significantly predict mortality.
Críqui, McDermott et al.40						

Results given with 95% confidence intervals. Abbreviations: XLFPD; cross-linked fibrin degradation products, IC; intermittent claudication, PAD; peripheral arterial disease, MI; myocardial infarction, CHD; coronary heart disease, F; fatal, NF; non-fatal, TIA; transient ischemic attack, AP; angina pectoris, IHD; ischaemic heart disease; PAOD; peripheral obstructive arterial disease, FS; Fontaine stage, ABI; ankle-brachial index, CVD; cardiovascular disease, CA; coronary angiography, CV; cardiovascular disease, CBVD; cerebral vascular disease, PPTF; peak posterior tibial flow velocity, RR; risk ratio, HZ; hazard ratio, DD; D-dimer, PY; packyears, TC; Total cholesterol.

There was variability in the set exclusion criteria concerning previous procedures, disease severity, cardiovascular morbidity and medication for the in this review included studies. Also, the follow up duration differed; three studies had a follow-up of 1 to 2 years, five studies had an average follow-up of three to four years while two studies had a mean follow-up of more than six years. Four studies were performed in the United States of America (Chicago and Oregon), the other six studies were centered in Europe (Royat, Milan, Edinburgh, Moscow and Skopje). A point of practical interest is the type of D-dimer assay used. Nine of the ten studies included in this review made use of a quantitative D-dimer assay; only in one study a semi-quantitative assay was used (Table 5.3).

Table 5.3 D-dimer tests used in the assessed studies.

Authors	Product	Supplier
Fowkes et al.	Elisa	Agen
Smith et al.	Elisa	Agen
Cortellaro et al.	Dimertest®	Ortho
Boneu et al.	Asserachrom® D-dimer	Stago/Roche
Komarov et al.	Elisa	Boehringer-Mannheim
Bosevski et al.	Turbiquant®	Dade-Behring
Musicant et al.	Electro immunoassay	Trinity Biotech
Vidula et al.	Asserachrom® D-dimer	Stago/Roche
Vidula et al.	Asserachrom® D-dimer	Stago/Roche
Criqui et al.	K-assay D-dimer kit	Kamiya

Study quality

Overall the studies were of acceptable to good quality: the overall reporting of the studies, the study design and selection of study population and statistical analysis were adequate. Since all studies were observational no randomization and blinding reports were applicable. All studies reported the results in sufficient detail and drew conclusions based on the stated results. The main weakness of some of the studies was the number of included patients, which was often too small to yield an adequate number of outcomes (Table 5.1).

Analysis

The data representation of five out of ten of the included studies did not allow us to make 2x2 tables or use the generic inverse variance method. The mean estimated risk for an increased D-dimer for the prediction of arterial thrombotic events could therefore only be calculated for a subgroup of studies. We pooled the data of four studies,^{3,38,40-42} based on the fact that they were similar for duration of follow-up (short to intermediate), patient population (ABI 0.58-0.64 or Fontaine IIB), all analyses were adjusted for confounders, and the outcome measures could be considered similar. All

studies reported on (combined) fatal and non-fatal cardiovascular events except for the study by Vidula et al. that solely reported cardiovascular mortality. However, in excluding the data from this study, the heterogeneity increased, resulting in an I^2 of 40%, $p=0.19$. Therefore we decided against excluding this study. The meta-analysis now includes data of four studies. Pooling the RRs resulted in an overall relative risk of 2.30 (95% CI 1.43-3.68) with an I^2 of 16% ($p=0.31$) (Figure 5.2).

a. Meta-analysis with the 2010 Vidula study excluded

b. Meta-analysis with the 2010 Vidula study included

Figure 5.2 Meta-analysis. A) 2010 Vidula study (38) excluded. B) 2010 Vidula study (38) included.

Study results

The first study that assessed the value of D-dimer as an independent predictor for arterial thrombotic events was performed in 1993 by Fowkes et al.⁴⁰ followed by a report on the extended follow-up of six years for the same study population in 1997 by Smith et al. In the first report, results of 617 consecutive patients diagnosed with PAD were published. In accordance with findings of the previous Northwick Park study, plasma fibrinogen concentration and age were the most important independent predictors of coronary events. In addition, the plasma concentrations of cross-linked fibrin degradation products (XL FDP) were independently associated with coronary heart disease events (combined fatal and non-fatal), as well as progression of PAD, the latter being measured by a decrease in ABI. Relative risks for combined CHD-events were shown as quintiles of baseline-XL FDP expressed relative to the lowest quintile. An increased baseline-XL FDP was independently associated with a fall in ABI (indicating further progression of PAD) as well, even after adjustment for baseline ABI.

In 1997, the long-term outcomes for the remaining PAD-patients ($n=607$) were presented.³ The prospective cohort showed that baseline levels of fibrin D-dimer were higher in claudicants who subsequently developed ischaemic heart disease or stroke

during this longer follow-up when compared to the group that did not develop events. However, after adjusting for confounding factors (Table 5.2) none of the haemostatic factors were significant predictors anymore. The authors concluded that haemostatic factors, one of which being D-dimer, *might* have a possible contributory role in the prediction of CVD.

In 1994 the Italian group of Cortellaro et al. found in a cohort of 50 patients with leg atherosclerosis, combined in 40% of the cases with coronary and/or cerebral atherothrombotic disease, that the subset of patients that experienced subsequent thrombotic events had higher D-dimer level at baseline than the patients remaining free of thrombotic events during the follow-up of 1 year.³⁷ A major weakness of this study was however an insufficient sample size and therefore the data could not be subjected to multiple regression analysis.³⁷

In the study by Boneu et al. patients with moderately severe peripheral arterial disease (n=324) were included while on annual stay in the thermal resort of Royat.⁴² Blood samples were taken during the first ten days of the patients stay with follow-up examinations performed at one and two years during subsequent visits. D-dimer levels were associated with a slight, progressive increase in risk of a vascular event across successive higher quartiles (quartile 4 versus quartile 1, $P=0.02$), however these results became non-significant when major vascular events (vascular death, cerebral ischaemia, cardiac events, acute peripheral ischaemia) were considered separately. When the relative risks were adjusted for potential confounders, D-dimer levels were no longer predictive of a vascular event ($RR=1.7$, with 95% CI 0.86-3.47: $P=0.12$). As potential explanations for these findings the authors suggested that the included patients only presented with PAD of moderate severity, while subsequent life style modification may have further attenuated the risk of thrombotic events.

In 2002, Komarov et al. intended to evaluate the frequency of thrombotic events with peripheral arterial occlusive disease during a follow-up period of 3-5 years and determine whether the baseline levels of haemostatic factors, including D-dimer, were related to the risk of future thrombotic events.⁴¹ During the mean observation period, from entry to final evaluation (average of 4.2 years) there were 54 validated outcome events. The main findings were that PAD-patients who had D-dimer levels that exceeded the upper limit of normal were at increased risk of developing thrombotic events, it was shown that a baseline D-dimer level in the highest quintile (>861 ng/ml) was associated with arterial thrombotic events compared to a baseline D-dimer level of the lowest quintile (<280 ng/ml). This finding was independent of conventional cardiovascular risk factors and baseline clinical manifestations of cardiac, cerebral and peripheral atherosclerosis. The authors emphasized the relevance of fibrinolytic activity

in relation to the D-dimer increase, in showing an inverse correlation between D-dimer and PAI-1 activity.

Bosevski and colleagues (2005) examined whether the value of D-dimer in plasma could predict mortality in patients with both PAD *and* coronary artery disease (CAD). In the 90 patients with coexistent PAD and CAD, the subset stratified for an elevated plasma level of D-dimer >300 ug/ml showed a higher mortality. The authors concluded from this, that D-dimers are a strong predictor of mortality in patients with PAD and CAD supporting the concept that activated thrombogenesis dictates the clinical outcome.³⁹

A study performed around the same time by Musicant et al. assessed the relationship between elevated baseline levels of D-dimer (and biomarkers of inflammation) and the progression of PAD in patients with symptomatic PAD.⁴⁵ Remarkably, patients were only included in this study if they had undergone vascular surgery in at least one leg or carotid artery. The hypothesis that D-dimer was associated with progression of PAD and/or CAD was not confirmed, as only a significant association between baseline D-dimer and myocardial infarction was found ($P=0.04$). Explanations for the fact that no other associations were found were the limited sample size as well as the length of follow-up (average of 38.4 months (± 20.4 months)). The authors drew the overall conclusion that, even though the D-dimer levels are significantly associated with time to subsequent myocardial infarction, there is little role for the use of D-dimer as initial screening test for the presence of PAD or for the risk of progression of symptomatic PAD.

Vidula et al. published two studies on D-dimer and PAD.^{9,38} The objective of the first study, published in 2008, was to determine whether elevated levels of D-dimer were more closely associated with short-term rather than long-term mortality in patients with PAD ($n=377$) and whether greater increases in biomarker levels were associated with higher mortality rates during the first year after the increase than during the later years. It was found that higher levels of D-dimer were associated with higher all-cause mortality among patients who died within one year, and in the time span between one to two years after biomarker measurement. Elevated levels of D-dimer were not associated with greater all-cause mortality two to three years after the measurement. Among the deceased, it was found that D-dimer levels were highest at the last visit prior to death. Higher D-dimer levels during each follow-up interval were statistically significantly associated with cardiovascular mortality. The results did not substantially vary when analyses were repeated and adjusted for covariates including age, sex, race, diabetes, number of cardiovascular diseases, smoking and the ABI. A greater *increase* in D-dimer level between baseline measurement and one-year follow-up measurement was associated with a significantly increased risk for all-cause mortality and CVD-mortality during the first year after the first annual follow-up visit. *Increase* in D-dimer

level between baseline and two-year follow-up measurement was only associated with a higher all-cause mortality, but not CVD-mortality, during the following year after the increase. According to the authors short-term increases in D-dimer levels may reflect temporary increases in atherosclerotic plaque remodelling. A limitation of the study was that especially for CVD-deaths sample sizes were too small to confidently predict mortality. Comparing their study to previous ones, the authors mentioned that cardiovascular disease accounted for fewer than 50% of the deaths, something that possibly could be attributed to improved treatment of atherosclerotic risk factors.

In the study performed by Vidula et al. (2010) the primary objective was different to that of the 2008 study: to determine whether statin use was associated with cardiovascular mortality in PAD-patients according to presence or absence of increased D-dimer levels. The included patient population of this study was, according to the authors (Dr. McDermott, personal communication), partially identical to that of the 2008-study. The median level of D-dimer was 650 ng/ml (measured in baseline samples of 587 patients). During the mean follow-up of 3.7 years, 43 of the in total 129 deaths were attributable to CVD. Of the patients with a D-dimer level above 650 ng/ml 28 died due to cardiovascular causes, of the patients with a D-dimer below 650 ng/ml only fourteen died due to CVD. Statin users had a significantly lower all-cause and CVD-related mortality compared to statin nonusers. Since no statistically significant interaction was found between the high versus low D-dimer level and statin use in the analyses of statin use with mortality it was concluded that participants with baseline D-dimer values >650 ng/ml did not have significantly greater benefit from statin use than those with baseline D-dimer values <650 ng/ml.

The main objective of Criqui et al. (2010) was to determine whether novel biomarkers, including D-dimer, could improve risk prediction of (CVD-) mortality beyond the standard risk factors in PAD. Noteworthy in this study is the definition of PAD, which was set as a peak posterior tibial flow velocity of <11cm/sec, a previous leg revascularization (angioplasty or surgery) or an ABI of <1.00, or >1.40. Cox-proportional hazard models were fitted for the mortality endpoints, being all-cause mortality, CVD mortality and non-CVD mortality for two years and at long-term follow-up. Beside CRP, none of the other biomarkers, including D-dimer, significantly predicted any kind of mortality. According to the authors however, at a full follow-up, the already known, standard risk factors dominated the c-statistic. This led to the conclusion that the morbidity and mortality in PAD-patients may depend on the baseline risk composition of the cohort and the length of the risk period.

Discussion

We conducted this systematic review of the literature to identify the value of D-dimer as a predictor of adverse cardiovascular events in PAD-patients. Ten studies from six different Western countries were included in the review. To the best of our knowledge this is the first systematic review featuring all available evidence on D-dimer and the follow-up of patients with PAD exclusively.

We found that increased D-dimer was independently associated with a two times increased risk of near-term cardiovascular events (RR 2.30 (95% CI 1.43-3.68)). However, formal meta-analysis was only feasible for four out of ten included studies. We were able to pool data for these studies^{3,38,40-42} because they were similar for duration of follow-up (short to intermediate), patient population (ABI 0.58-0.64 or Fontaine IIB) and outcome measures.

Overall, we encountered several sources of heterogeneity, making it difficult to perform a meta-analysis for most study data. In some studies patients were included directly upon diagnosis of PAD, while in other studies patients that had already been diagnosed with PAD during the past decade could be included. In the majority of the studies, the duration of PAD was not clearly described making it difficult to draw proper conclusions. While diagnostic criteria upon inclusion in general will adjust for major differences in severity of PAD, the use of slightly deviant criteria in some studies may have had some impact on the study results, but also on the baseline D-dimer levels. Extent of the disease was difficult to assess as study populations differed in apparent duration of disease at baseline.

Concomitant vascular disease, another possible indicator of the extent of the disease, was quite variable in the different studies. In the study performed by Bosevski, for example, only patients with concomitant coronary and PAD were included, whereas in the Boneu study all patients with concomitant vascular disease were excluded from participation. Comorbidity, such as cancer or other (inflammatory) diseases could also have influenced baseline D-dimer levels, as well as study outcomes. Several studies therefore excluded patients with cancer or evidence of diseases with a possible fatal prognosis in the following two years. In the Vidula studies however, the percentage of included patients with cancer and pulmonary disease was quite high (up to 23% and up to 38%, respectively). The average baseline D-dimer level found in these populations was therefore probably also relatively high (>950 ng/ml), clearly higher in comparison to the baseline levels of the other studies. Unfortunately, in most of the included studies cancer or inflammatory disease rates were not even assessed and made it therefore unclear what the influence was on the baseline D-dimer levels.

The percentage of patients with diabetes and arterial hypertension varied from 8% to 55.5% and from 20% to 91%, respectively. Previous studies have not observed consistent significant correlations of D-dimer with diabetes mellitus (type 2) and systolic blood pressure.^{46,47} However it is known that diabetes and hypertension can

contribute to cardiovascular mortality. The difference in included patient populations, although probably not contributing to differences in baseline D-dimer levels, might have significantly influenced the study results. As most studies were performed around the end of the 20th century, the treatment of PAD for all patients of the studies included in the review can be considered rather well comparable with similar attention for lifestyle changes, antiplatelet therapy and lipid control. Nevertheless, in the majority of studies the statin and antiplatelet administration was not described making it difficult to estimate the influence on outcome and D-dimer levels.

Comparing the ten studies, a division can be made into three different groups: studies with a short term follow-up of one year, studies with an intermediate follow-up of 2 to 4 years and studies with a long follow-up duration of over six years. The studies performed by Fowkes and Cortellaro, both with a follow-up of one year showed that fibrin D-dimer predicted both deterioration of PAD and subsequent thrombotic events. When the duration of the follow-up of the Fowkes-cohort increased to over six years, D-dimer levels lost their independent significant association after adjustment for confounders. Also in the study by Criqui 2010, D-dimer was not able to predict neither all-cause-, nor CVD-mortality. Nevertheless, the three studies with an intermediate follow-up that could be pooled do show a significant positive association with increased D-dimer. The study performed by Boneu et al. with a two-year follow-up, showed no predictive value of D-dimer in arterial events when adjusted for confounders such as smoking etc. The average onset of disease however was 9.3 years before inclusion in the study.

A pathophysiological explanation for the association between high plasma D-dimer levels and the increased risk of arterial thrombotic events in the *near-term* period following the measurement can possibly be found in the fact that D-dimer is part of the so-called inflammation-coagulation-axis. The role of inflammation in atherosclerosis progression and plaque destabilization has been clearly established (increase in CRP and other pro-inflammatory mediators like IL-1 and IL-6), while the role of coagulation in this process is not yet fully clarified.¹⁴ In previous studies it has been found that an increased CRP and atherosclerotic progression are positively associated with an increase in fibrinogen and a procoagulant response causing an increase in the formation of cross-linked fibrin.²⁹ It has been suggested that the generation of several haemostatic enzymes (e.g. thrombin) and the increase in fibrin contribute to atherosclerosis progression and modulation of the plaque phenotype. Indeed, cross-linked fibrin is a detectable component of the atherosclerotic plaque.¹⁶ It may be assumed that a greater vascular burden of fibrin generates a larger amount of fibrin cleavage fragments, due to ongoing fibrinolysis, in patients with extensive atherosclerosis.¹⁶ Supporting this model is the fact that with an increased severity and larger extent of PAD, and subsequently a higher fibrin level, also higher D-dimer levels are found.¹¹ In a general population with presumed lower disease severity a recent

meta-analysis showed that D-dimer was indeed more modestly, but still significantly associated with first CHD events.⁴⁷

An important question that remains is whether D-dimer is: **a.** causally related to thrombogenesis through temporary increase in plaque remodelling leaving a plaque more prone to rupture or **b.** solely an indicator of increased fibrinolysis. However in both cases it seems likely that the fibrinolytic system is a fast acting system that responds to the acute situation (as needed in the normal situation to preserve haemostasis) and therefore a good predictor of near-term events. The traditional atherosclerotic risk factors (smoking, diabetes etc) predict long-term cardiovascular events and mortality better than D-dimer levels.⁹

Variation in D-dimer tests has been an issue of debate because of the potential discrepancies between test outcomes due to differences between calibrators and different manufacturer cut-off values.⁴⁸ However, Boeer et al. showed that even though there was no standardization among the commercially available D-dimer assays, most assays showed similar characteristics concerning the detection of D-dimer fragments.⁴⁹ The largest factor influencing the D-dimer outcome was suggested to be the cut-off values used by the manufacturers in relation to the study population. In the assessed studies self-set cut-off values for high vs. low levels of D-dimer based on the baseline measurements of the study population and not on the manufacturers cut-off values were used. Manufacturer influences on cut-off values are therefore not to be expected. Just as with follow-up duration, comparing the ten studies for D-dimer levels, a division can be made into three different groups: studies with a low D-dimer cut-off (<200 ng/ml), studies with an intermediate D-dimer cut-off value (200-<900 ng/ml) and the studies with a high D-dimer cut-off value (>900 ng/ml). These variations might be due to concomitant diseases in the different study populations, influencing the height of the mean baseline D-dimer levels. Both in studies with a high mean baseline D-dimer level and in studies with a lower mean baseline D-dimer level significant associations were found between D-dimer and arterial thrombotic events.

This review supports the hypothesis that plasma D-dimer is a potential risk predictor for cardiovascular events. However, it remains unclear whether D-dimer, or the formation of the precursor product, cross-linked fibrin, plays a causal role in the pathophysiology of these adverse events, or whether D-dimer is simply a marker of the extent of the disease and therefore related to the event.⁵⁰

Conclusion

Elevated levels of D-dimer are associated with a two times increased risk of arterial thrombotic events and CVD mortality in the time period following this measurement in

patients with PAD and could therefore be applied as a simple tool to identify patients at increased risk. Elevated D-dimer seems to be a better predictor for the short-term (<4 years) rather than the long-term risk of arterial thrombotic events and CVD-mortality.

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CHAPTER 6

Hypercoagulability does not predict short-term cardiovascular complications in patients with peripheral arterial disease

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Submitted

Abstract

Background

Thrombogenic and inflammatory risk factors have been implicated in both the pathogenesis of peripheral arterial disease (PAD) and the occurrence of cardiovascular complications in PAD-patients.

Design and methods

Between 2009 and 2013, 301 consecutive newly diagnosed PAD-patients were enrolled in a prospective study evaluating the association of various haemostatic and rheological factors (D-dimer, fibrinogen, von Willebrand factor (vWF) and thrombin generation testing by means of the Calibrated Automated Thrombogram (CAT)-assay on platelet poor plasma) on the progression of PAD, the occurrence of cerebro- and cardiovascular endpoints and a composite endpoint of both progression of PAD and cerebro- and cardiovascular endpoints. The relationship between time to the various endpoints and baseline haematological and rheological variables was examined by univariate analysis and Cox proportional hazards analysis.

Results

Adequate baseline samples were available in 280 subjects (mean age 66 ± 8.4 yrs; 65.4% men) with the majority of patients classified as Fontaine IIa (58.1%) or Fontaine IIb (33.6%). The mean follow-up time was 319 days (range 1-770 days). Two patients reached a cardiovascular endpoint and subsequently died, three patients died due to non-vascular causes. Mean baseline levels of D-dimer, fibrinogen and vWF were at the upper limit of the reference interval (454 ng/ml, 3.6 g/l and 150%, respectively) as was the normalized peak of the thrombin generation curve (132%). Progression of PAD occurred in 37.1% of patients, 6.8% of patients experienced a cerebro- or cardiovascular endpoint during follow-up. Diabetes was the only risk factor significantly associated with a cerebro- or cardiovascular endpoint during follow-up in the univariate analysis. None of the biomarkers for hypercoagulability, including thrombin generation, predicted vascular outcomes or progression of PAD, except for fibrinogen levels exceeding 3.6 g/l that had a significant inverse association for progression of PAD (hazard ratio (HR) 0.65 (95% CI: 0.43 to 0.98, $p=0.04$)). In the cox proportional hazard regression diabetes remained an independent predictor of cerebro- and cardiovascular events with a HR 5.37 (95% CI 1.79 to 16.11, $p=0.003$). A fibrinogen level exceeding 3.6 g/l significantly decreased the risk of progression of PAD with a HR of 0.61 (95% CI 0.39 to 0.95, $p=0.03$). Surprisingly, a haematocrit level of >0.43 L/l was inversely associated with the composite endpoint with an HR of 0.49 (95%CI 0.24 to 0.98, $p=0.043$).

Conclusions

The risk of atherothrombotic events in the short term was not associated with any of the studied markers of coagulation, except for fibrinogen of which higher levels (>3.6 g/l) were associated with a 39% decreased risk of progression in patients with PAD. Apparently, plasma hypercoagulability is not a predominant factor in the aetiology of short-term cardiovascular complications in patients with PAD.

Introduction

Peripheral arterial (occlusive) disease (PAD) is a common manifestation of atherosclerotic vascular disease and associated with significant morbidity and mortality. This increased morbidity and mortality is mainly due to cerebrovascular and cardiovascular events. The annual event rate of major cardiovascular events (comprising myocardial infarction, ischemic stroke and vascular death) in PAD-patients is approximately 5-7% and is related to the severity of the disease.¹ PAD is a growing clinical problem as the prevalence of PAD increases with age and the proportion of aged persons in the world will increase steadily over the next century.² Beside the morbidity of cerebro- and cardiovascular events, PAD itself has been shown to confer a negative effect on quality of life and health status, comparable to that of heart failure.³ Considering this burden of PAD there is a need to identify novel risk factors as that may allow earlier detection and an improved understanding of disease aetiology and progression, which could potentially lead to therapeutic targets or even prevention of PAD.

Well-known risk factors that favour the development and the occurrence of cardiovascular complications in PAD include among other things smoking, hypertension, and hypercholesterolemia. Additionally, thrombogenic and inflammatory risk factors have been implicated in both the pathogenesis of PAD and likewise the occurrence of cardiovascular complications.⁴⁻⁸ Various studies show that there is a *systemic* increase in the levels of various haemostatic and rheological factors indicating a hypercoagulable state in PAD-patients including von Willebrand factor (vWF), prothrombin fragment 1.2 (F1.2), thrombin-antithrombin (TAT) complexes, fibrinogen and D-dimer.^{7,9-17} Furthermore, in atherosclerosis, there is not only a *systemic* increase of coagulation activity, yet also *locally*, within the atherosclerotic vessel wall, there appears to be increased activity of the coagulation system. It has been suggested that this local coagulation activity may play an important role in the onset of atherothrombosis and in atherogenesis itself.¹⁸ Measurement of (one of) the aforementioned thrombotic markers could therefore be of help to identify patients at risk of accelerated progression of arterial disease or the risk of developing a cerebro- or cardiovascular event.¹⁶ In recent years test systems have been developed that can assess the *in vitro* potential of plasma to generate thrombin: as thrombin is a key enzyme in the coagulation cascade these thrombin generation assays potentially provide us with useful information about the (pro-) thrombotic propensity of the individual patient.

A number of studies have shown that increased levels of haemostatic markers, such as fibrinogen and D-dimer, are linked with the earlier appearance of PAD and are predictive of cardiovascular mortality in PAD-patients.^{11,19-22} For other haemostatic factors, such as vWF, this association has so far remained undetermined.^{14,23} Data on the predictive value of thrombin generation potential for progression of PAD and/or

occurrence of cerebro- or cardiovascular events in patients with PAD is still scarce. The aim of the present study was to assess whether elevated baseline levels of haemostatic factors, vWF and fibrinogen, in combination with the *in vivo* haemostasis activation marker D-dimer and the thrombin generation potential, are correlated to severity of PAD and subsequently associated with the short-term occurrence of cerebro- or cardiovascular events and progression of PAD in patients with newly (<3 months) diagnosed occlusive PAD of the lower extremities.

Patients and methods

Patients

This study was a follow-up investigation of a cohort of 280 consecutive patients with newly diagnosed PAD with ages ranging from 37 to 87 years (183 males and 97 females) from three different Limburg based hospitals (Zuyderland Medical Centre Heerlen and Sittard and the Maastricht University Medical Centre, Limburg, the Netherlands). Patients were included between January 2009 and November 2013 after written informed consent of the patients was obtained. The study protocol was approved by the institutional Medical Ethical Committees and conforms to the ethical guidelines of the 1975 Declaration of Helsinki (Seoul 2008).

The diagnosis of PAD was based on an in-hospital performed ankle brachial index (ABI) of ≤ 0.9 , following initial clinical assessment with history taking and physical examination performed by the treating physician. Exclusion criteria for participation in the study were the use of medication known to directly affect coagulation (e.g. coumadins, direct factor Xa-inhibitors and direct factor II-inhibitors, and heparin), known coagulation disorders, age under 18 years, the presence of active malignancy or chronic inflammatory diseases (patients with COPD Gold I and II were included as well as patients with a history of gout, as long as there were no recent exacerbations). Patients who underwent a cardiovascular intervention/ arterial (peripheral) surgery within the past six months and patients with an episode of (unstable) angina pectoris, myocardial infarction, stroke or heart failure within the past three months were excluded as well.

Sample size

In our PAD-population we anticipated to find a group with an accelerated progression of PAD with a high incidence of vascular events and a group with a slow and steady progression with a lower incidence of vascular events. The annual event rate at the start of our study was estimated at respectively 12.5% and 2.5%. Based on these values a power-analysis with a power of 0.90 and a significance level of 0.05 was performed which led to a required sample size of 280 patients.

Baseline examination

Patients were asked to participate in the study and were subsequently included within three months after the initial diagnosis of PAD. On entry patients were seen by a study physician or research nurse and were questioned about their medical (cardiovascular) history, intermittent claudication via the Edinburgh Claudication Questionnaire, smoking and drinking history and current medication. A sample of 30ml of venous blood was taken for analysis of haemostatic and rheological factors.

Prescription medication use, smoking status (including pack years) and family history of cardiovascular disease were self-reported. Previously diagnosed diabetes and hypertension at baseline were self-reported and checked in patient files. Previous cardio-vascular disease was defined by physician-diagnosed (stable) angina, myocardial infarction or cardiovascular intervention. Previous cerebro-vascular disease was defined as stroke or transient ischemic attack.

Blood collection and plasma preparation

Venous blood was collected in a 4.0 ml EDTA-tube (Vacutainer plastic, K2E (EDTA) 7.2 mg, Becton and Dickinson) and 4.5 ml 3.2% citrate tubes (Vacutainer glass, 0.105 M, Becton and Dickinson). Blood was drawn by clean venipuncture in the antecubital fossa and within one hour processed to obtain platelet-poor plasma (PPP). PPP was obtained by initial centrifugation at 2,500g for 5 minutes (18°C) followed by centrifugation at 10,000 g for 10 minutes (18°C) and stored at -80°C until use. All samples were thawed for 15 minutes at 37°C before testing.

Measurements

Conventional coagulation tests and hematologic parameters

Haemoglobin, haematocrit, erythrocyte sedimentation rate (ESR), thrombocyte count, leucocyte count and leucocyte differentiation, red blood cell distribution width (RDW) and mean platelet volume (MPV) measurements were performed on a Sysmex XN-9000 (Sysmex Corporation, Kobe, Japan) using blood from an EDTA-tube. The measurements of aPTT (Dade[®] Actin[®] FSL Activated PTT Reagent, Calcium Chloride Solution), PT (Dade[®] Innovin[®]), Fibrinogen (Dade[®] Thrombin Reagent), D-dimer (INNOVANCE D-Dimer), and vWF-antigen (vWF Ag), were performed on the Sysmex CS-2100i analyzer (Sysmex Corporation, Kobe, Japan) according to the manufacturer's instructions (all reagents by Siemens Healthcare Diagnostics Products, Marburg, Germany).

Thrombin generation measurements

Thrombin generation was measured by means of the validated Calibrated Automated Thrombogram method (Thrombinoscope BV, Maastricht, The Netherlands) in tissue factor-triggered platelet-poor plasma. Measurements were conducted on 80 µl human

platelet-poor-plasma with 20 μ l fluorogenic substrate, Calcium chloride (FluCa) and 20 μ L trigger reagent. In this study a final concentration of 1pM tissue factor (TF) was used as a trigger with 4 μ M phospholipids (at 20:20:60 mol% PS:PE:PC), experiments were also performed in the absence of TF. The CAT reagents were obtained from Thrombinoscope BV. Measurements were performed according to our standardised protocol as described earlier.²⁴ With this method thrombin activity is continuously monitored by a low affinity fluorogenic substrate for thrombin (Z-Gly-Gly-Arg-AMC). The Fluorescence is then read by a Fluoroskan Ascent reader with a 390/460 filter set (Thermo Labsystems OY, Helsinki, Finland). Thrombin generation curves are then calculated with Thrombinoscope software (Thrombinoscope BV). To correct for substrate consumption and inner-filter effects a calibrator sample from the same plasma is used to which a fixed amount of thrombin- α 2-macroglobulin (20 μ l Thrombin Calibrator) and 20 μ l FluCa is added. The lagtime, the endogenous thrombin potential (ETP), the normalized endogenous thrombin potential, the Peak Height, normalized peak height, time to Peak (ttP) and Velocity Index (VI) were the thrombin generation parameters chosen a priori to be the primary predictor variables. With Lagtime reflecting the initiation phase of coagulation (the moment at which 10 nM thrombin is formed), the Peak Height showing the maximum amount of formed thrombin expressed in nmol/l, the time to peak indicating the time in minutes required to reach maximum thrombin generation, the Velocity Index is calculated by dividing the peak thrombin concentration by the difference between time to peak and lag time and the endogenous thrombin potential reflecting the total amount of thrombin formed in time.

Antithrombin (AT)-inhibitor complexes assays

To establish AT-inhibitor complex levels Factor IXa-AT and factor Xa-AT in-house enzyme-linked immunosorbent assays (ELISAs) were developed. Sheep anti-human factor IX or X (Affinity Biologicals Inc., Ancaster, Canada) was added to immunoplates at a final concentration of 2 μ g/ml (Nunc, Maxisorp, Thermo Fisher Scientific Inc., Waltham, MA, USA) and incubated overnight at 4°C. Following the incubation, the plates were washed with washing buffer and blocked, using a blocking solution (200 μ L/well), for 2 hours at 37°C. Then, plasma samples, which were diluted in blocking buffer, were added to the plate together with established control samples (factor IX-AT or factor X-AT) and incubated at 300 rpm for 2 hours at room temperature (RT). Subsequently, the plates were washed and incubated with a biotin-labelled sheep anti-human antithrombin detection antibody (100 μ l/well) (Haematologic Technologies Inc.) for 1 hour at RT. Following this procedure, the plates were incubated for 30 min with Avidin-Horseradish Peroxidase (Affinity Biologicals Inc. Ancaster, ON Canada) washed once more and incubated again for 30 min, however now with the substrate solution (TMB Peroxidase Substrate, Lucron Bioproducts, Gennep, The Netherlands).

The absorbance was measured using a microplate reader at 450 nm (Bio-Rad Laboratories BV, Veenendaal, the Netherlands).

Definition of clinical endpoints

The clinical endpoints during follow-up were categorized as follows: 1. The composite of unstable angina pectoris, nonfatal myocardial infarction (with or without revascularization), nonfatal cerebrovascular attack, transient ischemic attack or other infarctions (e.g. retinal infarction) was defined as a “cerebro- and cardiovascular endpoint”. 2. The composite of progression of PAD based on a decrease of the ABI with ≥ 0.1 , a revascularization procedure or a newly diagnosed stenosis of a different artery (e.g. renal artery stenosis or subclavian artery) was defined as “progression of disease”. 3. The third endpoint was the composite of the mortality due to cerebro- or cardiovascular cause. If a patient had multiple events, the first occurring event was used in the analysis.

Statistical analysis

Baseline characteristics between PAD-patients without endpoint and patients with cerebro- and cardiovascular events or progression of PAD during follow-up were compared using general linear models for continuous variables (one way ANOVA) or Kruskal-Wallis test with, when applicable, the Bonferroni post-test and chi-square tests for categorical variables. Data are presented as mean with standard deviation when data were normally distributed, or as median with minimum and maximum values when data did not follow Gaussian distribution. The Pearson Correlation Coefficient or Spearman test was used to test for correlations, depending on the distribution of the data.

Univariate analysis

For the selected baseline laboratory values (including both classic haemostatic markers e.g. haematocrit, D-dimer, vWF and fibrinogen as well as new markers including thrombin generation parameters and IX-AT and X-AT complex levels), subjects with values in the upper 50% (mean or median values of controls were used as cut-off value, based on the normal distribution of the data) were compared with those of the lower 50%, for time to the (composite) endpoint.

Multivariate analysis

A Cox proportional hazards model was used to relate baseline laboratory markers to the occurrence of the different endpoints. The model was adjusted for age, gender, Fontaine classification, diabetes mellitus, (previous) tobacco use, antiplatelet therapy or cholesterol lowering medication, hypertension, and baseline cardio- or cerebrovascular morbidity. Results of the Cox logistic regression model were presented

as the hazard ratio (HR) and the 95% confidence interval (95% CI). A two-sided p-value <0.05 was considered statistically significant. All statistical analyses were performed using SPSS 22.0 (SPSS, Inc., Chicago, IL).

Results

Subjects

Initially 301 patients with PAD were included in the study and informed consent was obtained. However, subsequently 21 patients had to be excluded from further participation (Figure 6.1): in ten patients PAD was diagnosed more than three months prior to study-inclusion, two patients underwent a percutaneous transluminal angioplasty few days before study-venipuncture, one patient was on oral anticoagulants, in two patients the ABI was not ≤ 0.9 . In six patients venipuncture proved to be repeatedly impossible. Therefore blood samples were obtained from 280 PAD-patients. The mean age of the study-population was 66 ± 8.4 years and 65.4% of the included patients were men. The majority of included patients were classified as Fontaine IIa (58.1%) or Fontaine IIb (33.6%) with a mean resting ABI of 0.85 and 0.86 respectively. The baseline characteristics of the patients included in the study are listed in Table 6.1.

Figure 6.1 Inclusion of eligible PAD-patients.

Table 6.1 Methodological quality assessment of the included studies.

Characteristics	All PAD patients (n=280)	PAD patients with Cerebro- or cardiovascular endpoint during FU (n=19)	PAD patients with progression of disease during FU (n=104)	Patients without endpoint during FU (n=157)	P-value
Demographics					
Age (years: mean $\pm$ SD)	66 $\pm$ 8.4	69 $\pm$ 7.2	65 $\pm$ 9.5	67 $\pm$ 7.7	0.04
Male sex (%)	183 (65.4)	13 (68.4)	70 (67.3)	100 (63.7)	0.80
BMI (kg/m ² : mean $\pm$ SD)	26.5 $\pm$ 4.5	26.0 $\pm$ 4.5	26.3 $\pm$ 4.5	26.8 $\pm$ 4.4	0.62
Family history of CVD (% positive)	121 (43.2)	9 (47.7)	47 (45.2)	65 (41.4)	0.96
Medical history					
Smoking (%)					0.34
Never smoked	14 (5)	1 (5.3)	4 (3.8)	9 (5.7)	0.79
Active smokers	145 (51.8)	7 (36.8)	59 (56.7)	79 (50.3)	0.24
Stopped smoking >1yr ago	85 (30.4)	9 (47.4)	34 (32.7)	42 (26.8)	0.15
Stopped smoking <1yr ago	32 (11.4)	2 (10.5)	6 (5.8)	24 (15.3)	0.06
Unknown	4 (1.4)	0 (0)	1 (1.0)	3 (1.9)	0.71
Self-reported PY (years: median with minimum and maximum)	40 (0-102)	40 (1-60)	40 (5-90)	40 (0-102)	0.97
Diabetes Mellitus					0.03
Patients using oral antidiabetic medication (%)	35 (12.5)	5 (26.3)	16 (15.4)	14 (8.9)	0.05
Patients using insulin (%)	13 (4.6)	4 (21.1)	2 (1.9)	7 (4.5)	0.001
Hypertension (%)	158 (56.5)	11 (57.9)	56 (53.8)	91 (57.9)	0.61
Previous cardio-vascular disease (%)	101 (36.1)	9 (47.4)	36 (34.6)	56 (35.7)	0.56
Previous cerebro-vascular disease (%)	41 (14.6)	3 (15.8)	17 (16.3)	21 (13.8)	0.79
Using thrombocyte aggregation inhibitors (%)	238 (85)	16 (84.2)	90 (86.5)	132 (84.1)	0.16
Using cholesterol lowering medication (%)	227 (81.1)	14 (73.7)	83 (79.8)	130 (82.8)	0.58
Fontaine Classification at diagnosis (%)					0.05
I	10 (3.6)	2 (10.5)	2 (1.9)	6 (3.8)	0.17
IIa	165 (58.1)	10 (52.6)	53 (51.0)	102 (65.4)	0.07
IIb	94 (33.6)	6 (31.6)	43 (41.3)	45 (28.8)	0.10
III	7 (2.5)	0 (0)	4 (3.8)	3 (1.9)	0.48
IV	3 (1.1)	1 (5.3)	2 (1.9)	0 (0)	0.06

Table 6.1 (continued)

Characteristics	All PAD patients (n=280)	PAD patients with Cerebro- or cardiovascular endpoint during FU (n=19)	PAD patients with progression of disease during FU (n=104)	Patients without endpoint during FU (n=157)	P-value
Ankle-Brachial Index (mean $\pm$ SD)					
Right in rest	0.85 $\pm$ 0.2	0.79 $\pm$ 0.2	0.83 $\pm$ 0.2	0.88 $\pm$ 0.2	0.07
Left in rest	0.86 $\pm$ 0.2	0.93 $\pm$ 0.2	0.86 $\pm$ 0.2	0.86 $\pm$ 0.2	0.33
Right after treadmill	0.61 $\pm$ 0.3	0.49 $\pm$ 0.3	0.58 $\pm$ 0.3	0.65 $\pm$ 0.3	0.04
Left after treadmill	0.69 $\pm$ 0.3	0.70 $\pm$ 0.3	0.68 $\pm$ 0.3	0.68 $\pm$ 0.3	0.98

BMI, Body Mass Index; CVD, Cardiovascular Disease; PY, pack years; PAD, Peripheral Arterial Disease; FU, Follow-Up. Continuous data are expressed as mean with standard deviations or as median with minimum and maximum, based on the normal distribution of the data. Dichotomous data are shown as n (%). To detect differences the Pearson chi-squared was used for categorical variables, whereas the Oneway ANOVA or Kruskal-Wallis with Bonferroni post-testing were performed in continuous variables. No significant differences were found with the Bonferroni post-test.

Follow-up

No patients were lost to follow-up for non-medical reasons. The mean duration of follow-up, from entry to final evaluation, or to an end-point or death if earlier than evaluation, was 319 days (ranging from 1 day to 770 days) (Figure 6.2). A total of 19 patients (6.8%) experienced a cerebro- or cardiovascular endpoint during follow-up (6 patients a cerebrovascular endpoint, 13 patients a cardiovascular endpoint), 104 patients (37.1%) had significant progression of disease during follow-up. Two patients reached a cardiovascular endpoint and subsequently died, three patients died due to non-vascular causes (newly diagnosed metastasised small-cell lung carcinoma, metastasised urologic malignancy and one patient died most likely due to malignancy with lung metastases, primary tumour unknown). The mean duration from study-inclusion until occurrence of any endpoint was 135 days (with a minimum of 1 day and a maximum of 736 days).

Patients who experienced a cerebro- or cardiovascular endpoint were more likely to suffer from diabetes mellitus than the patients who did not reach an endpoint during the follow-up. There are no significant differences in smoking status, Fontaine classification or ABI between the groups (Table 6.1).

Figure 6.2 Flow diagram.
Abbreviation: IC, Informed Consent

General haematology and haemostasis parameters

Table 6.2 shows the general haematology and haemostasis parameters of the PAD-population as a whole and when divided in groups based on type of endpoint. It can be observed that the majority of laboratory values, except for the ESR, are within the normal reference range as set by our laboratory. The sole parameters that show significantly different results between groups are the haemoglobin and haematocrit level, which are significantly lower in PAD patients who reach a cerebro- or cardiovascular endpoint (8.6 ± 1.0 mmol/l and 0.40 ± 0.05 L/L, resp.) in comparison to the baseline levels of these parameters in patients without endpoint during follow-up (9.0 ± 0.7 mmol/l and 0.43 ± 0.03 L/L, resp.).

Table 6.2 Baseline haematological and haemostatic parameters of the study population.

Haemostatic parameters with reference values	All PAD patients (n=280)	PAD patients with Cerebro- or cardiovascular endpoint during FU (n=19)	PAD patients with progression of disease during FU (n=104)	Patients without endpoint during FU (n=157)	P-value
Haemoglobin (7.3-9.7 mmol/l)	8.9 ± 0.8	8.6 ± 1.0	8.8 ± 0.9	9.0 ± 0.7	0.03
Haematocrit (0.36-0.48 L/l)	0.43 ± 0.04	0.40 ± 0.05	0.42 ± 0.04	0.43 ± 0.03	0.002 [*]
ESR (0-14mm)	14 (2-72)	12 (5-72)	15 (2-60)	15 (2-60)	0.83
Thrombocytes (130-150x10E9/l)	258 ± 68	275.5 ± 113.3	263 ± 65	253.8 ± 63.3	0.25
RDW (11.4-14.5%)	13.5 (11.7-18.4)	13.5 (12.0-15.7)	13.5 (11.7-16.6)	13.5 (12.2-18.4)	0.78
MPV [#] (9.2-12.7fL)	10.5 (8.7-13.6)	10.8 (9.5-12.2)	10.5 (9.3-13.3)	10.5 (8.7-13.6)	0.86
Leukocytes (3.5-11.0x10E9/l)	7.8 (3.2-16.3)	6.8 (5.1-13.2)	7.9 (3.9-16.3)	7.7 (3.2-13.3)	0.71
Lymphocytes (15-48%)	29.1 ± 7.2	26.9 ± 6.9	30.1 ± 8.1	28.7 ± 6.6	0.17
Monocytes (4-11%)	8 (0-15)	9 (7-12)	8 (0-14)	8 (5-15)	0.33
Neutrophil granulocytes (40-70%)	59 (30-80)	59 (50-73)	59 (30-77)	59 (40-80)	0.40
Eosinophil granulocytes (0-10%)	2 (0-19)	3 (1-5)	2 (1-19)	2 (0-9)	0.98
aPTT (23-32 sec)	27 (21-46)	27 (24-40)	27 (22-46)	27 (21-39)	0.40
PT (9.9-11.5 sec)	10.5 (9.3-12.5)	10.5 (9.6-11.9)	10.4 (9.6-12.5)	10.5 (9.3-12.1)	0.93

Abbreviations: ESR, erythrocyte sedimentation rate; RDW, Red blood cell distribution width; MPV, Mean Platelet Volume; aPTT, activated partial thromboplastin time; PT, Prothrombin time. Values expressed as mean with standard deviations or as median with minimum and maximum values, based on the normal distribution of the data. P-values were calculated using either the one-way ANOVA or Kruskal-Wallis test with Bonferroni post-testing. ^{*} Indicates a significant difference in the Bonferroni post-test with the significant difference between the patients with no endpoint during follow-up and the patients experiencing a cerebro- or cardiovascular event during follow-up. [#]only measured in 161 samples.

Biomarkers of interest

Table 6.3 shows the median levels of haemostatic and coagulation parameters as measured at baseline in all patients and in the patients grouped according to the type of endpoint they experienced during follow-up, respectively. Overall, median values of all parameters are within normal reference ranges, however data are markedly skewed, as can be expected. Both the vWF-antigen and fibrinogen levels are at the upper limit of the reference interval. For D-dimer this increase is also noticeable, while some

patients show extreme outliers with D-dimer values reaching 10,000ng/ml. With the thrombin generation measurement an increased normalized peak is found for the study population, the normalized ETP values however are within normal ranges. Measurement of the antithrombin (AT)-inhibitor complex assays also showed no difference between patients with and without endpoint during follow-up. Concluding, none of the chosen biomarkers show a significant difference between groups.

Table 6.3 Tested biomarkers of the study population at baseline.

Haemostatic parameters with reference values	All PAD patients (n=280)	PAD patients with cerebro- or cardiovascular endpoint during FU (n=19)	PAD patients with progression of disease during FU (n=104)	Patients without endpoint during FU (n=157)	P-value
vWF-antigen (50-150%)	150 (76-323)	147 (102-265)	156 (76-323)	150 (77-317)	0.70
Fibrinogen (1.7-4.0 g/l)	3.6 (1.0-6.0)	3.6 (1.0-6.0)	3.4 (2.1-6.0)	3.7 (1.9-5.8)	0.08
D-Dimer (0-500 ng/ml)	454 (32-10000)	478 (200-10000)	439 (154-3230)	470 (32-7126)	0.72
Lagtime 1pM TF	4.7 (0-8.6)	5.0 (4.0-6.3)	4.7 (0-8.6)	4.6 (3.0-7.3)	0.21
ETP 1pM TF	1288 ± 284	1233 ± 334	1286 ± 274	1295.6 ± 284.3	0.66
Normalized ETP (%) 1pM TF	105.4 ± 25.8	97.2 ± 34.9	106.4 ± 24.5	105.7 ± 25.4	0.35
Peak 1pM TF	177 (0-301)	169 (40-267)	164 (0-301)	180 (45-286)	0.28
Normalized Peak (%) 1pM TF	131.7 ± 41.6	120.5 ± 42.95	130.2 ± 43.7	134.0 ± 39.9	0.37
Time to Peak 1pM TF	9.0 (0-14.7)	9.3 (7-12.7)	8.8 (0-14.7)	8.7 (5.3-13.3)	0.43
Velocity Index 1pM TF	44.4 ± 21.6	39.9 ± 22.7	43.3 ± 22.5	45.7 ± 20.8	0.43
Lagtime 0pM TF	3.3 (2.0-6.3)	3.3 (2.7-5.30)	3.3 (2.0-6.0)	3.3 (2.0-6.3)	0.41
ETP 0pM TF	1403 (0-2381)	1405 (885-1964)	1397 (0-2111)	1403 (868-2381)	0.80
Normalized ETP (%) 0pM TF	105 (0-175)	105 (0-150)	105 (0-163)	105 (0-175)	0.70
Peak 0pM TF	250.2 ± 56.8	231.2 ± 68.1	248.8 ± 60.4	253.3 ± 52.6	0.26
Normalized Peak 0pM TF	97.5 ± 21.9	94.1 ± 24.6	96.3 ± 24.6	98.8 ± 19.5	0.53
Time to Peak 0pM TF	6.3 (0-10.7)	6.7 (4.7-9.3)	6.3 (0-10.3)	6.3 (4.7-10.7)	0.50
Velocity Index 0pM TF	85.5 ± 33.2	79.9 ± 40.4	84.8 ± 37.0	86.7 ± 29.5	0.68
IX-AT (pM)	214 (73-839)	230 (139-483)	209 (90-558)	215 (73-839)	0.27
X-AT (pM)	424 (166-927)	436 (306-751)	417 (238-829)	426 (166-927)	0.78

vWF, von Willebrand factor; TF, Tissue Factor; ETP; endogenous thrombin potential; IX-AT, Factor IX-Antithrombin; X-AT, Factor X-Antithrombin. Values expressed as mean with standard deviations or as median with minimum and maximum values, based on the normal distribution of the data. P-values were calculated using either the one-way ANOVA or Kruskal-Wallis test.

Univariate analysis

Table 6.4 shows the cox regression analyses for the various risk factors and haemostatic and coagulation parameters in relation to occurrence of cerebro- or cardiovascular endpoints and progression of peripheral vascular disease. For this analysis we included a composite endpoint including both cerebro- and cardiovascular events together with progression of PAD as well.

Cerebro- or cardiovascular endpoint

None of the chosen baseline parameters was able to significantly predict a cerebro- or cardiovascular endpoint during follow-up except for previously diagnosed Diabetes Mellitus, which showed a Hazard Ratio (HR) of 4.8 (95% CI: 1.93 to 11.73, $p=0.001$) (Table 6.4).

Progression of PAD

An increased severity of PAD, as measured with Fontaine classification at baseline, proved to be a significant predictor for progression of PAD (HR 1.73, 95% CI: 1.27 to 2.36, $p=0.0001$). It should be taken into account however that, due to low numbers at baseline, patients with a Fontaine classification of III or IV were combined into one group. A fibrinogen-level exceeding 3.6g/l proved to be significantly associated with reduced progression of PAD (HR 0.65, 95% CI: 0.43 to 0.98, $p=0.04$).

Composite endpoint; progression of PAD combined with cerebro-or cardiovascular endpoint

A baseline haematocrit level of over 0.43 L/l proved to be a significant, negative predictor (HR 0.67, 95% CI 0.47-0.97, $p=0.03$) whereas the Fontaine classification showed a positive predictive value of 1.63 (95% CI: 1.23-2.16, $p=0.001$) for reaching the composite endpoint during follow-up. The other parameters evaluated in the study showed HR's ranging from 0.42 to 1.72, with p -values that did not reach significance (Table 6.4).

Multivariate analysis

A Cox proportional hazard regression analysis was performed. As aforementioned the model was adjusted for age, gender, Fontaine classification, diabetes mellitus, (previous) tobacco use, antiplatelet therapy and cholesterol lowering medication, hypertension, and baseline cardio- or cerebrovascular morbidity. Previously diagnosed diabetes mellitus at baseline independently increased the risk of cerebro- or cardiovascular events with a hazard ratio (HR) of 5.37 (95% CI 1.79 to 6.11, $p=0.003$). Adjusting for the same variables, but using progression of PAD as the censoring variable, increased severity of PAD as measured with the Fontaine classification increased the risk 1.66 times (1.19 to 2.30, $p=0.003$).

Table 6.4 Hazard ratios and 95% CI of baseline characteristics for the study endpoints in a univariate analysis.

Variable	Cerebro- or cardiovascular events			Progression of peripheral arterial disease			Composite endpoint		
	Hazard ratio	95% CI	P value	Hazard ratio	95% CI	P value	Hazard ratio	95% CI	P value
Age >66yr	1.05	0.99-1.11	0.12	0.91	0.61-1.34	0.62	1.03	0.72-1.46	0.89
Gender	1.02	0.63-1.66	0.93	1.04	0.84-1.28	0.71	1.05	0.87-1.26	0.64
Diabetes Mellitus	4.76	1.93-11.73	0.001*	1.14	0.69-1.90	0.61	1.52	0.99-2.31	0.051
Fontaine classification	1.17	0.55-2.53	0.68	1.73	1.27-2.36	0.0001*	1.63	1.23-2.16	0.001*
Using cholesterol lowering medication	0.69	0.25-1.92	0.47	1.01	0.62-1.67	0.96	0.82	0.54-1.25	0.35
Using platelet inhibitors	0.90	0.48-1.68	0.74	0.87	0.65-1.17	0.35	0.94	0.74-1.21	0.65
Hypertension	0.92	0.37-2.30	0.86	0.88	0.60-1.30	0.53	0.84	0.59-1.19	0.33
Previous cardiovascular disease	0.79	0.50-1.24	0.30	1.06	0.86-1.30	0.61	0.99	0.82-1.18	0.89
Previous cerebrovascular disease	1.21	0.35-4.19	0.76	1.20	0.71-1.02	0.49	1.22	0.76-1.94	0.41
Any tobacco use	1.09	0.15-8.19	0.93	1.51	0.56-4.01	0.42	1.49	0.61-3.65	0.38
Hemoglobin >8.09mmol/l	0.56	0.22-1.43	0.22	0.77	0.52-1.13	0.18	0.73	0.51-1.03	0.08
Hematocrit >0.43L/l	0.42	0.15-1.16	0.09	0.72	0.48-1.08	0.11	0.67	0.47-0.97	0.03*
Fibrinogen >3.6g/l	0.87	0.35-2.17	0.77	0.65	0.43-0.98	0.04*	0.76	0.53-1.09	0.13
D-Dimer >454ng/ml	1.12	0.45-2.78	0.80	0.89	0.61-1.32	0.57	0.94	0.67-1.34	0.74
vWF-antigen >150%	0.89	0.36-2.23	0.81	1.18	0.80-1.75	0.40	1.15	0.81-1.64	0.44
ETP 1pM TF >1288nM.min	1.01	0.41-2.50	0.98	0.87	0.59-1.29	0.49	0.86	0.61-1.23	0.41
Peak 1pM TF >177nM	0.78	0.31-1.95	0.60	0.94	0.64-1.39	0.77	0.88	0.62-1.25	0.46
IX-AT >214	1.72	0.68-4.37	0.25	0.84	0.57-1.24	0.39	0.97	0.69-1.38	0.88
X-AT >424	1.15	0.47-2.84	0.76	0.92	0.62-1.35	0.67	0.95	0.67	0.79

A fibrinogen level exceeding 3.6 g/l however significantly decreased the risk of progression of PAD with a HR of 0.61 (95%CI 0.39 to 0.95, $p=0.03$). In an analysis of the composite endpoint in the same study population an increased Fontaine classification increased the risk 1.52-fold (95% CI 1.13 to 2.04, $p=0.006$) whereas an increased haematocrit, of over 0.43L/l, decreased this risk by approximately half (HR 0.49, 95% CI 0.24-0.98, $p=0.043$). No other variables were found to independently increase or decrease the risk of one of the endpoints during the follow-up of the study (Table 6.5, Figure 6.3).

Figure 6.3a Cumulative hazard analysis for the composite endpoint comparing the groups based on Fontaine classification and on the Haematocrit level, with a cut-off value of 0.43 L/l.

Figure 6.3b Cumulative hazard analysis for the progression of PAD comparing the groups based on the fibrinogen level with a cut-off value of 3.6 g/l and the Fontaine classification.

Figure 6.3c Cumulative hazard analysis for the occurrence of a cerebro- or cardiovascular endpoint comparing the groups based on the presence of diabetes mellitus at baseline.

Table 6.5 Multiple regression coefficients of plasma markers with established cardiovascular risk factors.

Variable	Cerebro- or cardiovascular events			Progression of peripheral arterial disease			Composite endpoint		
	Hazard ratio	95% CI	P value	Hazard ratio	95% CI	P value	Hazard ratio	95% CI	P value
Age > 66yr	1.84	0.62-5.45	0.27	0.82	0.52-1.30	0.41	0.95	0.63-1.44	0.81
Gender	0.99	0.29-3.39	0.98	1.12	0.69-1.84	0.64	1.13	0.72-1.77	0.60
Diabetes Mellitus	5.37	1.79-16.11	0.003	1.06	0.59-1.91	0.85	1.50	0.92-2.45	0.10
Fontaine classification	1.25	0.55-2.85	0.60	1.66	1.19-2.30	0.003	1.52	1.13-2.04	0.006
Using cholesterol lowering medication	0.49	0.12-1.95	0.31	0.71	0.37-1.35	0.30	0.68	0.39-1.20	0.19
Using platelet inhibitors	0.69	0.14-3.42	0.65	0.63	0.29-1.37	0.25	0.74	0.38-1.44	0.37
Hypertension	0.73	0.23-2.29	0.59	0.90	0.58-1.39	0.63	0.85	0.57-1.26	0.41
Previous cardiovascular disease	1.28	0.34-4.81	0.71	1.53	0.87-2.69	0.14	1.45	0.88-2.41	0.15
Any tobacco use	0.85	0.13-4.26	0.75	1.44	0.71-2.95	0.31	1.31	0.70-2.47	0.41
Hemoglobin >8.9mmol/l	2.51	0.47-13.47	0.28	1.32	0.61-2.87	0.49	1.37	0.68-2.75	0.38
Hematocrit >0.43L/l	0.24	0.04-1.40	0.11	0.53	0.25-1.15	0.11	0.49	0.24-0.98	0.043
Fibrinogen >3.6g/l	0.79	0.28-2.29	0.67	0.61	0.39-0.95	0.03	0.69	0.46-1.03	0.07
D-Dimer >454ng/ml	1.03	0.33-3.19	0.96	1.11	0.70-1.74	0.66	1.10	0.73-1.66	0.65
WVF-antigen >150%	0.93	0.33-2.60	0.89	1.33	0.86-2.06	0.21	1.26	0.85-1.87	0.25
ETP 1pM TF >1288nM.min	1.76	0.45-6.92	0.42	0.88	0.50-1.55	0.65	0.98	0.58-1.63	0.92
Peak 1pM TF >177nM	0.51	0.14-1.85	0.31	1.04	0.58-1.87	0.91	0.90	0.53-1.52	0.68
IX-AT (Maastricht) >214	1.31	0.46-3.67	0.61	0.78	0.51-1.17	0.23	0.86	0.59-1.26	0.45
X-AT (Maastricht) >424	0.99	0.35-2.84	0.99	1.05	0.69-1.60	0.83	1.05	0.71-1.54	0.81

Discussion

The aim of our study was to establish the value of selected existing and novel markers of hypercoagulability, for predicting short-term cardiovascular events, in a PAD population with relatively recently established vascular disease. The rationale for evaluating laboratory markers of coagulation is the high incidence of cardiovascular (CV) events in these patients. This is linked to the epidemiological and experimental evidence of close ties between coagulation, atherosclerosis and thrombosis. Further support came from our recent systematic analysis, showing that an elevated d-dimer plasma level indicates a higher risk of CV events on the short to medium follow up.²² The available evidence assumes the presence of hypercoagulability in patients with PAD. A recent study showed that a residual thrombin generation potential was *inversely* linked to the occurrence of atherothrombotic events in PAD-patients.²⁵ It was hypothesized that this was due to exhaustion of thrombin generation; on-going thrombin generation *in vivo* would diminish the residual thrombin potential in *in vitro* experiments.

However, in our cohort of patients with recently diagnosed PAD, evidence for hypercoagulability is subtle. Overall, there were no substantial elevations in markers of coagulation activity. Indicative of hypercoagulability is the fact that in our population both the vWF-antigen and fibrinogen levels are at the upper limit of the reference interval, which is in accordance with published data where increased levels have been described.^{5,26,27} In accordance, most markers of coagulation, including the CAT-parameters as well as the results of the antithrombin (AT)-inhibitor complexes assays, were skewed towards high values; however, median levels were not discriminative for patients with or without CV complications. As a primary candidate marker of hypercoagulability, d-dimer was selected, as our recent meta-analysis showed that an increased D-dimer level was independently associated with an increased risk (2-fold) of cardiovascular events within 4 years after the D-dimer measurement in patients with known PAD.²² In the present study, we did not find any significant associations with one of the outcome measures for D-dimer. One of the most likely factors explaining this attenuated D-dimer effect is the use of medication including statins, which are known to attenuate coagulation activity.^{28,29} Despite the established guidelines for treatment of PAD, various studies have shown that a large proportion of PAD-patients are undertreated.³⁰ In the majority of the studies analysed for our systematic review no specific data was given on the use of statins in the selected study populations, except for the study by Vidula et al that stated that only 42% of the population used statins at baseline. Other studies, not included in the review, reported various numbers, varying from 25 to 67% of PAD-patients using statins at study inclusion.³⁰⁻³² With 81.1% of all patients included in our study using statins, this might have attenuated hypercoagulability as well as the potential to detect its effects in relation to clinical outcomes.^{30,33}

In this study we addressed the value of platelet poor plasma based coagulation assays, which may be a methodological concern as cellular elements like platelets and leukocytes are important players in atherothrombosis.³⁴ For thrombin generation, the use of the recently published whole blood TG assay may provide additional information.³⁵ At the same time, the absence of cellular components does not rule out the contribution of relevant risk factors in the plasma milieu. In fact, both fibrinogen and vWF have been repeatedly related to atherothrombotic disease.^{10,36} However, in our study none of these proteins showed any significant association with clinical outcomes or severity of PAD. In fact, of all laboratory markers tested, severity of PAD was, albeit weakly, *inversely* correlated to the haemoglobin and haematocrit level. The cause for this correlation remains unclear.

A peculiar finding was that an increased fibrinogen level of over 3.6 g/l was *inversely* associated with the 'progression of PAD'-endpoint, suggesting that an increased fibrinogen level would lead to a decreased progression of PAD during a short-term follow-up. This finding is rather surprising as in a number of previous studies it was shown that an increased fibrinogen level is linked with the earlier appearance of PAD and is predictive of cardiovascular mortality in PAD-patients.^{11,19-21}

To assess the prognostic value of patient characteristics and haemostatic markers for cerebro- or cardiovascular events and quick progression of atherosclerotic disease in PAD-patients we observed that the only parameter showing a positive association with the cerebro- or cardiovascular endpoint was the presence of diabetes which is in accordance with precedent findings.³⁷⁻³⁹ For none of the other risk factors or preselected parameters a correlation was found. Given the study population and the relatively short follow up, it may well be that only very strong risk conditions like diabetes have sufficient influence to determine the risk of events on a short term, in contrast to hypercoagulability markers.

Strengths and limitations

A possible limitation of this study is the relatively short follow-up as a result of which only a small number of (symptomatic) cerebro- and cardiovascular endpoints could be assessed. The study has however several strengths; one of the strengths of this study is that we were able to include a relatively large homogeneous group of patients within approximately the same stage of PAD, as all patients were included within 3 months after diagnosis. Furthermore this inclusion adhered to the pre-calculated sample size with no loss to follow up. A definite strength is the use of a concise and state of the art panel of plasma assays, including novel immunoassays for intrinsic coagulation activity.

Conclusion

In patients with recently diagnosed PAD, our chosen set of markers of coagulation activity reflecting either the actual status (d-dimer) or the clotting potential (CAT) does not indicate the risk of atherothrombotic events in the short term. In this cohort, only diabetes predicted outcomes within the one-year follow up period. This is possibly due to the fact that patients in this study already received appropriate cardiovascular protective drugs, including platelet inhibitors and statins. Further work is needed to develop clinically useful markers, or novel assay platforms, that combine biomarkers representing not only plasma but also cellular components of mechanisms involved in atherothrombosis.

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PART II

Haemostatic changes caused by severe liver disease

CHAPTER 7

Cirrhosis patients have a coagulopathy that is associated with decreased clot formation capacity

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Abstract

Background

The coagulopathy in cirrhosis is associated with thrombosis and bleeding.

Objectives

To gain better insights into the coagulopathy in patients with cirrhosis, we evaluated plasma thrombin generation and whole blood clot formation in a cross-sectional study.

Methods

Blood was collected from 73 patients with all-cause cirrhosis (Child-Pugh-A n=52, B n=15, C n=6) and 20 healthy controls. Activity of the coagulation pathways were measured with assays for factor VIIa and factor IXa-antithrombin and factor Xa-antithrombin complexes, respectively. Thrombin generation by Calibrated Automated Thrombography was determined in platelet poor plasma using a 1 or 5 pM tissue factor trigger with/without thrombomodulin. ROTEM-measurements were performed in whole blood triggered with 35 pM tissue factor without/with 175ng/ml tissue Plasminogen Activator (the latter referred to as 'tPA-ROTEM').

Results

We observed an increased generation of factor VIIa and a moderately elevated amount of factor IXa (in complex with antithrombin) without apparent increase in factor X activation in patients with cirrhosis. In accordance with this prothrombotic state, markers of thrombin generation potential were also increased upon increasing severity of cirrhosis. In the whole blood clotting assay we observed delayed clot formation and decreased clot strength associated with increased cirrhosis severity. No significant differences were found for tPA-ROTEM parameters of clot degradation.

Conclusion

These results indicate that cirrhosis patients have an overall procoagulant plasma milieu but a decreased whole blood clot formation capacity with an apparently unaltered resistance to clot lysis.

Introduction

In patients with cirrhosis substantial changes in the haemostatic system occur, making them not only prone bleeding but also to thrombotic events.¹⁻³ These haemostatic changes include thrombocytopenia combined with platelet function defects, decreased levels of pro- and anticoagulant factors and decreased levels of various fibrinolysis proteins.^{4,5} Although such changes would suggest a bleeding tendency, a number of recent studies demonstrate that patients with cirrhosis have a 'rebalanced' haemostatic system with a normal, or even increased degree of coagulability when measured with more global coagulation tests such as the thrombin generation measurement.⁶⁻⁸ This reset balance is possibly due to the fact that reduced synthesis of pro-coagulant factors by the diseased liver is compensated by a concomitant decrease of anti-coagulant proteins.⁹ Although the thrombin generation assay is able to take anti-coagulant forces into account, this is generally performed in platelet poor plasma, thus lacking information on protein-cell interactions.¹⁰ Thromboelastometry, on the other hand, allows for continuous registration of the viscoelastic changes in whole blood which is an indication of the function of plasma, blood cells and platelets in the context of clot formation and lysis.¹¹ The fibrinolytic system is also known to be disturbed in cirrhosis, yet whether this disturbance leads to increased clot degradation, as has been assumed for years, has recently come under debate.¹² Recently, Lisman et al. found no signs of increased global fibrinolysis, whereas other recent studies demonstrated a hyperfibrinolytic state in the majority of patients with cirrhosis.¹³⁻¹⁵ One of the reasons for these contradicting findings may lie in the lack of appropriate global laboratory assays to evaluate the overall fibrinolytic activity.^{12,16}

In order to gain better insights into the haemostatic profile of patients with increasing severity of cirrhosis, we performed a cross-sectional study in which we included the thrombin generation assay (TGA) combined with novel assays for quantifying extrinsic (FVIIa), intrinsic (FIXa-antithrombin (AT) complex) and common pathway (FXa-AT complex) elements of the coagulation cascade. Moreover, we tested clot lysis in whole blood samples using a modified tissue factor-triggered rotational thromboelastometry to which tPA was added. This 'tPA-ROTEM' assay was recently developed to assess the global clot formation and clot lysis in whole blood, in order to better approximate the *in vivo* situation.

Patients and methods

Patients

From 2011 to 2013, 73 consecutive patients from the gastroenterology departments of two hospitals in South-Limburg, the Netherlands, with all-cause cirrhosis (48 males and

25 females) with ages ranging from 20 to 79 years were enrolled after written informed consent was obtained. The diagnosis of cirrhosis was based on clinical, laboratory, ultrasound, gastroscopy and/or histological evidence. Patients with various stages of cirrhosis severity as estimated according to the Child-Pugh-Turcotte Classification were enrolled: Child-Pugh A ($n=52$), Child-Pugh B ($n=15$), Child-Pugh C ($n=6$). The severity of cirrhosis was also assessed by calculating the MELD-score (Model for End-stage Liver Disease) with the formula $9.57 \times \ln(\text{creatinine}) + 3.78 \times \ln(\text{bilirubin}) + 11.2 \times \ln(\text{INR}) + 6.43$.¹⁷

Exclusion criteria were the use of medication known to affect coagulation (e.g. cumadins, direct factor Xa- or thrombin inhibitors, or heparin), documented congenital coagulation disorders, age under 18 years. The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki (Seoul 2008) and was approved by the local medical ethics committee (METC azM / UM).

Healthy controls

Twenty healthy individuals were enrolled in the study, as sex- and age matched healthy controls (HC), after we obtained informed consent (mean age of 50 years, 55% males). Exclusion criteria were the use of medication known to affect coagulation, known coagulation disorders and age under 18 years.

Pooled normal plasma

Pooled normal plasma (stored frozen at -80°C) used for the normalization of thrombin generation samples was prepared by pooling plasma from 80-90 apparently healthy volunteers. Volunteers on anticoagulation and women using oral contraceptives were not recruited.

Blood collection and plasma preparation

Venous blood was collected in 4.5 ml 3.2% citrate tubes (Vacutainer glass, 0.105M, Becton and Dickinson). Blood was drawn by clean venipuncture and within one hour processed to platelet-poor plasma (PPP). PPP was obtained by initial centrifugation at: 2000g for 15 minutes (18°C) followed by centrifugation at 11.000g for 10 minutes (18°C) and stored at -80°C until use. All samples were thawed for 15 minutes at 37°C before testing. One 4.5ml 3.2% citrate tube (Vacutainer glass, 0.105M, Becton and Dickinson) was used to collect whole blood for the 'tPA-ROTEM'.

Measurements

Conventional coagulation tests and hematologic parameters

Measurements of aPTT (Dade® Actin® FSL Activated PTT Reagent, Calcium Chloride Solution), PT (Dade® Innovin®), Fibrinogen (Dade® Thrombin Reagent), Antithrombin (Berichrom® Anti-Thrombin III (AT)), D-dimer (INNOVANCE D-Dimer), Plasminogen (Berichrom Plasminogen), vWF-antigen (vWF Ag), Protein C (Berichrom Protein C), were performed on the Sysmex CA-7000 analyzer (Sysmex Corporation, Kobe, Japan) according to the manufacturer's instructions (all reagents by Siemens Healthcare Diagnostics Products, Marburg, Germany).

Thrombin generation

Thrombin generation in tissue factor-triggered platelet-poor plasma was measured by means of the Calibrated Automated Thrombogram method (Thrombinoscope BV, Maastricht, The Netherlands). Measurements were conducted on 80 µl platelet-poor plasma with a final concentration of 1 or 5 pM tissue factor (PPP Reagent Low; Thrombinoscope BV) and in the presence of 4 µM phospholipids. The measurements were conducted in the absence or presence of 0.56nM recombinant soluble thrombomodulin (Thrombinoscope). Measurements were performed as described earlier.¹⁸ Each thrombin generation measurement includes pooled normal plasma; all parameters (Lag time, Endogenous thrombin potential (ETP), Peak height and Time to peak) are expressed as the ratio of value_{patient}/value_{pooled normal plasma}, expressed as percentages. To assess the effect of thrombomodulin (TM) on the thrombin generation measurement, the ETP ratio was calculated by dividing ETP *with* TM by ETP *without* TM (ratio ETP = ETP +TM / ETP – TM).

Rotational thromboelastometry

The tissue factor triggered ROTEM (EXTEM based) and the 'tPA-ROTEM' give a graphic representation of the clot formation process, and due to the addition of tPA also include the subsequent (resistance to) clot lysis. Both the ROTEM and the 'tPA-ROTEM' were carried out by means of the 4 channel ROTEM® Gamma device operated according to manufacturer instructions using tissue factor (Innovin) and tPA (Actilyse). Recalcified citrated blood (300 µl) was incubated, within one hour after venipuncture, at 37°C in a preheated cup. For the purpose of this study the ROTEM was determined in recalcified citrated whole blood incubated with two different triggers: 35 pM tissue factor in the presence or absence of 175 ng/ml tissue plasminogen activator (tPA). To assess the tissue factor concentration, we performed kinetic factor Xa generation measurements in which tissue factor activity in diluted Innovin was determined. Extem reagent tissue factor activity was subsequently estimated against the diluted Innovin in clotting and Xa generation measurements.

The following numerical parameters investigated on whole blood were (1) α-Angle (α) defined as the speed (sec/min) at which the clot is formed; (2) Clot Formation Time

(CFT), defined as the time (sec) from initiation of clotting until a clot firmness of 20mm is detected; (3) Maximum Clot Firmness (MCF) defined as the maximal amplitude (mm) of the clot; (4) Lysis Onset Time (LOT) defined as the time (sec) until 15% reduction of the MCF; (5) Lysis Time (LT) defined as the time (sec) until 90% reduction of the MCF and (6) Fibrinolysis velocity (FV) defined as the decline in % per minute between the LOT and the LT. We assessed the reproducibility for the tPA-ROTEM parameters with 16 repeated measurements with the blood of a healthy volunteer. This showed intra-assay coefficients of variation of 12.1%, 4.9%, 16.6% and 17.1% for CT, MCF, LOT, and LT, respectively, at the 175 ng/ml rt-PA concentration.

Quantification of FVIIa

Levels of FVIIa were measured using a homemade VHH against FVIIa and a HRP conjugated polyclonal antibody against FVII (1 µg/ml; SAFVII-IG, Stago, France) in a semi-automated ELISA on a TEC-AN Freedom EVO robot (Tecan, Switzerland) as described.¹⁹

Quantification of FIXa-AT and FXa-AT complexes

FIXa, FXa, and AT were obtained from Haematologic Technologies (Essex Junction, VT, USA). FIXa-AT and FXa-AT complexes were prepared by incubating FIXa or FXa (2 µM) with AT (5 µM) and heparin (50 µM; UFH, Leo Pharma, Ballerup, Denmark) for 3 hours at 37°C in Tris 20 mM, NaCl 150 mM, EDTA 1 mM, pH 7.5. Complete inhibition was confirmed by assessing the amidolytic activity of FIXa or FXa for S-2366 (Chromogenix, Mölndal, Sweden) or SpecXa (American Diagnostica, Greenwich, CT, US), respectively. Given the 1:1 stoichiometry of the protease-AT complexes, the final complex concentration was assumed to be equal to the protease concentration (2 µM). Complexes were aliquoted (50 nM in PBS 10 mM, tween 0.1%, ovalbumin 2.5%, pH 7.4) and stored at -20°C. Protease-AT complexes were assayed using an ELISA set-up. Dilutions (150-fold in NaHCO₃ 100 mM, NaCl 500 mM, pH 9.6) of polyclonal anti-FIX-IgG (CL20041K, Cedarlane, Burlington, ON, Canada) or anti-FX-IgG (CI20245K) were immobilized overnight at 4°C. Wells were washed with PBS 10 mM, tween 0.1%, pH 7.4 and incubated with PBS 10 mM, tween 0.1%, ovalbumin 1% for 1.5 h at room temperature. Samples were diluted (7.5-200-fold) in PBS 10 mM, tween 0.1%, ovalbumin 0.05%, EDTA 10 mM, benzamidin 10 mM, pH 7.4 and incubated overnight at room temperature. After washing, peroxidase-labelled anti-AT-IgG (800-fold diluted in PBS 10 mM, tween 0.1%, pH 7.4; CL20018K) was incubated for 1.5 h at room temperature. Peroxidase was detected using 3,3',5,5'-tetramethylbenzidine. Concentrations were calculated from plots of log absorbance vs. log concentration employing FXIa-AT (20-700 pM) or FXa-AT (10-700 pM) complexes as reference.

Statistical analyses

Statistical analysis was performed using the GraphPad Prism version 6.00 software (GraphPad Software, Inc. For Mac OS X, San Diego, CA, USA). Normality of the data was tested with the D'Agostino & Pearson omnibus normality test. According to the distribution of the variables data are expressed as median $\pm$ interquartile ranges (IQR). Differences between the results of the healthy controls and patients with cirrhosis in the three Child-Pugh groups were analysed with the Kruskal-Wallis test with Dunn's post-test. The Mann-Whitney U test was used to test for differences between median values of two separate groups. The associations between FVIIa, FIXa-AT, FXa-AT, thrombin generation measurement, AT and D-dimer plasma levels were assessed using Spearman correlation coefficient. Statistical significance was considered as a two-tailed probability <0.05 .

Results

The main characteristics of the patient population are reported in Table 7.1.

Table 7.1 Demographic and laboratory characteristics of the patient population.

Characteristics (reference values)	Child-Pugh A (n=52)	Child-Pugh B (n=15)	Child-Pugh C (n=6)
Age (yr)	61 (54-67)	53 (51-66)	55 (40-62)
Sex (male, %)	31 (59.6%)	12 (80.0%)	5 (83.3%)
Aetiology:			
Alcoholic (n)	26	10	4
NASH (n)	4		
Haemochromatosis (n)	4		
AIH/PBC (n)	6		
Toxic/Medication (n)	3		1
Hep B (n)	1		
Hep C (n)	1	3	
Congenital Fibrosis (n)		1	1
Unknown origin (n)	7	1	
Child-Pugh score	5 (5-5)	8 (7-8)	10 (10-11)
MELD score	7 (7-9)	13 (11-16)	15 (13-18.5)
INR (0.80-1.20)	1.06 (1.02-1.12)	1.21 (1.15-1.30)	1.38 (1.25-1.60)
Albumin (32.0-47.0 g/l)	40.5 (37.9-43.7)	32.6 (30.0-34.3)	26.8 (25.2-31.9)
Bilirubin ($<20.0 \mu\text{mol/l}$)	14.5 (9.8-21.9)	54.5 (40.3-61.7)	75.7 (48.0-128.4)
Creatinine (60-115 $\mu\text{mol/l}$)	75.0 (63.0-83.0)	76.0 (69.0-90.0)	66.0 (51.5-86.0)

Values expressed as median with interquartile ranges. Abbreviations: NASH, Non-Alcoholic Steatosis Hepatitis; AIH, Auto-immune Hepatitis; PBC, Primary Biliary Cirrhosis; Hep B, Hepatitis B virus; Hep C, Hepatitis C virus.

Inspection of the individual clinical records showed that 19 patients had a history of bleeding (14 patients experienced a gastrointestinal bleeding, three reported a

bleeding after an operation, two experienced an intracranial haemorrhage), three had a history of (arterial) thrombosis (two patients a transient ischemic attack, one a minor stroke), one patient had both a bleeding and a thrombosis. Patients did neither have a recent bleeding episode, nor received recent platelet or plasma substitution (<1 week prior to inclusion). Table 7.2 shows the haematological and haemostatic parameters of the study population. In line with previous findings, median outcomes of the classic coagulation tests PT, INR and aPTT were significantly prolonged in patients with CP-C cirrhosis in comparison to healthy controls and patients with CP-A cirrhosis ($P<0.0001$). The majority of the haematological and haemostatic parameters were significantly decreased in patients with CP-C cirrhosis ($P<0.05$). The D-dimer and vWF-antigen levels were however significantly increased in CP-C patients ($P<0.0001$).

Table 7.2 Haematological and haemostatic parameters of the study population.

Haemostatic parameters with reference values	Healthy controls (n=20)	Child-Pugh A (n=52)	Child-Pugh B (n=15)	Child-Pugh C (n=6)	P value
Haemoglobin (7.3-9.7 mmol/l)	8.8 (8.6-9.6)	8.7 (8.0-9.4)	7.6 (7.2-8.6)	7.15 (6.3-7.7)	$P=0.002$
Haematocrit (0.36-0.48 L/l)	0.40 (0.40-0.44)	0.41 (0.38-0.44)	0.36 (0.34-0.41)	0.36 (0.30-0.38)	$P=0.0008$
Erythrocytes (3.7-5.0x10 ¹² /l)	4.6 (4.4-5.0)	4.5 (4.2-4.7)	3.8 (3.4-4.4)	3.2 (2.7-4.3)	$P<0.0001$
Leucocytes (3.5-11.0x10 ⁹ /l)	6.6 (5.7-8.7)	6.6 (5.1-8.0)	5.5 (3.8-7.4)	5.1 (4.8-6.0)	$P=0.0986$
Thrombocytes (130-150x10 ⁹ /l)	254 (225-291)	171 (106-213)	81 (54-103)	102 (66-120)	$P<0.0001$
aPTT (23-32 sec)	27 (26-29)	28 (27-31)	31 (29-32)	33 (31-36)	$P<0.0001$
PT (9.9-11.5 sec)	10.6 (10.4-11.0)	11.6 (11.2-12.5)	13.3 (12.6-14.5)	15.3 (13.8-18.2)	$P<0.0001$
INR (0.8-1.2)	0.95 (0.94-0.99)	1.06 (1.02-1.12)	1.21 (1.15-1.30)	1.38 (1.25-1.60)	$P<0.0001$
vWF-antigen (50-150%)	136 (104-164)	215 (160-302)	410 (331-465)	525 (334-633)	$P<0.0001$
Fibrinogen (1.7-4.0 g/l)	3.2 (2.7-3.9)	3.3 (2.6-3.8)	2.70 (2.0-3.8)	1.1 (0.9-2.7)	$P=0.0099$
Antithrombin (80-120%)	104 (96-108)	86 (77-99)	50 (42-74)	40 (24-57)	$P<0.0001$
Protein C (70-140%)	117 (108-129)	88 (76-108)	48 (35-70)	36 (21-46)	$P<0.0001$
Plasminogen (80-130%)	115 (109-120)	100 (88-112)	77 (63-86)	68 (37-89)	$P<0.0001$
D-Dimer (0-500 ng/ml)	323 (228-398)	434 (298-838)	1032 (752-2195)	6309 (1504-9091)	$P<0.0001$

Values expressed as median with interquartile ranges. The P-values were calculated using the Kruskal-Wallis with Dunn's post-test.

Plasma levels of (activated) coagulation factors

Assessment of the extrinsic coagulation pathway revealed a significant 3.2-fold increase of plasma FVIIa levels in patients with CP-A cirrhosis as compared to healthy controls ($P<0.0001$, Figure 7.1). Furthermore, comparison of the median FVIIa plasma levels of CP-A patients with those of CP-B (1.7-fold) and CP-C (0.7-fold) may suggest that the severity of cirrhosis is inversely correlated with FVIIa levels, which was significant for, albeit only five, CP-C patients ($P<0.05$). Analysis of the intrinsic pathway via protease-AT measurements indicated a 2.2-fold increase of FIXa-AT complex levels in CP-A and 3.7-fold in CP-B patients in comparison to controls, yet this increase is only significant for CP-B patients in comparison to healthy controls. ($P=0.0053$, Figure 7.2A). In a number of CP-A and CP-C patients the FIXa-AT complex values were found to be outside of the calibration range, these were set at 20 or 700 pM. For plasma values of FXa-AT complexes, we observed no significant differences between the patients and healthy controls, nor between the Child-Pugh classified patient groups (Figure 7.2B). However, FIXa-AT plasma levels significantly correlated with those of FXa-AT for the patient groups (Spearman correlation coefficient $r=0.585$; $P<0.0001$).

Figure 7.1 Plasma factor (F) VIIa levels in healthy controls and cirrhosis patients. Plasma FVIIa levels of healthy controls and cirrhotic patients were determined relative to the median value of the 20 age and sex-matched healthy individuals, which was set at 1 (dotted line). Data are presented as median with interquartile ranges and were analyzed statistically as described, with * $P<0.05$, *** $P<0.0001$.

Figure 7.2 Plasma factor (F) IXa-AT and FXa-AT complex levels in healthy controls and cirrhosis patients. Plasma FIXa-AT (A) and FXa-AT (B) levels of healthy controls and cirrhotic patients were determined relative to the median value of 20 age- and sex-matched healthy individuals, which was set at 1 (dotted line). Individual patient values represent the mean of duplicate measurements. The values are presented as median with interquartile ranges and were analyzed statistically as described, with * $P < 0.05$.

Thrombin generation

Global coagulation was measured in platelet poor plasma triggered with 1pM tissue factor (Figure 7.3). Whereas thrombin generation proceeded as anticipated in normal pooled plasma, an increasing severity of cirrhosis resulted in a reduced lag time and increase in thrombin peak. All parameters derived from the thrombin generation curve are shown as normalized values in more detail in Figure 7.4 (A-D). The lag time significantly shortened with increasing Child-Pugh score (CP-A: 6.83 min vs. CP-B: 4.91 min and CP-C: 3.84 min, $P < 0.05$). For the normalized peak height, which indicates the maximal thrombin formation, high median values were found and a significant difference was observed between the healthy controls and the CP-A and CP-B group (CP-A: 201%, vs. CP-B: 278% and CP-C: 229%, $P < 0.05$). We found no significant differences between the CP-groups for the endogenous thrombin potential (ETP), however the median ETP of all three groups was relatively high in comparison to normal pooled plasma (CP-A 146%, CP-B 169% and CP-C 161%). Furthermore, the ETP was significantly increased for all CP-groups in comparison to the ETP of healthy controls (HC 109%). The time till maximal thrombin formation is reached, represented by the 'time to peak', was significantly shorter with increasing CP-score (CP-A: 10.7 min vs. CP-B: 8.0 min and CP-C: 6.7 min, $P < 0.0001$). In order to attenuate the possible effects of contact activation on thrombin generation, thrombin generation was also assessed in the presence of a 5pM tissue factor trigger; the normalized parameters of this curve are depicted in Figure 7.4 (E-H). Similar to our previous findings, both lag time and time to peak decreased significantly with increasing cirrhosis severity, whereas the endogenous thrombin potential and peak height were clearly increased in

comparison to the values obtained with normal pooled plasma; however we observed no significant differences between the cirrhosis groups.

Figure 7.3 Thrombin generation in cirrhosis patients and normal pooled plasma. Shown are the thrombin generation curves based on the mean values of all included patients. Thrombin generation was measured following a 1 μ M tissue factor trigger in the presence of 4 μ M phospholipids in platelet-poor normal pooled plasma ('NPP'), or platelet-poor plasma of CP-A, CP-B or CP-C patients.

To evaluate the activity of the anticoagulant protein C pathway, the same experiments were performed in the presence of 0.56nM thrombomodulin. This concentration was chosen as such to inhibit thrombin generation in normal pooled plasma by 50%.¹⁸ As anticipated, addition of thrombomodulin reduced the endogenous thrombin potential and subsequently the ETP ratio (Figure 7.5). In the CP-B and CP-C group a significantly higher ETP-ratio, and thus lesser reduction of the ETP, as compared to the healthy controls and CP-A groups was observed (ETP-ratio: HC 0.66, CP-A 0.77, CP-B 0.90, CP-C 0.96).

Figure 7.4 Parameters of thrombin generation in healthy controls and cirrhosis patients. Thrombin generation was measured following a 1 pM (A, B, C, D) or 5 pM (E, F, G, H) tissue factor trigger in the presence of 4 IM phospholipids in platelet-poor normal pooled plasma ('NPP'), or platelet-poor plasma of healthy controls, CPA, CP-B or CP-C patients. Shown are the normalized thrombin generation parameters lag time (A, E), endogenous thrombin potential (ETP) (B, F), peak height (C, G) and time to peak in percentages (D, H). Data are presented as median with interquartile ranges and were analyzed statistically as described, with * $P < 0.05$, ** $P < 0.001$, *** $P < 0.0001$.

Figure 7.5 The endogenous thrombin potential (ETP) ratio. Thrombin generation was measured following a 1 pM tissue factor trigger in the presence of 4 IM phospholipids and the presence or absence of 0.56 nM thrombomodulin in platelet-poor plasma of healthy controls, or CP-A, CP-B or CP-C patients, or normal pooled plasma (dotted line). Data are presented as median with interquartile ranges and were analyzed statistically as described, with * $P < 0.05$, ** $P < 0.001$, *** $P < 0.0001$.

Rotational thromboelastometry

To assess the global clot formation, we performed rotational thromboelastometry measurements on whole blood samples and analyzed the thromboelastometry parameters (α -angle, clot formation time and maximum clot firmness, Figure 7.6A-C). For the α -angle, denoting the speed at which a clot forms, increasing severity of cirrhosis correlated with a reduction in α -angle, which was significant between CP-A patients and CP-C patients (78° vs. 70.5° , $P = 0.0015$). In accordance with this, the clot formation time was significantly prolonged with increasing cirrhosis severity (CFT: HC 51 sec, CP-A 63 sec, CP-B 90 sec and CP-C 104 sec). Furthermore, upon blood clot formation, the maximum clot firmness, reflecting the absolute strength of the fibrin and platelet clot, significantly decreased with increasing CP-score (MCF: HC 69 mm, CP-A 63 mm, CP-B 49 mm and CP-C 40 mm). When comparing the clot formation results of cirrhosis patients with the sex- and age-matched healthy controls, it can be noticed that median CP-C values are significantly decreased for the α -angle and MCF ($P < 0.0001$) and significantly prolonged for the CFT ($P < 0.001$).

tPa-induced fibrinolysis by rotational thromboelastometry

Standard thromboelastometry parameters representing fibrinolysis of a blood clot are set as the lysis onset time (LOT) and lysis time (LT). The fibrinolysis velocity (FV) calculates the decline of the clot between the LOT and LT in % per minute. In order to achieve full lysis after two hours of measuring, the same measurements were performed with the addition of 175 ng/ml tPA. As can be seen in Figure 7.6 (D-F) all median parameters of clot lysis show no significant differences between the three groups of cirrhotic patients, nor between the patients and the healthy controls.

Figure 7.6 Rotational thromboelastometry parameters of clot formation and fibrinolysis in cirrhosis patients. Rotational thromboelastometry was assessed on whole blood of healthy controls, or CP-A, CP-B and CP-C patients in the absence (EXTEM based, A,B,C) or presence of 175 ng ml⁻¹ tPA ('tPA-ROTEM', D,E,F). Data are presented as median with interquartile ranges and were analyzed statistically as described, with * $P < 0.05$, ** $P < 0.001$, *** $P < 0.0001$.

It can be observed that especially the Child-Pugh C patients show some extreme values in some of the performed measurements. These values were not all within one patient; the extreme FIXa-AT and FXa-AT levels occurred in three different patients. The extreme LOT and LT values however are within the same two patients. The patient that showed extremely increased LOT and LT values, showed the highest clot formation

capacity of the Child-Pugh C group. The patient that had an extremely decreased LOT and LT on the other hand, showed a significantly decreased clot formation capacity as well. This last-mentioned patient was overall severely ill with a MELD-score of 21 and significantly reduced antithrombin, fibrinogen and platelet levels.

We furthermore assessed whether the patients with a bleeding phenotype (n=19) were the same patients that showed signs of decreased clot formation capacity. There were no significant differences in the parameters of clot formation and clot lysis, nor in the parameters of the thrombin generation measurement, however between patients that experienced a bleeding and the patients without bleeding (data not shown).

Discussion

Recent work established that even in cirrhosis the equilibrium in coagulation is maintained, albeit that the balance may easily be perturbed.^{9,20} In order to explore the mechanisms underlying this balance, we assessed the coagulation-, clot formation- and clot lysis-status in patients with cirrhosis with a concise and state of the art panel of assays.

A first novel finding is a rather pronounced activation of the extrinsic coagulation system, indicated by elevated FVIIa levels in patients with cirrhosis (except for the CP-C patients, possibly due to a depletion of zymogen as FVII is the first clotting factor to fall in cirrhosis.²¹). This increased activity of the extrinsic pathway through FVIIa may be due to the increased levels of circulating microparticle tissue factor activity in cirrhotic patients that has recently been described by Rautou et al.²² Subsequently, this increased FVIIa may contribute to an increased activation of factor IX, through the Jossen loop, explaining a moderate increase in FIXa-AT complexes. Apparently, this is not sufficient to further drive the downstream factor X activation, which did not show statistically significant changes in FXa-AT complexes. Thus, a primary activation through the extrinsic tissue factor-FVIIa route seems to be an active pathway in patients with liver cirrhosis.

Thrombin generation analysis further corroborates the potential to hypercoagulability in liver cirrhosis, indicated by shortened lag times, a decreased time to peak and increased peak and ETP levels. In addition, we found that in patients with increasing severity of cirrhosis, in line with their significantly decreased protein C levels, the reduction in endogenous thrombin potential by thrombomodulin was significantly diminished, contributing to the hypercoagulability.

Our finding that patients with cirrhosis show a prothrombotic state in plasma even in the measurements conducted without thrombomodulin is a new observation. Tripodi et al. showed that thrombin generation without thrombomodulin was impaired, yet when the test was modified by adding thrombomodulin, patients generated as much thrombin as controls. A possible explanation for these contradictory results can be

found in the concentration of added phospholipids to the thrombin generation measurement. In the tests performed by our lab we used 4 μM phospholipids while Tripodi et al. used 0.5 μM phospholipids. Recombinant tissue factor itself does not carry enough procoagulant phospholipids to establish optimal prothrombin conversion. Additional phospholipids are therefore required. As the thrombin generation measurement appears to be phospholipid dependent in the 0-2 μM range, and only reaches a plateau at 3 μM , this could have caused the observed differences.²³ This is further supported by the thrombin generation measurements in cirrhosis patients performed by Gatt et al. who also used 4 μM phospholipids. Although Gatt did not find an increased thrombin generation in cirrhosis patients (without thrombomodulin), the ETP-levels of patients and controls were comparable, and not lower, in the measurements performed without thrombomodulin.⁸

A second new finding is that in spite of the overall procoagulant plasma milieu, the ROTEM analysis showed a significant reduction in clot formation capacity, while the addition of tPA did not reveal any significant alterations in resistance of the clot to lysis in patients with cirrhosis.

Using a novel 'tPA-ROTEM' application, we found, despite an increased thrombin generation potential, a significant decrease of the parameters representing the formation of a blood clot between CP-A and CP-C patients. In accordance with the decreased velocity of clot formation, the clot formation time was prolonged, indicating that in patients with increasing severity of cirrhosis it takes longer before a clot is formed. When finally the clot is formed, the absolute strength of this platelet and fibrin clot is diminished. These findings are in line with the findings of Tripodi et al. who compared 'classic' thromboelastometry results of apparently healthy controls with results of cirrhotic patients of combined CP-classes.¹¹

Possible disturbances in fibrinolysis due to cirrhosis might also contribute to an increased risk of bleeding.¹⁵ In accordance with the debate around a 'rebalanced' coagulation system in cirrhosis, a similarly 'rebalanced' fibrinolytic system has been proposed.¹³ So far, only a limited number of studies on fibrinolysis in cirrhosis have been performed mostly employing plasma clot lysis assays, or the measurement of levels of individual components of the fibrinolytic system. With our in house developed 'tPA-ROTEM' we were able to measure the resistance of the clot against lysis in undiluted whole blood. In this study the median of all three parameters of clot lysis (the onset of lysis, the lysis time and the velocity of fibrinolysis) showed no differences between patients with cirrhosis and healthy controls, nor between patient groups. This indicates that with increasing severity of cirrhosis no altered resistance of the clot to fibrinolysis was observed. This finding is supported by the findings of Lisman et al. who observed that the reduction of antifibrinolytic factors in cirrhosis is compensated by the concomitant reduction of profibrinolytic factors.¹³ Our results are in contrast with those of Rijken and Colluci, who observed a significant decrease in clot lysis time pointing to

an increase of fibrinolytic potential.^{14,15} The reason for the disparity between these results is likely due to methodologic differences and/or differences in the selection of patients.¹⁵

This study has several limitations. The major limitation of our study is the limited number of CP-C patients. This small number is due to the fact that patients with CP-C cirrhosis were often not able to give informed consent, or presented themselves in an emergency situation that required direct treatment with either blood products or albumin subsequently changing their CP-classification and coagulation status and were therefore not eligible for study participation.

A limitation of the tPA-ROTEM measurement is the large concentration of exogenous tPA (175 ng/ml) that has to be added to achieve full lysis within a reasonable timeframe. It is known that large concentrations of exogenous tPA, can make an assay less sensitive to the balance of the endogenous levels of tPA and PAI.¹⁵ As it has been suggested that hyperfibrinolysis in cirrhosis is mainly caused by significantly elevated tPA plasma concentrations, this cannot be detected in the tPA-ROTEM.^{24,25} The tPA-ROTEM therefore does not accurately assess the fibrinolytic potential, yet yields information on the resistance of the clot to fibrinolysis.

In conclusion, our data show a slightly dominant extrinsic route of coagulation activation, in conjunction with an increased thrombin generating potential in patients with cirrhosis. This accelerated thrombin generation translates into increased *plasma* clot formation, combined however with an attenuated fibrin clot firmness with seemingly unaltered resistance to fibrinolysis in whole blood. These complex alterations in blood coagulation may contribute to the delicate balance in tendency to bleeding and thrombosis in patients with cirrhosis.

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CHAPTER 8

**Decreased prothrombin conversion and thrombin
inactivation result in rebalanced thrombin generation
in liver cirrhosis**

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Abstract

Impaired coagulation synthesis in liver cirrhosis causes a reduction of most pro- and anticoagulant factors. Cirrhosis patients show no clear bleeding or thrombotic phenotype when they are in a compensated state, although they are at risk for both types of haemostatic event because of a limited coagulation factor reserve. Thrombin generation (TG) is a global test of coagulation and its outcome depends on underlying pro- and anticoagulant processes which are be disturbed in cirrhosis. We aimed to quantify the pro- and anticoagulant processes that underlie TG to investigate abnormalities in prothrombin conversion and thrombin inactivation in cirrhosis.

TG, prothrombin conversion, and thrombin inactivation were quantified in 30 healthy subjects and 73 cirrhosis patients, classified according to Child-Pugh as A (n=52), B (n=15) and -C (n=6). Both prothrombin conversion and thrombin inactivation are reduced in liver cirrhosis patients. The effect on pro- and anticoagulant processes partially cancel each other out and as a result TG is surprisingly comparable at 5 pM tissue factor between healthy subjects and patients. This supports the hypothesis of rebalanced haemostasis. In silico analysis shows that normalization of either prothrombin conversion or thrombin inactivation to physiological levels would cause an elevation of TG, whereas the normalization of both simultaneously maintains a balanced TG.

In conclusion, TG in cirrhosis patients remains within the normal range, despite large changes in prothrombin conversion and thrombin inactivation, indicating that indeed coagulation is rebalanced in cirrhosis. Nevertheless, the changes in the underlying TG indicate that cirrhosis patients might require adapted haemostatic treatment.

Introduction

Coagulation in liver cirrhosis is disturbed because of reduced clotting factor production by the liver and alterations of platelet function and number.¹ The production of both procoagulant (FII, FV, FVII, FIX, FX, and FXI) and the anticoagulant factors (protein C, protein S, and antithrombin) is reduced.²⁻⁵ Coagulation factor levels in liver cirrhosis patients can be as low as the levels found in congenital deficiency, without the symptoms that are generally seen in deficient patients.²⁻⁵ The most commonly reported haemostatic problems in liver cirrhosis are bleeding from ruptured oesophageal varices, bruising, bleeding after invasive procedure, but also deep venous thrombosis, pulmonary embolism and intrahepatic thrombus formation.⁶⁻¹¹ Although both bleeding and thrombosis symptoms have been reported in cirrhosis, routine clinical test such as the prothrombin time (PT) and the activated partial thromboplastin time (aPTT) indicate an increased bleeding risk.^{3,12} The PT is often prolonged because of a reduction of procoagulant factor levels and if the PT is modified to include the effect of anticoagulant factors, no difference is found between liver cirrhosis patients and healthy subjects.^{1,12,13} During the last decade, a new view of coagulation in liver cirrhosis patients emerged: the rebalanced coagulation system.¹ It was hypothesized that a reduction of both the pro- and anticoagulant pathways results in a newly found balance in coagulation.^{1,6,14} This paper lends quantitative support to this concept and suggests that the new balance may be more precarious than the normal one is.

Whereas routine coagulation tests do not correlate with the bleeding nor thrombotic risk in liver cirrhosis, mainly because they only reflect part of the coagulation system, the thrombin generation test (TG), which represents the complete system, correlates better with the haemostatic situation in cirrhosis patients.^{2,4,5,12,15,16} Thrombin generation in plasma is dependent on two underlying processes, the production of thrombin (i.e. prothrombin conversion) and the removal of thrombin from the clotting plasma (thrombin inactivation).¹⁷ Prothrombin conversion is affected by the levels of all procoagulant factors, but also by the levels of proteins C and S. The latter factors destroy FVa and FVIIIa and thereby decrease prothrombin conversion.¹⁸ The major players in thrombin inactivation are antithrombin (AT) and α_2 -macroglobulin (α_2 M).¹⁹ We have recently developed a method to determine the prothrombin conversion and thrombin inactivation curves from a TG curve by an approach based on computational modelling of thrombin inactivation.²⁰⁻²² In this way we can investigate prothrombin conversion and thrombin inactivation separately. In this study we aim to investigate the changes in prothrombin conversion and thrombin inactivation in liver cirrhosis. Additionally, we aim to use computational modelling to investigate the individual contribution of changes in prothrombin conversion and thrombin inactivation to the differences in TG between patients and healthy subjects, and to investigate the consequences of these changes in pro- and anticoagulant processes for the bleeding management in cirrhosis patients.

Materials and methods

Sample collection and handling

The population tested in this paper is the same as described by Kleinegris et al.¹⁶ The study was approved by the local medical ethics committee and healthy volunteers and patients gave full informed consent for blood donation, according to the Helsinki declaration. All-cause liver cirrhosis patients were enrolled after diagnosis based on clinical, laboratory, ultrasound, gastroscopy and/or histological evidence. They were classified as Child-Pugh A (n=52), B (n=15), and C (n=6). Exclusion criteria were the use of medication that affects coagulation, documented coagulation disorders and age below 18 years. Blood was collected on 3.2% citrate in a 9:1 ratio for the preparation of platelet poor plasma. Platelet poor plasma was prepared by centrifuging twice at 2821 g for 10 minutes and was stored at -80°C until further use.

Materials

Chromogenic thrombin substrate S2238 was synthesized in house. Unfractionated heparin and bovine serum albumin were purchased at Sigma-Aldrich (Zwijndrecht, the Netherlands). Bovine thrombin was purified in house as described by Church²³ and bovine antithrombin according to the protocol of Thaler.²⁴ Staphylocoagulase was purified in house as described by Hendrix et al.²⁵ Reagents for thrombin generation were purchased from Thrombinoscope bv (Maastricht, the Netherlands) and recombinant soluble thrombomodulin was a gift from Thrombinoscope bv.

Measurement of coagulation factor levels

Functional AT, α_2 M and prothrombin levels were measured as previously described.²⁰ Plasma fibrinogen levels were measured by the Clauss method.²⁶

Thrombin generation

Calibrated Automated Thrombinography (CAT) was performed as previously described.¹⁷ All wells contained 80 μ l plasma and 20 μ l of PPP reagent LOW (1 pM TF, 4 μ M phospholipids) or PPP reagent (5 pM TF, 4 μ M phospholipids). To calibrator wells, 20 μ l of calibrator was added instead. Thrombin generation (TG) was initiated by the addition of 20 μ l of ZGGR-AMC (417 μ M) and CaCl_2 (16.7 mM). Recombinant soluble thrombomodulin (0.56 nM f.c.) was added to PPP reagent LOW to test for APC resistance. The TG fluorescence data was converted to thrombin generation curves, as described elsewhere²⁷ and used to perform additional computational analysis to extract prothrombin conversion curves.

Calculation of prothrombin conversion and thrombin inactivation

The thrombin generation curve is the net result of prothrombin conversion and thrombin inactivation, and therefore the course of prothrombin conversion can be calculated if thrombin generation and thrombin inactivation are known. Thrombin inactivation was predicted by the previously described and validated computational model based on the plasma antithrombin, α_2 -macroglobulin and fibrinogen level, and was used to determine prothrombin conversion curves from thrombin generation data.²⁰

In silico experimentation

Thrombin generation is the net result of the underlying processes of prothrombin conversion and thrombin inactivation. Therefore, TG can be calculated if the courses of prothrombin conversion and thrombin inactivation are known. Thrombin inactivation was predicted for each sample by a computational model as described previously²⁰, and prothrombin conversion curves were obtained from the original experimental data. This methodology makes it not only possible to predict a TG curve, but also to perform *in silico* experimentation to determine the effect of changes in prothrombin conversion or thrombin inactivation in cirrhosis patients.

Prothrombin conversion and/or thrombin inactivation were normalized to average healthy values to test the hypothesis that TG is rebalanced in liver cirrhosis, and that normalization of either prothrombin conversion or thrombin inactivation would result in a deviant haemostatic state in cirrhosis patients. The contribution of changes in prothrombin conversion in liver cirrhosis to TG were determined by simulating TG curves based on the average prothrombin conversion curves of healthy controls and the thrombin inactivation system as found in each individual cirrhosis patient. The effect of acquired AT deficiency in cirrhosis was determined by simulating TG curves based on prothrombin conversion curves as found in cirrhosis patients with the average AT level found in healthy controls (2.08 μ M). The role of α_2 M levels was investigated in the same manner.

The administration of prothrombin complex concentrate (PCC) with or without antithrombin supplementation was simulated *in silico* by increasing the prothrombin conversion curve (and the AT levels where indicated) to 110%, 120% and 130%, and the effects on TG were analyzed.

Statistics

The Statistical Package for the Social Sciences (SPSS) was used to determine the statistical significance of the results. The distribution of the data was tested with a Shapiro-Wilk test and the statistical significance of differences between the groups was determined with ANOVA or Kruskal-Wallis analysis accordingly.

Results

Plasma samples from 30 healthy subjects and 73 liver cirrhosis patients, classified as Child-Pugh (CP)-A, CP-B or CP-C, were collected (Table 8.1). Thrombin generation was measured at 1 pM TF (Figure 8.1) and 5 pM TF (Supplementary Figure S8.1). The lagtime and endogenous thrombin potential (ETP) were comparable between healthy subjects and cirrhosis patients, whereas the peak height and velocity index were significantly elevated in patients, irrespective of the TF concentration used. Subsequently, the time-to-peak was shorter in patients than in healthy subjects.

Table 8.1 Patient characteristics.

Characteristic	Child-Pugh A (n=52)	Child-Pugh B (n=15)	Child-Pugh C (n=6)
Age (years)	61 (54-67)	53 (51-66)	55 (40-62)
Sex (male, %)	31 (60%)	12 (80%)	5 (83%)
Child-Pugh score	5 (5-5)	8 (7-8)	10 (10-11)

Values are expresses as medians with interquartile ranges.

Plasma antithrombin, α_2 -macroglobulin, prothrombin and fibrinogen levels were measured to further characterize the haemostatic state of the subjects and to enable the calculation of prothrombin conversion and thrombin inactivation parameters (Table 8.2). Plasma AT levels were significantly decreased and some patients had AT levels as low as the levels found in heterozygous AT deficiency (50%). Plasma prothrombin levels were significantly reduced in cirrhosis patients as well. On the contrary, α_2 M levels were significantly increased in CP-A patients. Fibrinogen levels were elevated in CP-A patients, but diminished in CP-C patients.

Table 8.2 Coagulation factor levels.

Coagulation factor	Healthy subjects (n=30)	Child-Pugh A (n=52)	Child-Pugh B (n=15)	Child-Pugh C (n=6)
Prothrombin, μ M ($\pm$ SD)	1.13 ($\pm$ 0.25)	0.91 ($\pm$ 0.20)***	0.61 ($\pm$ 0.15)***	0.49 ($\pm$ 0.22)***
Antithrombin, μ M ($\pm$ SD)	2.08 ($\pm$ 0.37)	1.65 ($\pm$ 0.57)**	0.99 ($\pm$ 0.49)***	0.81 ($\pm$ 0.59)***
α_2 -macroglobulin, μ M ($\pm$ SD)	3.21 ($\pm$ 0.64)	3.66 ($\pm$ 0.75)*	3.31 ($\pm$ 0.86)	3.17 ($\pm$ 0.69)
Fibrinogen, g/L ($\pm$ SD)	2.67 ($\pm$ 0.40)	3.27 ($\pm$ 0.79)**	2.83 ($\pm$ 0.92)	1.77 ($\pm$ 0.94)***

*p<0.05, **p<0.01, ***p<0.001 compared to healthy subject values.

Figure 8.1 Thrombin generation in cirrhosis patients and healthy subjects. (A) Mean thrombin generation curves in healthy subjects (○), Child-Pugh A patients (●), Child-Pugh B patients (△), and Child-Pugh C patients (▲) measured at 1 pM TF. (B) Lag time, (C) peak height, (D) endogenous thrombin potential, (E) time-to-peak and (F) velocity index were quantified from the TG curves. *p<0.05, **p<0.01, ***p<0.001 compared to healthy subject values.

To study the significance of these differences in coagulation factor levels to the thrombin generation profile, we determined the courses of prothrombin conversion and thrombin inactivation during TG. Figure 8.2 and Supplementary Figure S8.2 show that there is a significant difference in the course of prothrombin conversion between patients and healthy subjects, triggered with 1 and 5 pM TF, respectively. The total amount of prothrombin that is converted during thrombin generation is significantly reduced in cirrhosis patients (15-43%, depending on the disease severity; Figure 8.2B), which corresponds to the reduced prothrombin plasma levels found in patients (Table 8.2). The maximal rate of prothrombin conversion (Figure 8.2C) is higher in patients, indicating that the concentration and/or activity of the prothrombinase complex is increased. In addition to the reduction of prothrombin conversion, we found a marked reduction of thrombin decay capacity in cirrhosis patients (Figure 8.3). The amount of thrombin-antithrombin complexes that were formed during TG was significantly lower in patients compared to healthy subjects (Figure 8.2D). On the contrary, thrombin- α_2 -macroglobulin complex formation is comparable in patients and healthy subjects, but the relative amount of thrombin inhibited by α_2 M is elevated in patients (Figure 8.2E-F). The function of the APC system was tested by measuring thrombin generation in the presence or absence of 0.56 nM thrombomodulin (Figure 8.4). In healthy subjects, TM caused a significant reduction of thrombin generation ($\pm 40\%$ reduction of the ETP), whereas significantly less TG inhibition was detected in cirrhosis patients (Figure 8.4A+C). In addition, prothrombin conversion was attenuated the most in healthy individuals (25%), and the least in CP-C patients (5%). The level of inhibition of TG and prothrombin conversion by TM addition decreases with the severity of the disease. Interestingly, TM decreases thrombin generation by reducing the total amount of prothrombin that is converted throughout the experiment, rather than decreasing the maximum rate of prothrombin conversion.

The thrombin generation profile of cirrhosis patients differs from that of healthy subjects because of (1) changes in prothrombin conversion, (2) antithrombin levels, and (3) α_2 -macroglobulin levels. We have performed *in silico* analysis to determine the individual contribution of these three changes to the overall process of TG (Figure 8.5). Firstly, we determined the effect of the reduction of prothrombin conversion in liver cirrhosis on TG by substituting prothrombin conversion curves from liver cirrhosis patients by healthy control prothrombin conversion curves (Figure 8.5A-B). If prothrombin conversion would be in the normal range in liver cirrhosis, this would cause the patient to be in a more procoagulant state, as thrombin generation would be markedly increased, especially in more severe cases of cirrhosis. Next, we determined the effect of acquired AT deficiency in cirrhosis patient by simulating TG curves as if patients had physiological AT levels (2.08 μ M). Figure 5C-D shows that an increase in AT level in liver cirrhosis significantly decreases TG in patients, but not in healthy subjects.

Figure 8.2 Prothrombin conversion in cirrhosis patients and healthy subjects. (A) Mean prothrombin conversion curves in healthy subjects (○), Child-Pugh A patients (●), Child-Pugh B patients (Δ), and Child-Pugh C patients (▲) triggered with 1 pM TF. (B) Total prothrombin conversion, (C) maximal rate of prothrombin conversion, (D) thrombin-antithrombin formation, (E) thrombin- α_2 -macroglobulin formation and (F) the percentage of thrombin inhibited by α_2 -macroglobulin were quantified from the TG curves. *p<0.05, **p<0.01, ***p<0.001 compared to healthy subject values.

Figure 8.3 Thrombin inactivation capacity in cirrhosis patients and healthy subjects. The thrombin decay capacity was calculated based on the antithrombin, α_2 -macroglobulin and fibrinogen level of the sample. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to healthy subject values.

Finally, we tested whether an increase of α_2 M levels in cirrhosis patients has a protective function as it might counteract the pro-coagulant effects of an acquired AT deficiency, by predicting TG as if the α_2 M level of each patient was equal to the average healthy subject α_2 M (3.21 μ M). Even though α_2 -macroglobulin plays a bigger role in thrombin inhibition in liver cirrhosis (Figure 8.4), this does not contribute significantly to thrombin generation in this context (Figure 8.5E-F).

Cirrhosis patients undergoing surgery often require transfusion products according to their clotting time (PT or aPTT). In Figure 8.6 we present an *in silico* prediction of thrombin generation after the administration of prothrombin complex concentrate by increasing the prothrombin conversion capacity of each subject to 110%, 120% and 130%. An increase of the prothrombin conversion capacity elevates ETP and peak height significantly (Figure 8.6C+E) in healthy subjects and cirrhosis patients. In addition to PCC administration alone, we also predicted the effect of *in silico* administration of PCC in combination with antithrombin (Figure 8.6B). The combined administration of pro- and anticoagulant factors also increase the ETP and the peak height significantly, but to a lesser extent than PCC alone (Figure 8.6D+F).

Figure 8.4 APC sensitivity in cirrhosis patients and healthy subjects. (A-B) Thrombin generation and prothrombin conversion curves measured at 1 pM TF in the absence (gray) or presence of 0.56 nM thrombomodulin (black) in healthy subjects (o), Child-Pugh A patients (●), Child-Pugh B patients (Δ), Child-Pugh C patients (▲). (C-F) The percentage of the ETP, peak height, total prothrombin conversion and the maximum prothrombin conversion rate in plasma with thrombomodulin compared to plasma without thrombomodulin. *p<0.05, **p<0.01, ***p<0.001 compared to healthy subject values.

Figure 8.5 The contribution of changes in prothrombin conversion, antithrombin levels and α_2 -macroglobulin levels to the altered thrombin generation profile in cirrhosis patients. Prothrombin conversion (A-B), antithrombin levels (C-D) and α_2 -macroglobulin levels (E-F) were normalized *in silico* to the average level found in the healthy subjects group. Thrombin generation was simulated (black) in healthy subjects (○), Child-Pugh A patients (●), Child-Pugh B patients (△), Child-Pugh C patients (▲) and compared to the experimental values found in the same group of subjects (grey). The simulated and experimental TG curves in the healthy subject group are almost completely superimposed. The ratio of simulated and experimental ETP values were compared between patients and healthy subjects. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to healthy subject values.

Figure 8.6 The *in silico* effect prothrombin complex concentrates with or without co-supplementation of antithrombin in cirrhosis. *In silico* experimentation was performed to predict the effect of prothrombin complex concentrate administration on thrombin generation in cirrhosis patients in the absence (A) or presence (B) of antithrombin supplementation. Prothrombin conversion curves were increased *in silico* to 110% (---), 120% (- -), ad 130% (- · -) with or without co-administration of 100%, 120%, or 130% AT. The effects of PCC with or without antithrombin were quantified by the ETP (C-D) and the peak height (E-F). Healthy subjects are shown in black, CP-A patients in red, CP-B patients in blue and CP-C patients in green. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to healthy subject values.

Discussion

Our study confirms previous reports that AT and prothrombin levels are reduced in patients with liver cirrhosis^{2-5,28} and that α_2 M levels are increased.²⁹⁻³² Thrombin generation was found to be elevated at both low and high tissue factor concentrations, but the ETP was unchanged, as was reported by others.^{12,15,28,33-36} Studies showed that liver cirrhosis patients do not suffer from unidirectional haemostatic problems (either bleeding or thrombosis) because their coagulation systems are rebalanced, as both their pro- and anticoagulant capacity decreases.^{1,6,37} This would explain the comparability of thrombin generation profiles between healthy subjects and cirrhosis patients. It is hard to imagine that the profound alteration of the coagulation factor profile in liver cirrhosis would not affect the processes of thrombin production and thrombin inactivation, which underlie the course of thrombin generation itself.

We showed that the total amount of prothrombin converted during TG is significantly reduced in liver cirrhosis patients, although the maximal rate of prothrombin conversion is increased in patients.²⁰ The reduction in prothrombin conversion is likely to be caused by a lower availability of prothrombin. The increased maximal rate of prothrombin conversion is dependent on the concentration or activity of the prothrombinase complex, as previously reported by Chandler et al.³⁸ Potential mechanisms could be the reduction of antithrombin levels, which cause reduced inhibition of FXa and thrombin or the attenuation of the inhibitory APC pathway. It is in our opinion unlikely that this effect is mediated via the APC pathway, as the effect is seen in TG measured in the absence of thrombomodulin and TM is required for the functioning of the APC pathway during TG.^{39,40} In addition, the reduction of AT levels (29%) in patients is reflected in a reduction of the thrombin decay capacity (30%). Even though α_2 M levels are increased in patients, α_2 M is incapable of restoring the thrombin decay capacity or thrombin generation itself in cirrhosis patients, but the percentage of thrombin that was inhibited by this secondary thrombin inhibitor was increased up to 3-fold compared to the values found in healthy subjects. Thus, in patients, less thrombin is formed, but it is formed faster and inactivated slower, causing an elevation of the thrombin generation peak height, but not the ETP. Nevertheless, we show that prothrombin conversion and thrombin inactivation are severely attenuated in cirrhosis, which provides experimental evidence that supports the hypothesis of rebalanced thrombin generation in cirrhosis. Similar to the prothrombin-antithrombin balance, we reckon with the possibility that the decrease of the factor V is compensated for by the decrease of the delimiting factors protein C and protein S. This is supported by the finding of thrombomodulin resistance in liver cirrhosis patients.^{34,35,41}

The coagulatory state is somewhat comparable to that of children. In the paediatric population, coagulation factor levels are reduced as well⁴²⁻⁴⁴ and we have recently shown that this causes thrombin generation to be lower due to a reduction of prothrombin conversion. The difference between children and cirrhosis patients is that

the maximum prothrombinase is reduced in children, whereas it is increased in cirrhosis patients.²² Children however show a similar newly formed balance of thrombin inactivation, in which α_2 -macroglobulin partly takes over the function of antithrombin, indicating that α_2 -macroglobulin is a back-up inhibitor for antithrombin on more than one occasion.

In silico experimentation pointed out that an increase of prothrombin conversion in liver cirrhosis patients to a physiological level will provoke a procoagulant state in CP-B and CP-C patients (ETP 300% of baseline on average), whereas the normalization of AT levels will cause a severe reduction of the ETP (half of all patients would have an ETP lower than 75% of normal). This indicates that indeed the coagulation system is rebalanced in liver cirrhosis patients, and that restoration of the prothrombin conversion capacity (e.g. based on a prolonged PT measurement) or thrombin decay capacity would result in an imbalanced coagulation system. Furthermore, we hypothesized that the increase in α_2 M level in liver cirrhosis could compensate for the AT deficiency to some extent. *In silico* experimentation shows that the increase of α_2 M levels in liver cirrhosis significantly improved the thrombin decay capacity, although it could not be restored to the level found in healthy subjects. However, the effect of elevated α_2 M levels in cirrhosis patients on TG itself was negligible and statistically insignificant. This is in line with the fact that AT is a much more potent thrombin inhibitor than α_2 M⁴⁵ and that a large increase in α_2 M level is needed to compensate a small loss of AT.

Even though a new haemostatic equilibrium seems to be established in liver cirrhosis, a higher incidence of bleeding and thrombotic episodes has been reported.⁶⁻¹¹ This may partly be due to local causes (e.g. varices), but also to a loss of buffer capacity, in the sense that small variations of a pro- and anticoagulant protein will result more easily in an unbalanced situation. Therefore, we can expect that patients react differently to anticoagulation treatment than healthy controls, as shown in recent literature.⁴⁶ The most obvious case is the decreased effectiveness of treatment with heparins, because heparin acts by increasing the AT activity.⁴⁷⁻⁴⁹ Also the effects of direct FXa or thrombin inhibitors can be expected to show altered dynamics. Indeed, it was recently published by Potze et al. that the *in vitro* anticoagulant effect of rivaroxaban is decreased in patients with liver cirrhosis.⁵⁰

In addition, the transfusion of fresh-frozen plasma and other blood products based on a prolonged PT measurement has been under debate in liver cirrhosis.^{15,51,52} Tripodi et al. showed that, although the administration of normal plasma shortened the PT, thrombin generation remained unchanged in liver cirrhosis patients.¹⁵

We performed *in silico* experiments to investigate the effect of the transfusion of procoagulant factors alone (PCC) or in combination with AT (to mimic PCC containing AT or fresh frozen plasma transfusion). We show that both transfusion protocols significantly increase TG, albeit PCC alone increases TG to levels associated with thrombosis, whereas PCC in combination with AT does not. This indicates that boosting

the procoagulant system by administration of procoagulant factors based on an increased PT can even evoke a procoagulant state in liver cirrhosis patients. This indicates that pre-procedural correction of the PT with blood products may not be necessary, is potentially harmful,^{51,52} and stresses that the PT is not a global indicator of the coagulant state and reflects primarily procoagulant pathways. A global haemostasis test such as the thrombin generation measurement would be a better alternative to assess the haemostatic state of cirrhosis patients.

Computational simulation of thrombin generation can be profitably used for 'what-if' analysis. In complex diseases, such as liver cirrhosis, coagulation is altered at many points which have opposite effects on thrombin generation (e.g. increase of α_2 M and FVIII, and a decrease of FII and AT). This makes the integration of these changes and their net effect on the outcome hard to predict. Here we showed that, computational simulation can be used to determine the net outcome of the effect of a change of one or more factors in the system, which can be a physiological change or the administration of a drug.⁵³ This is especially useful to select optimal treatment option in liver cirrhosis patients.

This study has two limitations, of which the first is that thrombin generation was not measured in platelet rich plasma and whole blood due to logistic difficulties. In addition, we currently only have a validated model for the interactions of thrombin with its inhibitors antithrombin and α_2 -macroglobulin.²⁰ An application of this model is that it can be used to split a thrombin generation curve into its underlying processes of prothrombin conversion and thrombin inactivation (the latter of which can be completely modelled). Unfortunately such a computational technique is not yet available for the APC system or the effects of FVIII, and therefore we could not yet investigate the role of the APC system or FVIII *in silico* in the current study.

In conclusion, we present experimental evidence that liver cirrhosis patients indeed have rebalanced thrombin generation. However, there are vast but concealed differences in the underlying mechanisms of prothrombin conversion and thrombin inactivation. This indicates that cirrhosis patients cannot be treated as haemostatically normal individuals. Caution is warranted in applying regular transfusion and anticoagulation protocols, especially if these procedures are based on a PT measurement, which overestimates the role of the procoagulant pathway in liver cirrhosis.

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Supplemental figures

Figure S8.1 Thrombin generation in cirrhosis patients and healthy subjects. (A) Mean thrombin generation curves in healthy subjects (○), Child-Pugh A patients (●), Child-Pugh B patients (Δ), and Child-Pugh C patients (▲) measured at 5 pM TF. (B) Lag time, (C) peak height, (D) endogenous thrombin potential, (E) time-to-peak and (F) velocity index were quantified from the TG curves. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to healthy subject values.

Figure S8.2 Prothrombin conversion in cirrhosis patients and healthy subjects. (A) Mean prothrombin conversion curves in healthy subjects (o), Child-Pugh A patients (●), Child-Pugh B patients (Δ), and Child-Pugh C patients (▲) triggered with 5 pM TF. (B) Total prothrombin conversion, (C) maximal rate of prothrombin conversion, (D) thrombin-antithrombin formation, (E) thrombin- α_2 -macroglobulin formation and (F) the percentage of thrombin inhibited by α_2 -macroglobulin were quantified from the TG curves. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to healthy subject values.

CHAPTER 9

Von Willebrand factor and D-dimer versus global coagulation assays, as predictors of bleeding, thrombosis and liver-disease related mortality in cirrhosis

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Submitted

Abstract

Background and objectives

Patients with cirrhosis have a fragile coagulation system, which is associated with both thrombosis and bleeding. It is clinically important to distinguish those patients that will bleed and those that develop a thrombosis. Therefore we initiated a prospective study to investigate the value of several haemostatic parameters for the prediction of coagulation related events and liver related mortality in patients with cirrhosis.

Methods

Blood was collected from patients with all-cause cirrhosis. Various coagulation parameters were measured, including D-dimer and vWF (von Willebrand Factor). Thrombin generation by Calibrated Automated Thrombography was determined in platelet poor plasma using a tissue factor trigger with/without thrombomodulin. The 'tPA-ROTEM' was determined in whole blood triggered with tissue factor with/without tissue Plasminogen Activator. During follow-up the composite of major bleeding, thrombotic event in any vessel and liver related mortality was recorded as an event.

Results

A total of 73 patients with cirrhosis (Child-Pugh A, n=52, Child-Pugh B, n=15 and Child-Pugh C, n=5) were followed for an average of 29 months (range 1-45months). During the follow-up fourteen patients experienced a bleeding, three a thrombo-embolic event and nine died from to liver-related causes. Univariate Cox regression analysis showed that an increased PT (Prothrombin Time) >12.2sec, INR (International Normalized Ratio) >1.10, a vWF >264%, D-dimer >621.5 ng/ml and a decreased ROTEM-MCF (Maximal Clot Firmness) < 61mm, decreased thrombocyte count <125x10⁹/l and antithrombin level >81% were significantly associated with the occurrence of a clinical endpoint. In the Multivariate Cox Proportional Hazard analysis only the vWF and D-dimer remained significant predictors of clinical events independent of age, the Child-Pugh score and the MELD-score with a hazard ratio (HR) of 4.07 (95% CI 1.43-11.54, p=0.008) and a HR of 3.31 (95% CI 1.13-9.65, p=0.028) respectively.

Conclusion

Both D-dimer and vWF have independent value as predictors of coagulation related events and liver related mortality in patients with cirrhosis. General coagulation assays such as the thrombin generation measurement and (tPA-) ROTEM were of no added value for risk prediction.

Introduction

Patients with cirrhosis have a fragile haemostatic system; changes include a reduced platelet count, platelet function defects and decreased plasma levels of coagulation factors and inhibitors of fibrinolysis.^{1,2} Consequently, it was assumed that patients with liver cirrhosis had an increased bleeding diathesis. Patients would therefore receive platelet transfusions and fresh frozen plasma prior to invasive procedures such as liver biopsies³ and surgical interventions. Accumulating evidence from studies that demonstrated more complex alterations in the haemostatic system has recently refuted this assumption and suggests a so-called 'rebalanced' haemostasis. A finding which is explained by a concomitant decrease in pro- and anti-coagulant factors, the latter not being detected by conventional coagulation tests such as PT and APTT. This 'rebalanced' haemostatic system can induce normal or even increased coagulability, but has proven to be easily disturbed and thereby giving rise to either bleeding or thrombotic events.⁴⁻⁷

During the past decades there has been a discrepancy between the information retrieved from clotting tests and the evidence regarding bleeding and/or thrombotic events in patients with cirrhosis. The PT (Prothrombin Time) and its derived measure the INR (International Normalized Ratio) for example, one of the laboratory variables in the Child-Pugh and MELD-score, are used to estimate not only the severity and prognosis of cirrhosis but also to assess the bleeding risk. As the PT can inform us on deficiency in one or more procoagulant factors, it cannot tell whether this deficiency is counterbalanced by a concomitant decrease in anticoagulant factors.⁸ Various studies state that the INR cannot be used in patients with chronic liver disease, unless an adjusted calibration is established based on plasma of these patients.² Furthermore, the PT and INR as well as other conventional coagulation tests are poorly correlated with the risk of bleeding or bleeding-related clinical events in patients with cirrhosis.⁹⁻¹¹

Lately more sophisticated overall coagulation assays, such as the thrombin generation assay and thromboelastometry have been used to assess the coagulation status in patients with cirrhosis. However, so far no studies have directly determined whether these tests are useful in predicting the bleeding or thrombosis risk in patients with cirrhosis. Previously, we performed a cross-sectional study using a combination of both conventional coagulation tests and overall coagulation assays like the thrombin generation assay and the 'tPA-ROTEM' assay to gain better insight into the complete haemostatic profile of patients with increasing severity of cirrhosis.¹² The aim of the current, prospective study was to investigate the relationship between these haemostatic tests and the occurrence of bleeding, thrombo-embolic events and/or liver-related mortality in the cohort of cirrhotic patients.

Methods

Patients

Patients with all-cause liver cirrhosis were included at the gastroenterology departments of two hospitals in Limburg, the Netherlands. Patients, aged 20-79 years, were consecutively included from 2011 to 2013 after informed consent was obtained. The diagnosis of liver cirrhosis was based on a combination of clinical, laboratory, ultrasound, gastroscopy and/or histological evidence. Patients with variable stages of liver cirrhosis were included; the severity of disease estimated using the Child-Pugh-Turcotte Classification and by calculating the MELD-score (Model for End-stage Liver Disease: $9.57 \times \ln(\text{creatinine}) + 3.78 \times \ln(\text{bilirubin}) + 11.2 \times \ln(\text{INR}) + 6.43$).¹³

Patients were excluded when they used medication known to affect coagulation, in case of documented congenital coagulation disorders and when aged less than 18 years. Follow-up information of included patients was obtained in March 2015, using SAP and Medview; the local hospital patient file systems. The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki (Seoul 2008) and was approved by the local medical ethics committee (METC azM / UM: 09-2-119).

Collecting blood and plasma preparation

To obtain blood samples, venous blood was collected in citrate tubes (4.5 ml 3.2%, vacutainer glass, 0.105 M, Becton and Dickinson, Breda, The Netherlands). Blood was collected through direct, sterile venipuncture in the antecubital fossa. Subsequently, the blood was processed into platelet poor plasma (PPP) by an initial centrifugation step at 1750 g (at 18°C) for 15 minutes and a second centrifugation step at 11000g (at 18°C) for 10 minutes. Samples were then stored at -80°C until use. Before further testing, the samples were thawed for 15 minutes at 37°C. For the whole blood 'tPA-ROTEM'-analysis one 4.5 ml 3.2% citrate tube (Vacutainer glass, 0.105 M, Becton and Dickinson, Breda, The Netherlands) was collected and used directly.

Coagulation tests and measurement of hematologic parameters

Measurements of coagulation were performed on the Sysmex CA-7000 analyzer (Sysmex Corporation, Kobe, Japan) as instructed by the manufacturer (reagents from Siemens Healthcare Diagnostics Products, Den Haag, the Netherlands); PT (Dade® Innovin®), aPTT (Dade® Actin® FSL Activated PTT Reagent, Calcium Chloride Solution), Fibrinogen (Dade® Thrombin Reagent), Plasminogen (Berichrom Plasminogen), Antithrombin (Berichrom® Anti-Thrombin III (AT)), vWF-antigen (vWF Ag), D-dimer (INNOVANCE D-Dimer) and Protein C (Berichrom Protein C).

Thrombin generation measurement via the CAT-method

The validated Calibrated Automated Thrombogram method was used to measure thrombin generation (Thrombinoscope BV, Maastricht, The Netherlands) in tissue factor-triggered platelet-poor plasma. Measurements were conducted on 80 μ l human platelet-poor-plasma with 20 μ l fluorogenic substrate, Calcium chloride (FluCa) and 20 μ l trigger reagent. In this study we used a final concentration of 1pM tissue factor (TF) as a trigger with 4 μ M phospholipids (at 20:20:60 mol% PS:PE:PC), experiments were also performed in the presence of 0.56nM recombinant soluble thrombomodulin. All CAT reagents were obtained from Thrombinoscope BV. Measurements were performed according to our standardised protocol as described earlier.¹⁴ A priori we chose the parameters: lagtime, the endogenous thrombin potential (ETP), the normalized endogenous thrombin potential, the Peak Height, normalized peak height, time to Peak (ttP) and Velocity Index (VI) as the primary predictor variables. Lagtime reflects the initiation phase of coagulation (the moment at which 10 nM thrombin is formed), the Peak Height shows the maximum amount of formed thrombin expressed in nmol/l, the time to peak indicates the time in minutes required to reach maximum thrombin generation, the Velocity Index is calculated by dividing the peak thrombin concentration by the difference between time to peak and lag time and the endogenous thrombin potential reflecting the total amount of thrombin formed in time.

ROTEM-analysis

In this study the ROTEM (EXTEM based) and the 'tPA-ROTEM' were carried out by means of the 4 channel ROTEM[®] Gamma device operated according to manufacturer instructions using tissue factor (Innovin) and tPA (Actilyse), as described.¹⁵ In short, 300 μ l of recalcified citrated blood was incubated in a preheated cup, within one hour after venipuncture at 37°C.¹⁵ The tPA-ROTEM was determined in recalcified citrated whole blood incubated with two different triggers: 35 pM tissue factor in the presence or absence of 175 ng/ml tissue plasminogen activator (tPA). As the primary predictor variables we chose: the α -Angle (α) defined as the speed (sec/min) at which the clot is formed; the Clot Formation Time (CFT), defined as the time (sec) from initiation of clotting until a clot firmness of 20mm is detected; the Maximum Clot Firmness (MCF) defined as the maximal amplitude (mm) of the clot; the Lysis Onset Time (LOT) defined as the time (sec) until 15% reduction of the MCF; the Lysis Time (LT) defined as the time (sec) until 90% reduction of the MCF and the Fibrinolysis velocity (FV) defined as the decline in % per minute between the LOT and the LT.

Definition of clinical endpoints

Previous literature suggested that in cirrhosis there is a 'rebalanced coagulation', which can easily tip either way, towards bleeding or thrombosis. In a prospective study patients with liver cirrhosis were included in this study. Based on the outcome these

patients were divided in two groups: those with an event and those patients without an event during follow. An event comprised a composite of major bleeding (requiring medical attention in a hospital), a thrombotic event in any vessel or liver related mortality.

Statistical methods

Baseline characteristics between cirrhosis patients with and without event during follow-up were compared using the Kruskal-Wallis test, as data were not normally distributed (Shapiro-Wilk normality tests were performed). Data are presented as median with interquartile range (IQR).

Univariate analysis

For the selected baseline laboratory values subjects with the upper half of values (median values of the whole patient group were used as cut-off value) were compared with those of the bottom half of values, for time till event.

Multivariate analysis

A Cox proportional hazards model was used to relate these baseline laboratory markers to the occurrence of the event during follow-up. The model was adjusted for age, MELD-score, Child-Pugh score. Results of the Cox logistic regression model were presented as the hazard ratio (HR) and the 95% confidence interval (95% CI). A two-sided p-value <0.05 was considered statistically significant. All statistical analyses were performed using SPSS 22.0 (SPSS, Inc., Chicago, IL).

Results

A total of 73 consecutive patients with liver cirrhosis were included (48 male and 25 female) with a median age of 58 ± 14 years, (age range 20-79 years). Fifty-two patients were classified as Child-Pugh A, 15 as Child-Pugh B and 5 as Child-Pugh C. Table 9.1 shows demographic and laboratory characteristics of the patients. Data on previous bleeding- and/or atherothrombotic events were obtained from patient's individual medical records; 19 patients had a history of bleeding (two had an intracranial haemorrhage, fourteen had a gastrointestinal and one had a post-operative bleeding), three had thrombosis (two patients had a transient ischemic attack and one had a minor stroke), and one patient had a history of bleeding as well as thrombosis. None of the patients had a bleeding, received platelet or plasma substitution/replacement therapy within one week before inclusion.

Table 9.1 Baseline demographic and laboratory characteristics of the patient population

Characteristics (reference value)	
Age (year)	58 (54 - 72)
Sex (male, %)	48 (65.8)
Aetiology cirrhosis:	
Alcoholic	41
NASH*	4
Haemochromatosis	4
AIH/PBC	6
Toxic/Medication	4
Hepatitis B	1
Hepatitis C	3
Congenital fibrosis	2
Unknown origin	8
Child-Pugh classification:	
A	52
B	15
C	6
MELD-score	9 (3-16)
History of:	
Bleeding	19
Thrombosis	3
Albumin (32,0-47,0 g/l)	38.0 (33.8-41.6)
Bilirubin (<20µmol/l)	30.6 (11.8-39.8)
Creatinin (60-115 umol/l)	93.6 (64.3-82.8)
INR (0,80-1,20)	1.1 (1.03-1.19)

Values expressed as median with IQR. Abbreviations: NASH; Non alcoholic Steatohepatitis, AIH; Autoimmune hepatitis, PBC; Primary biliary cirrhosis, MELD; Model for End-Stage Liver Disease, INR; International Normalized Ratio.

In Table 9.2 the haematologic and haemostatic parameters at baseline of all included patients, and separately of those with or without endpoint during follow-up, are shown. During the follow-up fourteen patients experienced a bleeding (Oesophageal variceal bleeding (n=8), rectal bleeding (n=4), post operative bleeding (n=2), three patients experienced a thrombo-embolic event (portal vein thrombosis (n=1), tumour thrombosis portal vein (n=1), deep vein thrombosis (n=1)) and nine patients died due to liver-related causes (hepatorenal syndrome, bleeding, portal vein thrombosis, decompensated liver cirrhosis). Four patients died due to non-liver related events (one patient died due to small cell lung carcinoma, one to squamous-cell carcinoma of the gastro-intestinal tract, one to heart failure and one after rejection of a kidney transplant). Follow-up ended when an event occurred. Median event free survival was 29 months (ranging from 1-45 months). During the study there was a loss-to-follow-up of seven patients; at six months three patients decided to withdraw informed consent, at twelve months two patients had moved and two patients were lost-to-follow-up for unknown reasons. Median outcome parameters from patients between groups were compared. Thrombocytes, fibrinogen, antithrombin and protein C were significantly lower in the clinical-event group whereas the INR, PT, vWF and D-dimer were

significantly higher in the event group compared to the no-event group. For the aPTT and the plasminogen levels no significant differences were observed (Table 9.2).

Table 9.2 Hematological and hemostatic parameters of the study population at baseline.

	All cirrhosis patients	Cirrhosis patients with no endpoint during follow-up	Cirrhosis patients with endpoint during follow-up	p-value
	N=73	N=40	N=26	
Thrombocytes (130-150*10⁹/l)	125.0 (81–187)	169.0 (103.5–209.3)	96 (65.8–181.3)	p=0.036*
INR (0.80-1.2)	1.10 (1.03–1.19)	1.07 (1.02–1.16)	1.16 (1.07–1.26)	p=0.016*
aPPT (23-32 sec)	29.0 (27.3–32.0)	29 (27–32)	30 (28–32)	p=0.463
PT (9.9-11.5 sec)	12.2 (11.3–13.1)	11.9 (11.2–12.8)	12.9 (11.8–14.1)	p=0.005*
vWF (50-150%)	264 (177.5–364.0)	208.5 (160.0–303.5)	323.0 (273.5–450.5)	p=0.000*
Fibrinogen (1.7-4.0 g/l)	3.1 (2.4–3.8)	3.3 (2.58–3.8)	2.8 (2.2–3.8)	p=0.300
Antithrombin (80-120%)	81.0 (58.0–98.0)	82.0 (68.3–99.0)	69 (41.8–82.3)	p=0.008*
Protein C (70-140%)	79.0 (50.0–97.5)	82.0 (68.0–114.0)	60.5 (37.8–88.3)	p=0.008*
Plasminogen (80-130%)	91.0 (77.0–107.0)	100.0 (85.0–114.0)	87 (71–105)	p=0.145
D-dimer (0-500 ng/ml)	621.5 (325.8–1293.0)	398 (298.0–838.0)	891.5 (632.3–1705.3)	p=0.090*

Abbreviations: INR, International Normalized Ratio; aPTT, activated Partial Thromboplastin Time; PT, Prothrombin Time; vWF, von Willebrand factor.

The various parameters of both the thrombin generation measurement and the ROTEM-analysis at inclusion are depicted in Table 9.3. None of the parameters, for clot formation or for fibrinolysis, showed a significant difference between cirrhotic patients with or without endpoint during follow-up.

Univariate Cox regression analysis was performed using the median of the parameters from the complete study population as cut-off value. Various study-parameters, including an increased thrombocyte count, PT and INR, vWF, and D-dimer were significantly associated with the occurrence of a clinical endpoint (Table 9.4). Of the new coagulation tests included in this study only a Maximum Clot Firmness of >61sec was indicative of a clinical event, the other parameters showed no predictive value (Table 9.4).

In the subsequently performed multivariate analysis we decided to include age, Child-Pugh and MELD-score as covariates. Because of this we did not include the INR, PT, albumin and bilirubin levels into the analysis as these parameters are already included in either the MELD- or the Child-Pugh score. In the multivariate analysis a thrombocyte count of >125x10⁹/l, a vWF-level of >264%, an antithrombin-level of >81%, a D-dimer

level of >621.5ng/ml and Maximum Clot Firmness of >61.0mm were analysed together with the previously stated covariates as these parameters were all significantly associated with the occurrence of a clinical coagulation-related event in the univariate analysis.

Table 9.3 Thrombin generation and ROTEM analysis of the study population.

Parameters of the thrombin generation and ROTEM measurements (units)	All cirrhosis patients N=73	Cirrhosis patients with no endpoint during follow-up N=40	Cirrhosis patients with endpoint during follow-up N=26	p-value
Thrombin generation				
Lagtime 1pM TF (min)	5.8 3.3 – 8.4	6.8 3.9 – 10.7	5.3 3.0 – 7.6	p=0.4
ETP 1pM TF (nM.min)	1065.0 793.5 – 1336.5	1030 788.5 – 1271.5	1082.5 760.5 – 1404.5	p=0.6
ETP % 1pM TF (%)	148.2 94.6 – 201.8	151.2 101.2 – 201.4	147.4 92.2 – 202.6	p=0.5
Peak 1pM TF (nM)	163.2 84.2 – 241.3	155.2 72.8 – 237.6	188.3 116.2 – 72.1	p=0.2
Peak % 1pM TF (%)	210.7 105.2 – 316.2	210.7 102 – 319.4	231.7 96.8 – 366.6	p=0.8
ttP 1pM TF (min)	9.5 5.9 – 13.1	9.4 6.0 – 12.8	10.33 6.61 – 14.15	p=0.8
VI 1pM TF (nM/min)	49.0 10.5 – 87.5	45.1 6.1 – 84.1	56.0 19.7 – 92.3	p=0.3
Reduction-% with thrombomodulin (%)	17.3 -4.2 – 38.8	18.7 -1.6 – 39.0	16.4 -13.55 – 46.4	p=0.6
ROTEM				
α -angle (sec/min)	76.5 68.5 – 84.5	76.5 69.7 – 83.3	76.5 68.0 – 85.0	p=0.6
CFT (sec)	70.0 14.0 – 126.0	69.5 22.5 – 116.5	79.0 25.0 – 133.0	p=0.3
MCF (mm)	61.0 47.0 – 75.0	61.5 50.2 – 72.8	60.0 45.7 – 74.3	p=0.3
LOT (sec)	1521.5 921.7 – 2121.3	1633.0 956.2 – 2309.8	1600.0 1031.7 – 2168.3	p=0.2
LT (sec)	2164.0 1262.7 – 3065.3	2262.5 1225.5 – 3299.5	2114.0 1018.0 – 3210.0 -	p=0.2
FV (%/min)	7.0 1.8 – 12.2	6.9 3.2 – 10.6	7.2 1.2 – 13.2	p=0.4

In the Multivariate Cox Proportional Hazard analysis only vWF and D-dimer remained as significant independent predictors of clinical events after adjustment for age, the Child-Pugh and the MELD-score (Table 9.5). When a cut-off value of 621.5 ng/ml was used for D-dimer (based on the median cut-off of all included patients in the study) a HR of 3.31 (95% CI 1.13-9.65, p=0.028) was found for an event during follow-up. Using a cut-off value of 264% for vWF we found a HR of 4.07 (95% CI 1.43-11.54, p=0.008) (Figure 9.1). None of the other parameters of the conventional and overall coagulation tests were predictive for the occurrence of a coagulation related event or liver disease related death.

Table 9.4 Univariate Cox regression analysis.

Variable	Beta	Standard error of mean	Hazard ratio	95% CI-interval	p-value
Thrombocytes >125x10 ⁹ /l	-1.15	0.43	0.32	0.14-0.73	p=0.007*
INR >1.10	1.01	0.41	2.75	1.22-6.20	p=0.015*
PT >12.2sec	1.04	0.43	2.83	1.23-6.52	p=0.015*
aPTT >29sec	0.17	0.39	1.19	0.55-2.58	p=0.659
vWF >264%	1.59	0.47	4.91	1.96-12.2	p=0.001*
Fibrinogen >3.1g/l	-0.36	0.40	0.70	0.32-1.55	p=0.379
Antithrombin >81%	-0.96	0.47	0.38	0.15-0.95	p=0.039*
Prot. C >79%	-0.59	0.40	0.56	0.25-1.23	p=0.148
Plasminogen >91%	-0.78	0.41	0.46	0.21-1.02	p=0.057
D-dimer >621.5ng/ml	1.50	0.47	4.48	1.79-11.2	p=0.001*
Albumin >38g/l	-0.91	0.43	0.40	0.17-0.94	p=0.036*
Bilirubin >30.6μmol/l	1.16	0.41	3.19	1.44-7.05	p=0.004*
Creatinine >93.6μmol/l	-0.11	0.55	0.90	0.31-2.61	p=0.842
Lagtime >5.8min	-0.81	0.41	0.48	0.20-1.00	p=0.051
ETP% >148.2%	-0.51	0.40	0.60	0.27-1.32	p=0.205
Peak% >210.7%	0.16	0.40	1.17	0.54-2.55	p=0.688
ttP >9.5min	-0.12	0.40	0.89	0.41-1.93	p=0.76
Vel. Index >49nM/min	0.49	0.41	1.64	0.73-3.68	p=0.231
Reduction-% with TM >17.3%	-0.18	0.40	0.83	0.38-1.84	p=0.654
α-angle >76.5sec/min	-0.29	0.40	0.75	0.34-1.65	p=0.473
CFT >70sec	0.17	0.42	1.19	0.52-2.71	p=0.685
MCF >61.0mm	-0.87	0.43	0.42	0.18-0.98	p=0.045*
LOT >1521.5sec	-0.24	0.40	0.79	0.36-1.73	p=0.551
LT >2164sec	-0.61	0.41	0.55	0.25-1.21	p=0.135
FV >7%/min	0.70	0.43	2.0	0.86-4.67	p=0.105

Figure 9.1 Hazard functions of vWF and D-dimer.

Table 9.5 Cox proportional hazard analysis.

Variable	Beta	Standard error of mean	Hazard ratio	95% CI-interval	p-value
Thrombocytes >125x10 ⁹ /l	-0.80	0.46	0.45	0.18-1.11	p=0.081
vWF >264%	1.40	0.53	4.07	1.43-11.54	p=0.008*
Antithrombin >81%	-0.59	0.51	0.55	0.21-1.50	p=0.246
D-dimer >621.5ng/ml	1.20	0.55	3.31	1.13-9.65	p=0.028*
MCF >61.0mm	-0.50	0.49	0.61	0.23-1.59	p=0.311

Model adjusted for age, Child-Pugh and MELD-score.

Discussion

Coagulopathy in liver cirrhosis is a frequent and clinically relevant problem. At the same time, the occurrence of bleeding or thrombosis cannot yet be predicted with any test accurately. It is increasingly recognized that conventional coagulation tests cannot adequately predict the risk of bleeding or thrombosis and therefore should not guide clinicians in making decisions concerning pre-emptive interventions.^{16,17} There has been a need for studies addressing the value of routine and alternative tests mimicking more closely what occurs in coagulation in vivo such as the thrombin generation test and thromboelastometry and to determine their value in predicting coagulation related events. The aim of our prospective study was therefore to evaluate the value of various haematological parameters, including the capacity of coagulation tests to predict thromboembolic and/or bleeding complications and liver disease related mortality in patients with cirrhosis.

The first finding was that increased vWF and D-dimer levels were both independent predictors for the occurrence of thromboembolic/bleeding events and liver disease related mortality in patients with cirrhosis. A D-dimer level of >621 ng/ml showed an over 3-fold increased risk for the composite of bleeding/thromboembolic event or liver related mortality during a mean follow-up of 2.4 years. Previous research showed that D-dimer levels are progressively increased with an increasing Child-Pugh classification.^{12,18-20} In our study however this increased risk was independent of the Child-Pugh classification and the MELD-score. Recent research supported our findings, showing that an increased D-dimer level was an independent risk factor for the formation of portal vein thrombosis and proved to be a strong predictor of death in patients suffering from oesophageal varices bleeding.²¹ The reason for the independent association of D-dimer with thromboembolic/bleeding events or liver disease related mortality lies probably in the fact that D-dimer levels correlate with liver dysfunction in cirrhosis in a variety of ways. D-dimers are the end product of the coagulation cascade and reflect not only increased thrombin and clot formation, but also fibrinolysis. As the liver clears D-dimers, the D-dimer levels reflect impaired hepatic clearance capacity as well. This interesting finding helps to reflect the dynamic changes in the cirrhotic liver. Is for example the hepatic metabolic- and detoxification- function suddenly impaired due to an infection or drug therapy, this could lead to diminished D-dimer clearance. In this situation the patient could be prone to a thromboembolic or bleeding event. It would be therefore of interest to focus in future studies on the role of D-dimer as a biomarker that is able to predict the clinical outcome of a critically ill cirrhotic patient. An increased level of vWF-antigen of >264% also proved to be independently associated with an increased risk on the occurrence of a clinical endpoint during follow-up. vWF is an established marker of endothelial dysfunction and is significantly elevated in patients with liver disease, correlated to the severity of cirrhosis. This has been thought to be the result of a compensatory mechanism for the thrombocytopenia occurring in cirrhosis due to hypersplenism.²² It has been suggested that increased

levels of vWF in cirrhosis might contribute to hypercoagulability with subsequent microthrombotic events within the vasculature of the liver contributing to progression of decompensation and portal hypertension.²³ Over the past years there also have been raised alternative explanations for the increased vWF-levels in cirrhosis. First, an increased endothelial secretion of vWF due to endothelial damage based on bacterial derived endotoxins. Second, an increase in endothelial surface and subsequent vWF-synthesis due to collateralization and angiogenesis, and third a reduced clearance of vWF due to decreased levels of ADAMTS13 (**A**DAMTS13 **A**D **M**etalloproteinase with a **T**hrombo**S**pondin type 1 motif, member **13**) synthesized by the diseased liver.²³ Our study outcome is in line with previous studies that observed that vWF-antigen represents a valuable and non-invasive predictor of clinically significant portal hypertension, portal hypertension related events (such as ascites), the need for liver transplantation and mortality in cirrhotic patients.^{24,25} It should be noted however that in these studies, in comparison to our study, either all patients were known to have severe portal hypertension at inclusion or ascites was already seen as an endpoint, whereas specific coagulation related endpoints, such as variceal bleeding, were not assessed. Based on the aforementioned results we conclude that vWF not only appears to be a promising, non-invasive marker of decompensation, but also a good predictor of coagulation/bleeding related events in various patients with cirrhosis.

The second important finding of our study is that the global assays we tested did not have any predictive power for thrombotic or bleeding complications in our cohort. This suggests that these assays are of little value for risk prediction in cirrhotic patients possibly because they reflect the plasma clotting and lysis potential rather than the actual level of formed thrombin or fibrin. This conclusion is remarkable given the outcome of different studies, including our own, that documented the re-balanced haemostatic system in cirrhosis based on thrombin generation and/or ROTEM analyses. Apparently, the rebalanced coagulation system is of limited value in the intrinsic defence against thrombosis or bleeding in these patients. The explanation for bleeding and thrombotic events in cirrhotics may not be due to coagulation disturbances but lie elsewhere and should be sought in superimposed conditions like alterations of the haemodynamic state, bacterial infections and endothelial dysfunction.

However, one should take into account that in thromboembolic- and bleeding events cellular elements like platelets and leukocytes also play an important role. This role is not taken into account in the thrombin generation measurement used in this study, as it is a platelet poor plasma-assay only. The recently developed whole blood thrombin generation assay might provide additional information and potentially gives a better predictive value.²⁶ In the whole blood tPA-ROTEM measurement these cellular components are taken into account. This might be the reason that in the univariate analysis, the MCF, indicating the maximum firmness of a blood clot, showed a significant association with clinical outcome. However, as the clot firmness is most likely not the only determinant of coagulation related events and mortality, this marker lost its significance in the multivariate analysis. On the other hand, in a recent small

study it was shown that in 17 cirrhotics thromboelastometry was able to identify all patients who experienced bleeding after undergoing an invasive procedure. As in general practice most patients present themselves with spontaneous bleeding or thrombosis this test should be further investigated in patients at risk. More clinical studies with larger patient groups are therefore needed to identify the true value of the ROTEM in predicting coagulation related events, both spontaneous as well as after invasive procedures.²⁷

This study has several limitations. One factor is that due to the limited number of patients and therefore limited number of events we were not able to make a sub-analysis of the predictive value of the haemostatic parameters in bleeding- versus thrombotic events. Furthermore, some patients were included in the study during a hospital admission in a state of decompensated liver cirrhosis. As baseline parameters were therefore not obtained in a stable situation this may have caused biased results of blood parameters, Child-Pugh classification and MELD-score at inclusion. Finally, follow-up information of the included patients was retrieved from the hospital file systems and not by frequent, exclusive study check-ups with additional imaging. Patients were seen in the outpatient hepatic unit according to current follow up guidelines. As some patients were not compliant with routine follow-up this could have caused missing data points i.e. a portal vein thrombosis missed due to a lack of an abdominal ultrasound.

In conclusion, our study corroborates the potential of D-dimer and vWF in predicting bleeding, thrombosis and liver-disease related mortality in patients with cirrhosis. It also shows that the use of overall assays like thrombin generation in plasma is, at this stage of test development, of no added value yet for predicting incident bleeding (not related to surgery) and thromboembolism. Larger studies with ROTEM but also whole blood thrombin generation are worth doing to seek diagnostic improvement in cirrhotic patients.

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CHAPTER 10

Summary, general discussion and future prospects

Summary, general discussion and future prospects

The objective of this thesis was to explore the interaction between haemostasis and two chronic inflammatory diseases, PAD and liver cirrhosis, that affect and are affected by the haemostatic system. In this concluding chapter the main findings of this thesis are summarized and discussed.

Haemostasis and vessel wall damage

Part I of this thesis focuses mainly on the interaction between the damaged vessel wall, in PAD, and the coagulation system. In **chapter 2** a narrative review is presented on recent advances in research on the possible interactions between coagulation, the vascular endothelium and atherosclerosis and the potential consequences of these interactions for venous and arterial thrombosis. Various studies have shown over the past years that etio-pathogenic mechanisms behind venous and arterial thrombosis are considerably similar and traditional elements of Virchow's triad (blood components, state of the vessel wall and blood flow) are applicable to both arterial and venous thrombosis. The mediator between these components of Virchow's triad is the vascular endothelium, which should be regarded as a heterogenous conglomerate of cells with diverse structures and functions depending on the specific vascular site and health status of the patient.^{1,2} When vessel wall injury occurs, the damaged endothelium will react with a local prothrombotic, procoagulant and proinflammatory state leading to enhanced permeability and uptake of inflammatory cells and coagulation proteins essential for wound healing and tissue repair. As one of the functions of the coagulation system is to establish the interaction with the immune system, immune mediated coagulation responses may be regarded as a "response to injury"-element and pathological coagulation responses, including thromboembolism and DIC, can be seen as an excessive immune response to vessel wall injury. A link between blood coagulation and atherosclerosis has long been postulated as well. It has been observed in experimental data that thrombin is involved not only in the early stages of atherosclerotic plaque formation, yet in the advanced stages of atherosclerosis with plaque progression and destabilization as well.³⁻⁶ In clinical studies this association between hypercoagulability and development of atherosclerosis have so far shown conflicting results. Over the past few years an association between venous thromboembolism and atherosclerosis has been proposed as well. The nature of this association has not been clarified yet, however some of the risk factors (e.g. diabetes mellitus, hypertriglyceridemia and obesity) share effects on vascular endothelium. It has been suggested that the so-called "inflammation-coagulation crosstalk" may be at the basis of this observed risk interaction. As inflammation is also at the basis of atherosclerosis, it may be that in patients with (idiopathic) thrombosis the ongoing

inflammation may aggravate atherosclerosis. Suggested implications for both clinical research and medical practice therefore consider a potential role for prophylaxis with antiplatelet therapy and statins on top of modification of (atherosclerotic) life style counselling in patients whom experienced a VTE. Additionally, more extensive research should be done to study the effects of anticoagulant treatment (with either VKA's or NOAC's) on the inflammatory effects on the vessel wall, beyond the scope of preventing (recurrent) thrombosis.⁷

Not only the vessel wall but blood constituents like platelets take an important place in Virchow's triad. After damage to the vessel wall formation of the platelet plug through activation, adhesion and aggregation of the platelets is the first step in haemostasis. We performed a case-control study in order to study platelet reactivity in whole blood from patients with PAD. The results of this study are presented in **chapter 3**. Blood was obtained from 40 PAD-patients (of whom 38 were using aspirin) and 40 age-matched healthy controls. Subsequently we performed whole-blood flow cytometry and multiparameter thrombus formation under high-shear flow conditions via the newly validated Maastricht flow chamber.⁸

In the whole blood samples from the aspirin-treated patients flow cytometry experiments showed unchanged high platelet responsiveness when triggered with ADP. When glycoprotein VI was used as a trigger slightly elevated responsiveness was observed, while after stimulation with PAR-1 thrombin receptor decreased responsiveness of the platelets could be witnessed in comparison to the responsiveness of the platelets of control subjects. In the thrombus formation under flow experiments most parameters were equally high in both the patient- and the control group. Treatment with aspirin *in vitro* caused a significant reduction in thrombus formation, especially when performed on collagen surfaces.

When comparing intra-subject results it could be noted that the markers of the ADP- and collagen-stimulated activation in the flow cytometry experiments were strongly correlated with the results of the collagen-dependent thrombus formation under flow. This suggests there is a common individual-dependent regulation of these platelet activation processes. Based on the aforementioned results we concluded that despite the use of aspirin, most platelet activation properties of included PAD-patients (mostly with Fontaine II PAD) were still within the normal range indicating that there is a need for more effective antithrombotic pharmacoprotection in PAD. Subsequent studies on platelet reactivity under flow conditions in PAD with other thrombocyte aggregation inhibitors such as the P₂Y₁₂ antagonists (e.g. clopidogrel and prasugrel) and dipyridamol would therefore be of great interest.

In **chapter 4** we shifted the focus of our research on the coagulation status in PAD-patients from primary haemostasis to secondary haemostasis and fibrinolysis in order to gain more insights in the overall haemostatic profile. In the same patient and healthy

control population as in chapter 3 we performed thrombin generation analysis in both platelet poor plasma and whole blood samples. Additionally, modified ROTEM analyses were completed after triggering whole blood samples with TF in the presence and absence of tPA. We observed significantly increased levels of vWF-antigen, fibrinogen and D-dimer in PAD-patients, markers of the thrombin generation potential showed however no difference between the patient- and the healthy control group. As for the whole blood ROTEM clotting analyses we observed significantly faster clot formation and increased clot firmness in PAD-patients compared to healthy controls, yet no significant differences were found for parameters of clot degradation. In order to assess whether the haemostatic profile of PAD-patients that experienced a cardiovascular event within one year of follow-up after the initial diagnosis of PAD ('cases') versus PAD-patients that did not experience a CVD-event ('controls') we selected and subsequently divided the PAD-group in two and matched cases and controls based on age and sex. There were no significant differences in the hematologic and haemostatic markers between cases and controls neither were there differences in the thrombin generation measurements performed in platelet poor plasma. In the thrombin generation analyses in whole blood however it could be observed that ETP-values of cases were lower compared to the ETP-levels of the controls. In the modified ROTEM-measurements no differences between the patients with a CVD-event and the patients without a CVD-event were found.

Taking the aforementioned results into account we concluded that, despite the fact that thrombin generation potential *in vitro* is comparable between PAD-patients and healthy controls, patients with PAD do show an increased ability to form a stable clot in whole blood compared to healthy controls. This is most likely due to the increased fibrinogen levels related to inflammation in atherosclerosis. The unaltered capacity to generate thrombin *in vitro*, despite increased levels of previously formed thrombin, suggests that there is a consumption of coagulation proteins reducing the capacity to generate more thrombin subsequently. The difference in the ETP-values between PAD-patients with and without CVD-event in the whole blood thrombin generation analyses must have been related to the presence of cells in the whole blood assay. Possibly there is a more pronounced depletion of coagulation proteins present in whole blood in comparison to plasma, furthermore it cannot be excluded that there are direct effects of e.g. erythrocytes, leukocytes and platelets on thrombin generation. Recently, there has been an increased interest in role and function of erythrocytes in coagulation. Previously red blood cells were thought to be inactive bystanders in thrombin formation. Based on recent studies however it has been shown that erythrocytes contribute to coagulation through the exposure of phosphatidylserine (PS) on their exterior membrane making them more adhesive to endothelial cells as well as increasing their aggregation levels in obesity and diabetes for example.⁹⁻¹¹ For this reason, in order to mimic the coagulation status of (PAD-)patients as close to

physiology as possible, there still a need for (whole blood) assays that can measure thrombin and fibrin formation both under static and flow conditions.

Chapter 5 presents a systematic review and meta-analysis of the literature on D-dimer as a marker for cardiovascular and arterial thrombotic events in patients with PAD. Because patients with PAD have a high risk of cardiovascular death, yet there remains uncertainty regarding the individual risk profile of these patients, there was and is a need for simple (bio-)markers that can help identify patients at risk. We considered D-dimer, a fibrin degradation product and therefore a specific marker of hypercoagulability as well as of fibrinolysis, as one of these markers since it is a simple test and widely available. The search of the various databases yielded ten studies available for analysis, comprising 2420 patients with PAD with 9.77 cardiovascular events per 100 patient years. Due to extended heterogeneity of the included studies however only four studies remained feasible for inclusion in the meta-analysis as these studies were similar for duration of follow-up (short to intermediate), patient population and outcome measures. Pooling of these four studies revealed an independent association between an increased D-dimer and a two times increased risk of near-term cardiovascular events (RR 2.30, 95% CI: 1.43-3.68). Elevated D-dimer appeared to be a better risk predictor for the short term (<4 years) rather than the long-term risk of arterial thrombotic events and CVD-mortality. When the duration of the follow-up increased to over 4 years, the D-dimer levels lost their independent significant association after adjustment for confounders. As a pathophysiological explanation for the association between elevated plasma D-dimer levels and an increased risk of near-term arterial thrombotic events we suggested that this is due to the fact that D-dimer is part of the “inflammation-coagulation axis”. The role of inflammation in atherosclerosis and plaque destabilization is clearly established and previous studies have shown that increased inflammatory parameters and progression of atherosclerosis are positively associated with an increase in the formation of cross-linked fibrin.¹² As cross-linked fibrin is a detectable component of the atherosclerotic plaque it has been suggested that an increase in fibrin within the plaque contributes to atherosclerosis progression and modulation of the plaque phenotype.¹²⁻¹⁴ From this it may be assumed that with larger amounts of fibrin, stemming from greater vascular burden in patients with extensive atherosclerosis, a larger amount of fibrin degradation products due to ongoing fibrinolysis is detectable as well. Based on these results we support the hypothesis that plasma D-dimer is a potential risk predictor for cardiovascular events. Whether D-dimer plays a causal role in these adverse events’ pathophysiology or prevails solely as a marker of increased fibrinolysis remains uncertain and needs to be investigated.

As the several sources of heterogeneity made it difficult to perform a meta-analysis for most of the study data and in most of the included studies cancer or inflammatory

disease rates were not assessed making it unclear what the influence was of these on the baseline D-dimer levels we decided to set up our own prospective study. This prospective study is described in **chapter 6** and in addition to assessing the association of increased D-dimer, it also determines the correlation between increased levels of vWF-, fibrinogen- and thrombin generation potential by means of the CAT-assay with the near-term occurrence of cerebro- and cardiovascular events and progression of disease PAD-patients. Between 2009 and 2013 we included 301 patients with newly diagnosed PAD from the vascular surgery departments of 3 South-Limburg based hospitals. PAD was diagnosed through the ABI, and the majority of patients were classified as Fontaine IIa (58.1%) or Fontaine IIb (33.6%). From 280 patients we were able to obtain adequate baseline blood samples through direct venipuncture and these patients were followed-up for 319 days (range 1-770 days). During this follow-up time progression of PAD occurred in 37.1% of patients, while 6.8% of patients experienced a cerebro- or cardiovascular endpoint. Two patients reached a cardiovascular endpoint and subsequently died, three patients died due to non-vascular causes. At baseline the mean levels of D-dimer, fibrinogen and vWF were at the upper limit of the reference interval (454 ng/ml, 3.6 g/l and 150%, respectively) as was the normalized peak of the thrombin generation (131.7%). In the univariate analysis the only risk factor associated significantly with a cerebro- or cardiovascular endpoint during follow-up was the presence of diabetes mellitus. D-dimer-, fibrinogen-, vWF-levels and the parameters of the thrombin generation assay did not show significant associations with this endpoint. Remarkably, for the endpoint of progression of PAD an increased fibrinogen level (>3.6 g/l) showed a significant inverse association with a hazard ratio of 0.65 (95% CI: 0.43-0.98, $p=0.04$). In the cox proportional hazard regression analyses diabetes remained an independent predictor of cardio- and cerebrovascular events (HR 5.37; 95% CI 1.79-16.11, $p=0.003$) and a fibrinogen level over 3.6 g/l stayed associated with a decreased risk of progression of PAD (HR 0.61; 95% CI 0.39-0.95, $p=0.03$). Furthermore, and to our surprise, a haematocrit level of over 0.43 L/l proved to be inversely associated with the composite endpoint with an HR of 0.49 (95% CI 0.24-0.98, $p=0.043$). From these results we drew the conclusion, that except for fibrinogen, none of the studies markers of coagulation were associated with the risk of atherothrombotic events and progression of disease during a mean follow-up of 319 days. Allegedly, plasma hypercoagulability is not a predominant factor in the aetiology of short-term cardiovascular complications in newly diagnosed PAD-patients. We hypothesized that the reason for this seemingly lacking role of hypercoagulability was due to the fact that the over 81% of the patients included in our study used statins, which are known to attenuate hypercoagulability. This percentage is higher than has previously been reported in other studies. The peculiar finding that an increased fibrinogen level was inversely associated with the progression of PAD-endpoint and therefore suggesting that hyperfibrinogenemia will lead to a decreased progression of PAD during short-term follow-up is contrasting previous research on fibrinogen levels in PAD.¹⁵ An

explanation for this finding lies possibly in the fact that in our study only patients with newly diagnosed PAD (<3 months) were included instead of patients with longstanding PAD. Due to their recent diagnosis, therapeutic interventions had been newly implemented or were about to be implemented in the included patient group; lifestyle changes had been advised, patients had newly started with statins or platelet inhibitors and supervised walking programs were effectuated. As baseline blood samples were also obtained during the first three months after diagnosis the effects of these therapeutic interventions will not have been evident from the various laboratory parameters and it is possible that during the course of the follow-up these values would have fluctuated. Various studies have shown a decrease in plasma fibrinogen due to the use of statins and fibrates and supervised walking through treadmill training has shown to have a positive effect on endothelial function and decrease inflammation marker hs-CRP, IL-6 and fibrinogen.¹⁶⁻²² Investigating patients in a stable state of disease and with no drastic changes in their treatment regiments may have given more congruent results. On the other hand, this study was designed to find easy accessible and widely available haemostatic biomarkers that would help clinicians in identifying patients, in the early stages of disease, at risk for atherothrombotic events. Postponing the moment of venipuncture until the started treatment would have reached a steady state would therefore not have been feasible, not to mention that it would have been virtually impossible to estimate this exact timepoint for every individual patient. It would thus be of interest to prolong the follow-up duration of our study and at set time-points repeat the measurement of the biomarkers. This would allow one to study associations between the levels of the biomarkers at various disease stages and the occurrence of atherothrombotic events, progression of disease and the effect of the initiated treatment. Furthermore, it would give more insights into the intra-subject variability of haemostatic markers over time and their association with atherothrombotic events and disease related mortality.

The aim of the studies described above was to further elucidate the disease aetiology and progression of PAD on a haemostatic level and identify novel haemostatic risk factors that would allow earlier detection of adverse (atherothrombotic) events. The rationale for evaluating these markers of coagulation was the abundance of epidemiological and experimental evidence of close interactions between atherosclerosis, coagulation and thrombosis. Based on our data and data from previous studies it seems that no single haemostatic marker is adequate to characterize the complexity of PAD. Because of this a multi-marker approach of already existing and novel biomarkers is required to identify patients at risk for the progression of PAD and the prediction of cerebro- and cardiovascular events. The prerequisites of these markers should be that they are robust, standardized with acceptable intra- and inter-laboratory variability, easily measured, widely accessible and affordable and preferably

give information on all three components of Virchow's triad; the vessel wall, flow conditions and blood constituents.

Liver disease and coagulation

The second part of this thesis focuses on the coagulation abnormalities that occur when the liver function is disturbed due to cirrhosis. This because haemostasis is intimately related to the liver as most coagulation factors are synthesized by the livers' parenchymal cells and the liver's reticuloendothelial systems plays an important role in the clearance of the activation products of coagulation. Despite the progression that has been made in understanding the haemostatic derangements in cirrhosis in the past decades, it appeared that the used coagulation tests were not able to reflect these haemostatic alterations correctly. Various researchers expressed the need for new, overall tests to assess the true coagulation status. The collective objective of the studies of part II of this thesis was to further elucidate the pathophysiology of coagulation changes in patients with cirrhosis. In **Chapter 7** we describe a cross-sectional study that we performed with a concise and state of the art panel of assays to gain more insights into the various regulatory mechanisms of haemostasis: thrombin generation potential as well as the clot formation and clot degradation properties of cirrhotics. Blood was obtained from 73 patients with all-cause cirrhosis and 20 healthy controls and various coagulation tests were performed. Additional to measuring hematologic parameters and performing conventional coagulation tests, the activity of coagulation pathways was measured with assays for free factor VIIa, factor IXa-AT and factor Xa-AT complexes, thrombin generation by means of the CAT-assay was determined in platelet poor plasma and modified tPA-ROTEM analyses were performed in whole blood. We observed that classic coagulation tests (aPTT, PT and INR) were significantly prolonged with increasing cirrhosis severity whereas the majority of haematological and haemostatic parameters were significantly decreased (haemoglobin, thrombocytes, fibrinogen, antithrombin, protein C, plasminogen, $p < 0.05$). D-dimer and vWF-antigen levels however were significantly increased in Child Pugh C patients ($p < 0.0001$). Novel findings included an increased generation of factor VIIa and a moderate increase of the factor IXa-AT complex. There appeared to be no increase in factor X activation (as measured in complex with antithrombin) nonetheless. A possible explanation for the increased activity of the extrinsic pathway through factor VIIa, and subsequently the factor IXa-AT complexes through the Jost loop, may be the increased levels of circulating microparticle tissue factor activity in cirrhotics as has recently been described by Rautou.²³

In conjunction with these markers of a prothrombotic state, the thrombin generation parameters were increased as well upon increasing severity of cirrhosis. We observed that cirrhotics were able to generate as much, if not more, thrombin as healthy controls

even when the measurements were conducted in the absence of thrombomodulin. In comparison to previous studies this was a novel finding which possibly can be explained by differences in the laboratory execution of the CAT-assay.

Another novel finding was the observation that the modified ROTEM assay on whole blood showed signs of a delayed clot formation and decreased clot strength with increasing cirrhosis severity. The addition of tPA did not expose significant differences in the parameters of clot degradation. Due to the large quantity of exogenously added tPA to the modified tPA-ROTEM it is likely that this assay is less sensitive to the balance of the endogenous levels of tPA and PAI in cirrhotics. It can therefore not be used to accurately assess the fibrinolytic potential, it does however yield information on the resistance of the whole blood clot to fibrinolysis. Based on the preceding results we concluded that although cirrhotics have an overall procoagulant plasma milieu, they show a decreased clot formation capacity in whole blood. Furthermore, with increasing severity of cirrhosis there appears to be an unaltered resistance to clot lysis.

Generation of thrombin is depending on two underlying processes; on the one hand the production of thrombin due to prothrombin conversion and on the other hand the removal of thrombin from the plasma (thrombin inactivation). All procoagulant factors are of influence on the prothrombin conversion as are the anticoagulant factors protein C and S as well because they proteolytically inactivate factor Va and factor VIIIa and thus decrease the conversion of prothrombin. Thrombin inactivation is caused by direct inhibition by the serpin antithrombin and the proteinase inhibitor α_2 -macroglobulin (α_2 M). We aimed to quantify these processes separately through a recently developed approach based on computational modelling in order to investigate the changes in prothrombin conversion and thrombin inactivation in patients with cirrhosis (**chapter 8**).²⁴ We furthermore wanted to assess their individual contribution on the thrombin generation curves of both patients and healthy controls. Blood from the similar patient group with cirrhosis in various disease stages as in chapter 7 was obtained as well as that of 30 healthy controls. Functional antithrombin, α_2 M, prothrombin and fibrinogen level were measured and were significantly decreased with increasing severity of cirrhosis, except for α_2 M that proved to be significantly increased in CP-A cirrhosis. Thrombin generation was measured in the absence and presence of thrombomodulin. Considering that thrombin generation is the net result of prothrombin conversion and removal of thrombin from the plasma, the course of prothrombin conversion can be calculated when thrombin generation curves and thrombin inactivation parameters are known. Based on the plasma antithrombin, α_2 M and fibrinogen levels we predicted thrombin inactivation with the help of our computational model. Subsequently, with data from the original thrombin generation measurements we obtained prothrombin conversion curves that showed that the total amount of prothrombin converted is significantly reduced in patients with cirrhosis (15-43%, depending on the disease severity). This reduction is most likely due to a

lower availability of prothrombin due to a lesser synthesis by the liver. The maximal *rate* of prothrombin conversion however proved to be higher in cirrhotics, suggesting that either the concentration and /or the activity of the prothrombinase complex is increased.²⁵ A potential mechanism for an increased prothrombinase complex could be the decrease of antithrombin levels leading to a reduced inhibition of factor Xa. However, as in chapter 7 we did not observe increased Factor Xa levels in cirrhotics this implies that this may not be the appropriate explanation, further studies on this topic would therefore be essential.

Our experiments showed furthermore that the inactivation of thrombin was markedly reduced in cirrhosis. This was predominantly due to a significantly decreased amount of TAT-complexes as the thrombin- α_2 M complex formation proved to be comparable between healthy controls and patients with cirrhosis. This is in line with previous findings that AT is more potent than α_2 M and in order to compensate for a small decrease of AT, a relatively large increase of the α_2 M-level is required.²⁶

Based on these results we concluded that in patients with cirrhosis less thrombin is formed, it is formed faster and inactivated slower causing signs of hypercoagulability in the CAT-assay. Extended *in silico* experiments showed that adjusting prothrombin conversion, through the administration of prothrombin complex concentrate would lead to a *hypercoagulable* state. Normalizing antithrombin levels on the other hand could lead to a *hypocoagulable* state. This supports the hypothesis that the coagulation system in cirrhosis is rebalanced and adjusting it would likely lead to imbalances of the coagulation system and thus to possible clinical adverse events.

The second aim of part II of this thesis was to find (a concise set of) haemostatic markers or overall coagulation assays that can help clinicians in distinguishing what the actual coagulation status of the cirrhotic patient is and based on that status what the choice for the best treatment strategy should entail. The relatively high incidence of thrombotic events and bleeding complications in patients with cirrhosis shows us that the 'rebalanced coagulation system' is not as stable as that of healthy persons. Whether this is due to e.g. hemodynamic alterations, inflammation in spontaneous bacterial peritonitis or changes in the blood flow due to portal vein thrombosis or possibly a combination of all is not yet completely elucidated. The current available tests are however not able to assess the coagulation status and can therefore only be of limited help to distinguish which patients are at risk for either a thrombotic or bleeding-event. With more knowledge on specific aspects of the coagulation profile of patients with cirrhosis we decided to see whether some of the previously assessed coagulation markers could be useful in the prediction of coagulation related clinical events during follow-up. We designed a prospective study to investigate the value of both classic haemostatic parameters (including D-dimer and vWF) and new overall coagulation assays in assessing the prognosis of patients with cirrhosis (**Chapter 9**). The 73 patients with cirrhosis as previously described were followed-up for an average of

29 months and the composite of major bleeding, thrombotic events in any vessel and liver disease related mortality was recorded as an event. Multivariate analysis showed that only vWF and D-dimer were significant predictors of clinical events. The global coagulation assays such as the thrombin generation measurement and (tPA-)ROTEM proved to be of no added value for risk prediction. A possible explanation for the increased vWF-levels in cirrhosis, is threefold: 1) increased endothelial secretion due to damage caused by bacterial derived endotoxins, 2) portosystemic vessel collateralization and angiogenesis which increases the endothelial surface and thus vWF-synthesis and 3) a reduced clearance of vWF due to decreased levels of liver-synthesized ADAMTS13. With advanced cirrhosis and portal hypertension vWF-levels will therefore rise and can thus offer reliable information considering the impending (de-) compensated status of the patient with cirrhosis. The reason for the independent association of D-dimer with thromboembolic and/or bleeding events probably has a multifactorial origin. As the end product of the coagulation cascade D-dimer gives overall information on clot formation as well as clot lysis, and as it is cleared by the liver, it provides information on the hepatic clearance capacity as well. The global coagulation assays used in our study do not give information of the actual levels of formed fibrin, but inform us about the *potential* of the blood to clot or lyse. Our finding that the overall assays we tested did not have any predictive power for coagulation related events in this cohort therefore suggests that parameters of clot –formation and lysis *potential* are of little value predicting bleeding and thromboembolisms in cirrhotics. More prospective clinical studies with a larger amount of patients however are needed in order to confirm or contradict these findings.

Summarizing the data from our studies we can conclude that patients with cirrhosis, despite having lower levels of (inactive) pro- and anti- coagulants, have an overall procoagulant plasma milieu leading to an increased rate of prothrombin conversion and a reduced thrombin inactivation. Based on our data, combining classic with novel overall coagulation assays, we therefore support the hypothesis of a rebalanced coagulation system. This balance is however very fragile and can tip rather easily towards either bleeding or thrombosis. Because of this delicate balance it is difficult to choose the appropriate treatment strategy in cirrhosis; vitamin K antagonists not only decrease procoagulants but further reduce protein C and protein S as well possibly leading to an increased thrombotic risk, LMWH's need antithrombin in order to function and since AT levels are known to be lower in cirrhosis this may cause resistance to LMWH-treatment.²⁷ This was seen in our experiments as well; adjusting the prothrombin conversion with the help of prothrombin complex or antithrombin for example lead to either a hypercoagulable state or hypocoagulable state, respectively. The introduction of novel oral anticoagulants (FXa inhibitors like rivaroxaban and the thrombin inhibitor dabigatran) has led various researchers to explore these in cirrhotics with a VTE as well; these data are still limited and since these medications are partly

excreted by the liver the use of these agents is clearly not yet advisable.²⁸ Further research considering anticoagulant treatment is warranted, not only to reduce bleeding- and thrombotic events, but to study the effect of anticoagulants on the progression of fibrosis and consequently cirrhosis as well. Recent data of both human and animal studies suggests that a procoagulant imbalance in chronic liver disease favours the progression of fibrosis and anticoagulant treatment may slow this process down.²⁹⁻³¹

Thus, cirrhosis is associated with complex alterations in haemostasis and prognosis is affected by life threatening bleeding and thrombotic complications. Classic laboratory values (aPTT, PT and INR) are not able to discriminate between hypo- and hypercoagulability and furthermore have failed to predict coagulation related complications in cirrhotics.³² Our data showed that only vWF and D-dimer show an independent association with an increased risk of a coagulation-related events, or liver disease related mortality. None of the overall coagulation assays had any predictive power for coagulation related events in our cohort. It remains one of the challenges for the future to develop an overall assay that is able to recognize patients at risk for coagulation related complications. The measurement of individual components of the coagulation- and fibrinolysis pathways is however unlikely to help. Ideally, simple and well-defined overall tests representing both primary- and secondary haemostasis and fibrinolysis operating *in vivo* should be developed and investigated in clinical trials. Although this may currently be improbable, prospective studies on predicting bleeding and thrombosis in patients with cirrhosis should possibly entail a panel of combined coagulation tests; classic and novel, plasma and whole blood with the inclusion of point-of-care platelet function tests. It furthermore would be interesting to add vWF and/or D-dimer, possibly as a replacement for the INR, as separate clinical measures to the Child-Pugh and the MELD score to assess their value in these risk prediction models.

Finally, one of the aspects that should be taken into consideration is that in the studies performed so far on coagulation and cirrhosis, patients with cirrhosis form a very heterogeneous group. The majority of studies include patients with all-cause cirrhosis, not distinguishing between the different aetiologies. It is known that cholestatic liver disorders, compared to other chronic liver diseases, are associated with better outcomes of variceal bleeding and have less blood loss after liver transplantation. It has been suggested that this was due to the fact that platelet function differs between patients with cholestatic and non-cholestatic liver disease.³³ We therefore hypothesize that the cause of the liver disease does influence the specific coagulation pattern. It would be therefore of interest to study the coagulation changes in cirrhosis based on the separate aetiologies in order to define the optimal, personalized, treatment strategy.

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PART III

Addendum

Samenvatting

Samenvatting

In **hoofdstuk 1** wordt het onderwerp van dit proefschrift geïntroduceerd. Hemostase, in de volksmond ook wel de bloedstolling genoemd, is in het menselijk lichaam het mechanisme dat moet voorkomen dat er bloedverlies optreedt. Het is het proces waardoor bloed, dat in contact komt met andere oppervlakten dan de intacte wand van het bloedvat, gaat klonteren en vast wordt zodat een stolsel ontstaat. Verder heeft het ook de taak dat wanneer er een bloedstolsel nodig is, dit stolsel niet te groot wordt en daarmee een bloedvat geheel zou kunnen afsluiten. Hemostase is daarmee een complex proces dat goed uitgebalanceerd moet zijn. Dit systeem kan uit balans raken door bijvoorbeeld ontsteking of ziekte. In dit proefschrift wordt beschreven hoe twee verschillende ziektebeelden gepaard gaande met ontsteking, perifere arterieel vaatlijden (PAV) en levercirrose, van invloed zijn op de bloedstolling. Het eerste deel van dit proefschrift beschrijft de effecten van PAV op verschillende stappen van de bloedstolling en evalueert of een 'hypercoagulabele toestand' (een toestand waarbij er sprake is van een verhoogde stolbaarheid) kan voorspellen of PAV-patiënten een verhoogd risico op atherotrombotische complicaties hebben. Deel twee van dit proefschrift heeft als doel de hemostase van patiënten met cirrose beter in kaart te brengen met behulp van nieuw ontwikkelde stollingstesten die een volledig beeld van de stolling kunnen geven.

Deel 1 Interactie tussen de beschadigde vaatwand en het stollingssysteem

Hoofdstuk 2 beschrijft de recente ontwikkelingen op het gebied van de interacties tussen hemostase, het vasculair endotheel en atherosclerose en de mogelijke gevolgen van deze interacties voor zowel arteriële als veneuze trombose. Verscheidene studies hebben aangetoond dat de verschillende etio-pathogene mechanismes verantwoordelijk voor het ontstaan van zowel veneuze als arteriële stolsels, vergelijkbaar zijn en voldoen aan de gekende elementen van de triade van Virchow (1. bestanddelen van het bloed 2. de vaatwand 3. de stroming van het bloed). Het sturende onderdeel tussen deze elementen blijkt het vasculaire endotheel. Zodra beschadiging van de vaatwand optreedt reageert het beschadigde endotheel met een protrombotische, procoagulante en pro-inflammatoire staat waardoor er een verhoogde permeabiliteit van de vaatwand is en er een toename is van de opname van inflammatoire cellen en stollingseiwitten die nodig zijn voor wondgenezing en weefselreparatie. Aangezien een andere functie van het bloedstollingssysteem het activeren van het immuunsysteem betreft, kan gesteld worden dat pathologische stollingsziektebeelden zoals het optreden van een trombo-embolie of Diffuse Intravasale Stolling (DIS) een 'uit de hand gelopen' immuunreactie is op beschadiging van de vaatwand. Tussen hemostase en beschadiging van de vaatwand door atherosclerose lijkt eveneens een verband te bestaan; experimentele data tonen aan dat trombine betrokken is bij zowel de vroege als de late fases van het

atherosclerotisch proces. Ook het optreden van een veneuze trombose en atherosclerose wordt sinds kort met elkaar geassocieerd. De precieze aard van deze associatie is nog niet opgehelderd, echter lijkt verband te houden met de zogenaamde “inflammatie-coagulatie interactie” waarbij door aanhoudende ontsteking van de vaatwand bij een (idiopathische) trombose het atherosclerotisch proces wordt verergerd.

Na beschadiging van de vaatwand is de eerste stap die plaatsvindt in het hemostatische proces de formatie van een zogenaamde ‘platelet-plug’; een afsluitende prop van bloedplaatjes. Om de reactiviteit van trombocyten te bestuderen in patiënten met PAV werd in **hoofdstuk 3**, in een case-control studie, volbloed van 40 PAV-patiënten vergeleken met het volbloed van 40 gezonde controles. De reactiviteit van de trombocyten werd onderzocht middels flowcytometrie en ook werd de trombusformatie geobjectiveerd onder verschillende stroomcondities met een hoge afschuifspanning (‘shear’) met behulp van de recentelijk in Maastricht gevalideerde ‘flow chamber’. Patiënten die met aspirine behandeld werden toonden ten opzichte van de gezonde controles een onveranderde trombocyten-activiteit wanneer er getriggerd werd met ADP. Zodra er echter getriggerd werd met glycoproteïne VI werd een iets toegenomen reactiviteit geobjectiveerd en na stimulatie met de PAR-1 trombine receptor werd juist een afgenomen plaatjesreactiviteit waargenomen. In de ‘flow-experimenten’ werden ten aanzien van trombusformatie geen verschillen geobjectiveerd tussen PAV-patiënten en gezonde controles. Zodra er *in vitro* aspirine werd toegevoegd was er een significante afname van de trombusformatie, zeker wanneer deze experimenten werden uitgevoerd op oppervlakten bekleed met collageen. Aan de hand van deze resultaten concludeerden wij dat in PAV-patiënten, ondanks het gebruik van aspirine, de trombocyten-activatie onvoldoende onderdrukt is waardoor er nog steeds een risico bestaat op (ongewenste) trombusformatie. Derhalve bestaat de noodzaak tot een effectievere antitrombotische behandeling in patiënten met PAV.

In **hoofdstuk 4** verschuift de focus van ons onderzoek van primaire hemostase naar secundaire hemostase en stolselafbraak. In PAV-patiënten (dezelfde populatie als beschreven in hoofdstuk 3) bleken significant verhoogde spiegels van von Willebrand Factor-antigeen (vWF), fibrinogeen en D-dimeer aantoonbaar te zijn. De markers van trombine generatie experimenten toonden geen verschillen tussen PAV-patiënten en gezonde controles. Met behulp van gemodificeerde ROTEM-analyses bleek er wel een snellere stolselvorming en een toegenomen stevigheid van deze stolsels bij PAV-patiënten in vergelijking met de bloedstolsels van gezonde controles. Ten aanzien van de afbraak van de stolsels werden geen verschillen waargenomen. De 40 PAV-patiënten werden verder opgedeeld in twee groepen; patiënten die het afgelopen jaar een atherotrombotische complicatie doorgemaakt hadden (n=20) en een groep patiënten

zonder complicatie (n=20, gepaard op basis van leeftijd en geslacht). Tussen deze groepen bleken geen verschillen tussen hematologische, hemostatische parameters noch in de plasma trombine generatie resultaten. In volbloed bleken de Endogene Trombine Potentieel-waarden (ETP) van de patiënten met een complicatie echter lager dan de ETP-waarden van de patiënten zonder complicatie. In de ROTEM-analyses werd geen verschil tussen beide groepen geobserveerd.

Op basis van deze resultaten concludeerden wij dat: 1. Ondanks dat er in PAV-patiënten *in vivo* sprake is van een toegenomen doorgemaakte trombinevorming (blijkend uit het verhoogde D-dimeer) blijkt het *in vitro* trombine generatie potentieel niet verhoogd. Dit komt waarschijnlijk door het *in vivo* verbruik van de stofactoren waardoor verdere trombine-formatie *in vitro* afgenomen is. 2. Hoewel dat het *in vitro* trombine generatie potentieel in plasma dus vergelijkbaar is tussen PAV-patiënten en gezonde controles, blijkt de uiteindelijke stolselvorming in volbloed van patiënten wel toegenomen. We hypothetiseren dat dit komt door de verhoogde fibrinogeenspiegel die gerelateerd is aan de inflammatie bij atherosclerose.

Hoofdstuk 5 beschrijft een systematische review en meta-analyse ten aanzien de waarde van D-dimeer als marker voor cardiovasculaire en arteriële trombotische complicaties in patiënten met PAV. Tien studies werden getraceerd waarin de data van 2420 PAV-patiënten met gemiddeld 9,77 cardiovasculaire complicaties per 100-patiëntjaren werd beschreven. In verband met significante heterogeniteit bleek het uiteindelijk slechts mogelijk om vier studies in de meta-analyse te includeren. Pooling van deze vier studies toonde aan dat er een onafhankelijke associatie was tussen een verhoogde D-dimeer spiegel en een twee keer verhoogd risico op een cardiovasculaire complicatie op korte termijn (RR 2.30, 95% CI: 1.43-3.68). Een verhoogde D-dimeer waarde bleek een betere voorspeller van arteriële trombotische complicaties en cardiovasculaire mortaliteit op de korte termijn (<4 jaar) dan op de lange termijn (>4 jaar). Als pathofysiologische verklaring voor deze gevonden associatie hypothetiseerden wij dat dit het gevolg is van het feit dat D-dimeer deel uitmaakt van de zogenaamde “inflammatie-coagulatie-as”: zowel inflammatie als de bloedstolling spelen een duidelijke rol in de progressie van atherosclerose. Verscheidene studies hebben aangetoond dat een component van de atherosclerotische plaque fibrinedraden betreft. Het is voorts in de literatuur gesuggereerd dat ‘hoe groter het oppervlak van het atherosclerotisch vaatbed, en hoe meer fibrinedraden des te sneller de progressie van atherosclerose, met ook destabilisatie van de atherosclerotische plaque en dientengevolge waarschijnlijk dus cardiovasculaire events’. Aangezien D-dimeren het afbraakproduct van fibrinedraden zijn is het dus mogelijk dat toename van D-dimeer in het plasma een potentiële risico-voorspeller voor atherotrombotische complicaties is.

In **hoofdstuk 6** beschrijven we de resultaten van onze eigen prospectieve studie met betrekking tot de waarde van zowel D-dimeer, vWF, fibrinogeen en trombine generatie experimenten uitgevoerd met de CAT-assay in het voorspellen van cerebro- en cardiovasculaire complicaties en progressie van lokale ziekte in patiënten met PAV. Tussen 2009 en 2013 werden 301 patiënten met recent gediagnosticeerd PAV uit drie Zuid-Limburgse ziekenhuizen geïnccludeerd. Het merendeel van de patiënten had PAV Fontaine stadium IIa (58,1%) of Fontaine stadium IIb (33,6%). 280 van deze patiënten werden gedurende een gemiddelde van 319 dagen gevolgd in de tijd. Gedurende deze tijd bleek bij 37,1% van de patiënten sprake van duidelijke progressie van PAV terwijl 6,8% een cerebro- of cardiovasculaire complicatie doormaakte. De gemiddelde D-dimeer, fibrinogeen en vWF-waarden van het bloed afgenomen bij inclusie bleken binnen de bovenste limiet van het referentie interval (454 ng/ml, 3.6 g/l en 150%, resp.) net zoals de genormaliseerde piek van de trombine generatie meting (131,7%). In de univariate analyse bleek dat diabetes mellitus de enige risicofactor was die geassocieerd was met het optreden van een cerebro- of cardiovasculaire complicatie. Een fibrinogeen spiegel hoger dan 3.6 g/l bleef geassocieerd met een afgenomen risico op progressie van PAV (HR 0.61; 95% CI 0.39-0.95, $p=0.03$). De gekozen hemostase parameters toonden geen significante associaties met het optreden van een complicatie dan wel progressie van ziekte. In de multivariate analyse bleef de gevonden associatie onafhankelijk significant (diabetes: HR 5.37; (95% CI 1.79-16.11, $p=0.003$). Op basis van onze gevonden resultaten moeten we dus concluderen dat, behoudens fibrinogeen, geen van de gekozen hemostase parameters geassocieerd is met het optreden van atherotrombotische events en progressie van PAV gedurende de gekozen follow-up duur. Kennelijk speelt plasma hypercoagulabiliteit geen predominante rol in de etiologie van cardiovasculaire complicaties bij patiënten met recent gediagnosticeerde PAV.

Deel II Hemostase problemen veroorzaakt door leverziekten in hun eindstadium

Het tweede deel van dit proefschrift focust op de veranderingen in de bloedstolling die ontstaan wanneer de leverfunctie verstoord is door het optreden van cirrose. De lever synthetiseert niet alleen een groot deel van de stollingsfactoren echter neemt ook de klaring van de geactiveerde producten van de stolling voor rekening. De onderwerpen 'hemostase' en 'de lever' zijn op deze manier nauw met elkaar verbonden. Ondanks de vooruitgang op wetenschappelijk gebied betreffende de kennis over stollingsafwijkingen, die ontstaan bij het optreden van cirrose, blijken er geen goede hemostatische testen te bestaan die deze veranderingen nauwkeurig kunnen bepalen.

In **hoofdstuk 7** wordt een cross-sectionele studie beschreven waarin met een panel biomarkers de verschillende mechanismes van hemostase in kaart worden gebracht van patiënten met cirrose. Zowel de trombine genererende capaciteiten als de daadwerkelijke stolsel-opbouw en -afbraak worden van 73 cirrose-patiënten en 20

gezonde controles met verschillende testen in kaart gebracht en vergeleken. De klassieke stoltesten (aPTT, PT en INR) waren significant verlengd wanneer de ernst van de cirrose toenam, terwijl het merendeel van de hematologische en hemostatische parameters significant waren afgenomen (hemoglobine, trombocyten, fibrinogeen, antitrombine (AT), proteïne C en plasminogen, $p < 0,05$). De spiegels van D-dimeer en vWF-antigeen waren echter significant verhoogd in Child-Pugh C patiënten. Nieuwe bevindingen van deze studie waren de toegenomen factor VIIa-spiegels en een milde toename van het complex van factor IXa-AT. Er bleek geen toename van het factor Xa-AT complex. Een verklaring van deze toegenomen activiteit van de extrinsieke stolling middels factor VII en vervolgens het factor IXa-AT complex kan mogelijk worden gevonden in de toename van circulerende micropartikels met tissue factor zoals recent werd beschreven door Rautou et al. In samenhang met deze marker van een protrombotische status bleken ook de trombine generatie parameters verhoogd zodra de ernst van de cirrose toenam. Patiënten met cirrose bleken in staat evenveel, zo niet meer, trombine te kunnen genereren als de gezonde controles. Dit bleek ook het geval in de metingen uitgevoerd in de afwezigheid van trombomoduline. Deze laatste bevinding bleek nieuw ten opzichte van eerder uitgevoerd onderzoek en kan waarschijnlijk worden verklaard door een andere uitvoering van de CAT-assay. Ook de gemodificeerde ROTEM analyses in volbloed toonden nieuwe uitkomsten waarbij sprake was van een afgenomen snelheid van stolselvorming en een afgenomen stolselsterkte zodra de levercirrose ernstiger was. Er werden geen significante verschillen gevonden in stolselafbraak tussen patiënten onderling (op basis van de Child-Pugh score) en tussen patiënten en gezonde controles. Aan de hand van deze resultaten concludeerden wij dat patiënten met cirrose een procoagulant plasma milieu hebben, maar dat in hun volbloed juist sprake is van een afgenomen stolcapaciteit. Er zijn geen verschillen in weerstand tegen fibrinolyse naarmate de ernst van de cirrose toeneemt.

De hoeveelheid gevormd trombine is afhankelijk van twee processen; enerzijds de vorming van trombine door de omzetting van protrombine, anderzijds de klaring van trombine uit het plasma (trombine inactivatie). In **hoofdstuk 8** hebben wij gepoogd beide processen bij cirrose-patiënten in kaart te brengen door zogenaamd 'computational modelling'. Verder wilden we objectiveren wat de invloed van beide, individuele processen was op de trombine generatie curves van patiënten en gezonde controles. Bloed van dezelfde patiëntenpopulatie als in hoofdstuk 7 werd geanalyseerd evenals het bloed van 30 gezonde vrijwilligers. In dit bloed werden de functionele antitrombine, α_2M , protrombine en fibrinogeenspiegels gemeten; deze bleken significant verlaagd wanneer de ernst van de cirrose toenam. De α_2M -spiegel bleek echter significant verhoogd in patiënten met Child-Pugh A cirrose. Hierna werden ook de trombine generatie curves in de aan- en afwezigheid van trombomoduline gemeten. Vervolgens werd op basis van de gemeten antitrombine, α_2M en fibrinogeenspiegels de

trombine inactivatie berekend met behulp van een wiskundig model. Protrombine conversie curves toonden aan dat de hoeveelheid protrombine die omgezet wordt in patiënten met cirrose duidelijk is afgenomen naarmate de ernst van de cirrose toeneemt. Deze afname wordt meest waarschijnlijk veroorzaakt door een kleinere hoeveelheid door de lever gesynthetiseerde protrombine. De snelheid van de omzetting van protrombine naar trombine was echter hoger in patiënten met cirrose waardoor gesuggereerd kan worden dat óf de concentratie óf de activiteit van het protrombinase-complex is toegenomen. Onze experimenten lieten verder zien dat de inactivatie van trombine duidelijk was afgenomen bij verergering van de cirrose. Dit kan worden verklaard door de significant afgenomen hoeveelheid TAT-complexen aangezien het trombine- α_2 M complex vergelijkbaar was tussen patiënten met cirrose en gezonde controles. Op basis van de behaalde resultaten concludeerden wij dat ondanks dat in plasma van patiënten met cirrose minder trombine wordt gevormd, het trombine wel sneller gevormd wordt en minder snel wordt geïnactiveerd. In de CAT-assay ontstaat daardoor een 'hypercoagulabel beeld'. Verdere berekeningen toonden aan dat exogene aanpassing van de protrombine omzetting, ofwel door toevoeging van protrombine complex concentraat ofwel door antitrombine, zouden leiden tot een verlies van de stollingsbalans. Dit ondersteunt de eerdere hypothese dat het stollingssysteem van patiënten met cirrose in een wankel evenwicht verkeert. Aanpassingen hieraan zouden juist leiden tot het uit balans raken van dit systeem met als gevolg het optreden van nadelige klinische effecten.

In **hoofdstuk 9** worden de resultaten besproken van een prospectieve studie waarbij zowel de klassieke hemostase parameters als de nieuwe "overall" stollings-assays gebruikt worden om de prognose van patiënten met cirrose te beoordelen. De al eerder beschreven 73 patiënten werden gevolgd in de tijd, met een gemiddelde van 29 maanden, en het samengestelde eindpunt bestaande uit majeure bloeding, trombose in een willekeurig bloedvat en leverziekte gerelateerde mortaliteit werd geclassificeerd als complicatie. De multivariate analyse toonde aan dat enkel het vWF-antigeen en D-dimeer significante voorspellers waren van complicaties. De trombine generatie assay en de gemodificeerde ROTEM-assay hebben geen aanvullende waarde voor deze risico inschatting. Er zijn verschillende verklaringen voor het toegenomen vWF-antigeen in patiënten met cirrose: 1. Een toegenomen secretie door het endotheel door beschadiging veroorzaakt door bacteriële endotoxines, 2. Portosystemische collateraalvorming en angiogenese waardoor er een groter endotheel-oppervlakte is en dus meer vWF-secretie, 3. Een afgenomen klaring van vWF door een vermindering van de door de lever gesynthetiseerde ADAMTS13.

Naarmate de ernst van de cirrose en portale hypertensie toeneemt zal de vWF-spiegel dus toenemen en daardoor betrouwbare informatie geven over een op hand zijnde (de-) compensatie van de patiënt. De reden dat D-dimeer onafhankelijk geassocieerd is met het optreden van een trombose- dan wel bloedingscomplicatie komt doordat D-

dimeer zowel informatie over zowel stolselopbouw als stolselafbraak geeft, verder worden D-dimeren geklaard door de lever en de D-dimeerspiegel geeft daarmee ook informatie over de klaringscapaciteit van de lever. De “overall” trombine generatie- en ROTEM-assays die gebruikt werden in onze studie geven geen informatie over de daadwerkelijke hoeveelheid fibrine die gevormd is, maar informeren ons over de mogelijkheid dat het bloed nog kan stollen of lyseren (het potentieel). Dat deze overall testen geen voorspellende waarde bleken te hebben in dit cohort geeft daarom aan dat het stollings- en fibrinolyse*potentieel* op moment van bloedafname van weinig nut is voor het voorspellen bleedings-dan wel trombotische complicaties in patiënten met cirrose.

In **hoofdstuk 10** worden de resultaten van dit proefschrift besproken en bediscussieerd waarna aanbevelingen worden gedaan voor toekomstig onderzoek. Gebaseerd op onze data en de data van eerdere studies lijkt echter geen afzonderlijke hemostase marker in staat de complexiteit van PAV in kaart te brengen. Een zogenaamde “multi-marker aanpak” van reeds bestaande en nieuw te ontwikkelen biomarkers lijkt daarom vereist om patiënten te kunnen identificeren die risico lopen op cerebro- en cardiovasculaire complicaties en progressie van PAV. Uit het tweede deel van het proefschrift blijkt dat er bij patiënten met cirrose, ondanks dat zij lagere spiegels (inactieve) pro- en anticoagulante factoren hebben, er juist een overall procoagulant *plasma* milieu bestaat waardoor een snelle omzetting is van protrombine in trombine en een afname van de trombine inactivatie. Op basis van onze resultaten, waarbij we klassieke stollingstesten combineerden met nieuwe overall stollingstesten, moeten wij de hypothese van een gerebalanceerd stollingssysteem in patiënten met cirrose onderschrijven. Deze balans is echter fragiel en kan omslaan naar zowel de bleedings- als de trombose kant. Uit dit proefschrift blijkt eveneens dat de huidige overall stollingstesten geen voorspellende waarde hebben ten aanzien van trombotische- of bleedings-complicaties bij patiënten met cirrose. Het blijft daarom een uitdaging de ideale overall stoltest te ontwikkelen die de patiënten met een verhoogd risico op stollingsgerelateerde complicaties wel herkent. Deze test zou idealiter zowel de primaire, secundaire hemostase als de fibrinolyse in ogenschouw moeten nemen. Aangezien dit, op dit moment, echter nog onwaarschijnlijk is, is het daarom raadzaam in verdere prospectieve studies stoltesten te combineren: klassieke en nieuwe testen, plasma en volbloedtesten gecombineerd met point-of-care plaatjesfunctie testen. Voorts zou het tevens interessant zijn om vWF en/of D-dimeer, mogelijk zelfs als een vervanging van de INR, toe te voegen aan de Child-Pugh en de MELD-score om hun separate waarde in deze modellen voor risicobeoordeling vast te stellen.

Valorisatie

Valorisatie

Naast ‘onderwijs’ en ‘onderzoek’ heeft de Nederlandse overheid sinds enkele jaren ook ‘valorisatie’ als kerntaak gedefinieerd voor universiteiten¹. Valorisatie van wetenschappelijke kennis verwijst naar de mogelijkheid om de opgedane kennis in praktijk te brengen ten gunste van de maatschappij. Enerzijds door bijvoorbeeld het ontwikkelen van een product of medicijn, anderzijds door het toepassen of implementeren van de opgedane kennis in een (reeds bestaand) systeem of proces. Het doen van (goed) onderzoek verlegt de grenzen van onze wetenschappelijke kennis en dat heeft op zich reeds een grote maatschappelijke en culturele waarde. Echter met valoriseren wordt met name bedoeld dat de verworven kennis niet alleen bruikbaar is voor vakgenoten, maar ook voor andere belanghebbenden zoals bijvoorbeeld bedrijven of maatschappelijke organisaties². In het geval van deze thesis betreft het valorisatie-aspect met name het vergroten van de kennis op het gebied van de bloedstolling waardoor (op termijn) een bijdrage kan worden geleverd aan mogelijk betere diagnostiek en behandeling. Dit om onze voornaamste belanghebbende, ‘de patiënt’, en daarmee de maatschappij, hiervan te laten profiteren. In dit proefschrift zijn twee verschillende onderwerpen behandeld.

Deel 1 van dit proefschrift houdt zich bezig met de interactie tussen perifeer arterieel vaatlijden (PAV) en de bloedstolling. PAV, één van de manifestaties van atherosclerotisch vaatlijden, ontstaat door progressieve vernauwing van de arteriën naar de onderste extremiteiten. Hierdoor ontstaat hypoperfusie van het weefsel waardoor pijnklachten en uiteindelijk zelfs afsterving van het weefsel kunnen optreden. In uiteindelijk 1-5% van de gevallen is daardoor een amputatie nodig van de aangedane ledemaat³. Nog meer op de voorgrond staand is de verhoogde kans op atherotrombotische complicaties zoals een myocard infarct of een (ischemische) beroerte; binnen vijf jaar na de diagnose heeft ongeveer 20% van de patiënten een cardiovasculaire complicatie doorgemaakt en 10-15% van de patiënten is zelfs overleden^{4,5}. In 2007 werd het aantal patiënten met PAV in Europa en Noord-Amerika op 27 miljoen individuen geschat waarvoor jaarlijks 413.000 ziekenhuisopnames nodig waren⁶. Doordat het risico op PAV toeneemt met de leeftijd en er sprake is van vergrijzing van onze populatie is PAV dus een groeiend klinisch probleem⁷. Het eerste deel van dit proefschrift had als doel meer inzicht te krijgen in de invloed van hemostase op de etiologie en progressie van PAV en om nieuwe hemostatische risicofactoren te identificeren die ons in staat zouden stellen atherotrombotische complicaties sneller in het ziekteproces te kunnen identificeren. Buiten de traditionele risicofactoren zoals roken en diabetes lijken namelijk ook hemostase-gerelateerde risicofactoren van invloed niet alleen op het ontstaan van PAV, maar ook de daaraan verwante atherotrombotische complicaties. Gebaseerd op de gecombineerde data van eerdere studies (zoals onderzocht in **hoofdstuk 5**) concludeerden wij dat de simpele en

wereldwijd beschikbare D-dimeer test een redelijk goede voorspeller was van arteriële trombotische complicaties en cardiovasculaire mortaliteit op de korte termijn bij patiënten met PAV. Uit onze eigen prospectieve studie bleek deze voorspellende waarde van D-dimeer echter niet onafhankelijk aanwezig. Verschil met de eerdere studies was echter wel dat het hierbij patiënten betrof die pas recent met PAV waren gediagnosticeerd. Gebaseerd op de data gepresenteerd in **hoofdstuk 3,4 en 6** blijkt verder geen enkele afzonderlijke hemostase marker in staat de complexiteit van PAV in kaart te brengen. Ondanks dat de studies zoals beschreven in deze thesis dus niet hebben geresulteerd in de ontwikkeling van een nieuwe biomarker danwel predictiescore voor de prognose, hebben deze studies wel bijgedragen aan een verbeterd inzicht in de rol van de bloedstolling in PAV. Op basis hiervan adviseren wij dat ook tot het uitvoeren van nieuwe studies met een zogenaamde “multi-marker aanpak” van reeds bestaande en nieuw te ontwikkelen biomarkers om patiënten te kunnen identificeren die het risico lopen op cerebro- en cardiovasculaire complicaties en progressie van PAV. Deze markers zouden aan de voorwaarde moeten voldoen dat zij goedkoop, gemakkelijk te meten, gestandaardiseerd met een acceptabele “intra- en interlaboratory” variabiliteit en wereldwijd toegankelijk zouden moeten zijn. Ten aanzien van de behandeling van PAV komen wij, op basis van de resultaten uit **hoofdstuk 3**, tot de conclusie dat bij PAV-patiënten gebruik van enkel aspirine onvoldoende is. Bij de behandeling met aspirine is de trombocyten activatie onvoldoende onderdrukt waardoor aanvulling of verandering van de huidige therapie met effectievere antitrombotische medicatie dus noodzakelijk is. Echter ook hier zijn eerst aanvullende studies vereist waarbij rekening moet worden gehouden met o.a. een voldoende aantal PAV-patiënten, de ernst van de PAV van de geïnccludeerde patiënten en de gekozen antitrombotische therapie.

Deel twee van dit proefschrift beschrijft de veranderingen in de bloedstolling die ontstaan bij een verstoorde leverfunctie. Cirrose wordt over het algemeen gezien als het irreversibele eindstadium van een leverziekte. De levensverwachting van patiënten met cirrose is daarmee duidelijk afgenomen en de enige behandeloptie is een (zeer kostbare) levertransplantatie. De vier meest voorkomende oorzaken van cirrose in Europa zijn alcoholabusus, virale hepatitis B en C en het metabool syndroom gerelateerd aan overgewicht en obesitas. Ondanks dat er een duidelijke afname is van de virale hepatitisen in Europa is er juist een duidelijke toename van alcoholgebruik en obesitas. Beschikbare data toont bijvoorbeeld een prevalentie van “Non-alcoholic fatty liver disease” (NAFLD), een voorloper van cirrose, tussen de 2 en 44% in de Europese bevolking en zelfs 42,6 tot 69,5% in de personen met diabetes type 2⁸. Doordat het beginstadium van cirrose vaak lange tijd onopgemerkt blijft is de exacte prevalentie van cirrose niet bekend; wereldwijd, gebaseerd op autopsie-studies, wordt de prevalentie geschat op 4,5-9,5%⁹. Cirrose en de (ernstige) gevolgen daarvan zijn dus geen

geïsoleerde problemen, maar veelvoorkomend en met grote impact op de huidige maatschappij.

Doordat een groot deel van de stollingsfactoren door de lever gesynthetiseerd wordt is bij cirrose ook de hemostase aangedaan. Ondanks uitgebreid onderzoek is de pathogenese hiervan nog niet volledig opgehelderd. Lange tijd werd aangenomen dat er sprake was van een verhoogd bloedingsrisico was waardoor cirrose-patiënten standaard behandeld werden met plasma of (dure) procoagulante middelen voorafgaand aan invasieve procedures of operaties. Dit alom aanvaardde verhoogde bloedingsrisico werd recentelijk betwist toen er werd aangetoond dat patiënten met ernstige leverziekten in staat waren evenveel en mogelijk zelfs meer trombine te genereren dan gezonde personen. Ook bleek dat de tot nu toe gebruikte stollingstesten (zoals bijvoorbeeld de INR) niet in staat zijn om de hemostase van deze patiëntengroep goed weer te geven. Door ons onderzoek in **hoofdstuk 7-8**, waarbij we klassieke stollingstesten combineerden met nieuwe ‘overall’ stollingstesten, kregen we een beter inzicht in de bloedstolling van patiënten met cirrose. Op basis daarvan moeten wij de hypothese van een gerebalanceerd stollingssysteem onderschrijven en concluderen we het automatisch behandelen met procoagulante medicatie dus zeker niet altijd geïndiceerd is in deze groep. Omdat de stollingsbalans fragiel en kan omslaan naar zowel de bloedings- als de trombose-kant is het moeilijk de wel geschikte behandelstrategie te kiezen; zowel klassieke, en relatief goedkope, medicatie zoals vitamine K antagonisten als nieuwe, vrij dure, orale anticoagulantia (FXa-remmers zoals rivaroxaban en de directe trombine-remmer dabigatran) zouden theoretisch van pas kunnen ter voorkoming van complicaties in cirrose. Dit is echter nog maar mondjesmaat onderzocht. Verder onderzoek aangaande behandeling met antistollingsmedicatie en cirrose is daarom noodzakelijk.

Zoals hierboven reeds beschreven is levercirrose niet te genezen. Aangezien de lever een orgaan is met veel reservecapaciteit is het van groot belang het cirrose-proces tot stilstand te brengen. Wanneer echter een te groot deel van de lever cirrotisch is, is de enige behandeling een levertransplantatie. Gezien het tekort aan beschikbare donorlevers om alle patiënten direct te kunnen behandelen is er een wachtlijst. Om postmortale organen toe te kennen aan de patiënten die het meest behoefte hebben aan een transplantatie wordt de MELD-score (Mayo End-Stage Liver Disease) gebruikt. Deze score geeft aan welke patiënt in de drie komende maanden het grootste stervensrisico loopt. Voor het berekenen van de MELD wordt, naast bilirubine en creatinine, ook de INR gebruikt. Al sinds 2004 echter, kort na het begin van het gebruik van de MELD-score, is bekend dat gebruik van de INR door grote variabiliteit tussen laboratorium-waarden kan zorgen voor een 20% verschil in MELD-score¹⁰. Nu ook blijkt dat de INR geen betrouwbare meting is voor een goede weergave van de hemostase in cirrose-patiënten rijst de vraag of de INR überhaupt wel gebruikt zou moeten worden in deze zo belangrijke score. Op basis van de resultaten zoals beschreven in **hoofdstuk 9**

zou het mogelijk ook interessant zijn om vWF en D-dimeer toe te voegen aan de MELD-score (en Child-Pugh score) om zo hun aanvullende waarde te kunnen beoordelen in deze modellen voor risicobeoordeling. Verdere studies zouden ook kunnen evalueren of met het gebruik van D-dimeer in plaats van de INR er een verbetering van de MELD-score zou optreden; met andere woorden of het MELD-systeem effectiever zou worden op het voorkomen van overlijden vóór de transplantatie. Aangezien de positie van de patiënt op de wachtlijst uitsluitend gebaseerd is op de MELD-score zou een verbetering van dit scoresysteem uiteindelijk dus wel maatschappelijke gevolgen hebben.

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Curriculum vitae

Curriculum vitae

Marie-Claire Frederique Kleinegris was born in Terneuzen, the Netherlands, on September 24th 1984. After living one and a half year in Rastatt, Germany, she returned to Terneuzen in 1987 where she attended primary school. Subsequently, she completed her secondary education at the Zeldenrust-Steelantcollege in Terneuzen. After receiving her VWO-diploma in 2002, she studied at the Sir James Dunn Collegiate and Vocational School, Sault Ste. Marie (Ontario, Canada). In 2003 she started her studies of Medicine at Maastricht University where she participated in the 'International Track in Medicine'. During her medical training she embarked on traineeships abroad in Brussels (Belgium), Birmingham (United Kingdom), Stellenbosch (South-Africa) and Salta (Argentina). In 2009 she started with an elective clinical and scientific traineeship at the Division of Gastroenterology and Hepatology, Maastricht University Medical Center, where her interest in the interaction between coagulation and chronic liver disease was aroused. From January 2010 to June 2014, Marie-Claire worked as a PhD student affiliated to the department of Biochemistry and Internal Medicine at Maastricht University, under the supervision of Prof. dr. H. ten Cate, Dr. A. ten Cate-Hoek, Dr. G.H. Koek and Dr. B. de Laat. Her main research topic was coagulation testing in various patient groups including patients with extended atherosclerosis and patients with severe liver disease. This research was funded by the Center for Translational Molecular Medicine, project INCOAG. The results of her PhD are presented in this thesis. In July 2014, she has started her training to become a gastroenterologist under supervision of Prof. dr. A.A.M. Masclee (Gastroenterology, Maastricht University Medical Centre), Dr. J. Buijs (Internal Medicine, Zuyderland Medical Centre, Heerlen) and Dr. C.M. Bakker (Gastroenterology, Zuyderland Medical Centre, Heerlen).

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Dankwoord

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Marie-Claire