FIBRIN AND FIBRINOGEN DEGRADATION PRODUCTS IN PLASMA

Clinical and methodological studies using enzyme immuno assays

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FIBRIN AND FIBRINOGEN DEGRADATION PRODUCTS IN PLASMA

Clinical and methodological studies using enzyme immuno assays

FIBRINE EN FIBRINOGEEN AFBRAAKPRODUKTEN IN PLASMA

Klinische en methodologische studies met enzyme immuno assays

PROEFSCHRIFT

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INTRODUCTION

INTRODUCTION

The haemostatic mechanism is a dynamic balance which can basically be described as the equilibrium between fibrin formation (coagulation) and fibrin lysis (fibrinolysis). A schematic presentation of the haemostatic balance is given in Figure 1.

Activation of the coagulation cascade is a physiologic response to tissue injury, and results in the formation of fibrin. Fibrin formation and deposition are a fundamental process in the repair of injured tissue and protect the body against haemorrhage. Fibrin is formed from fibrinogen, a reaction catalyzed by thrombin (IIa) which is generated after contact activation or tissue activation of a cascade of proteolytic enzymes. The process of coagulation is controlled by coagulation inhibitors of which antithrombin III (AT-III) and protein C are the most important.

Normal fibrin formation and deposition are temporary reactions, which are curtailed or reversed when the initiating stimulus is removed, in order to restore normal tissue structure and function . Fibrin deposition is counterbalanced by the fibrinolytic system that lyses both fibrin and fibrinogen. Plasmin is the enzyme responsible for lysis of fibrinogen and fibrin. Plasmin is formed through activation of plasminogen by activators, such as tissue-type plasminogen activator (t-PA) and urokinase-type plasminogen activator (u-PA). Fibrinolysis is controlled by inhibitors of the plasminogen activators (PAI) and inhibitors of plasmin, such as α_2-antiplasmin, α_2-macroglobulin and C_1-inactivator.

Disruption of the haemostatic balance may occur when activation of coagulation is insufficiently counteracted by coagulation inhibitors or when reactive fibrinolysis is inhibited. Under those circumstances, patients are exposed to the risk of thromboembolism or multiple organ failure due to widespread intravascular deposition of fibrin. Consumption coagulopathy, due to increased turnover of plasma coagulation factors may, after a phase of hypercoagulability, change into hypocoagulopathy and haemorrhagic diathesis. Consumption coagulopathy may exist alone, it may be accompanied by slight secondary fibrinolysis or the secondary hyperfibrinolysis may predominate. The hyperfibrinolysis is a consequence of an excessive production of

plasmin with degradation of fibrin, fibrinogen and of coagulation factors and the appearance of fibrin and fibrinogen degradation products, which in turn inhibit fibrin polymerization and promote a haemorrhagic diathesis. Finally, lysis of fibrinogen (primary fibrinolysis of fibrinogenolysis) may occur alone, leading to a haemorrhagic state through formation of fibrinogen degradation products which have anticoagulant properties.

Analysis of the haemostatic balance in patients at risk for thromboembolism or bleeding is essential when treatment is considered. Disturbances of the haemostatic balance will be reflected by the concentration and composition of the products of the two opposing processes of coagulation and fibrinolysis - fibrin and fibrinogen derivatives - which can be used as markers of the status of the haemostatic balance.

With improved understanding of the basic mechanisms of thrombosis and haemostasis, there has been a rapid development of techniques to assess the haemostatic system and its associated disorders. One of the newer systems that has recently become available for the assessment of haemostatic disorders, both thrombotic and haemorrhagic, is the determination of fibrin degradation products (FbDP) and fibrinogen degradation products (FgDP) in plasma by enzyme immuno assays (EIA's) based on monoclonal antibodies.

This thesis comprises studies with monoclonal antibody-based plasma assays for derivatives of fibrin and fibrinogen in patients with diseases and conditions characterized by an activated state of coagulation and fibrinolysis.

The aims of the study were:

1. To review the clinical relevance of existing serum-based assays for fibrin/fibrinogen degradation products in comparison with the newly developed plasma-based assays for fibrin degradation products (FbDP, D-dimer), for the total of fibrin and fibrinogen degradation products (TDP) and for fibrinogen degradation products (FgDP), on the basis of published studies.

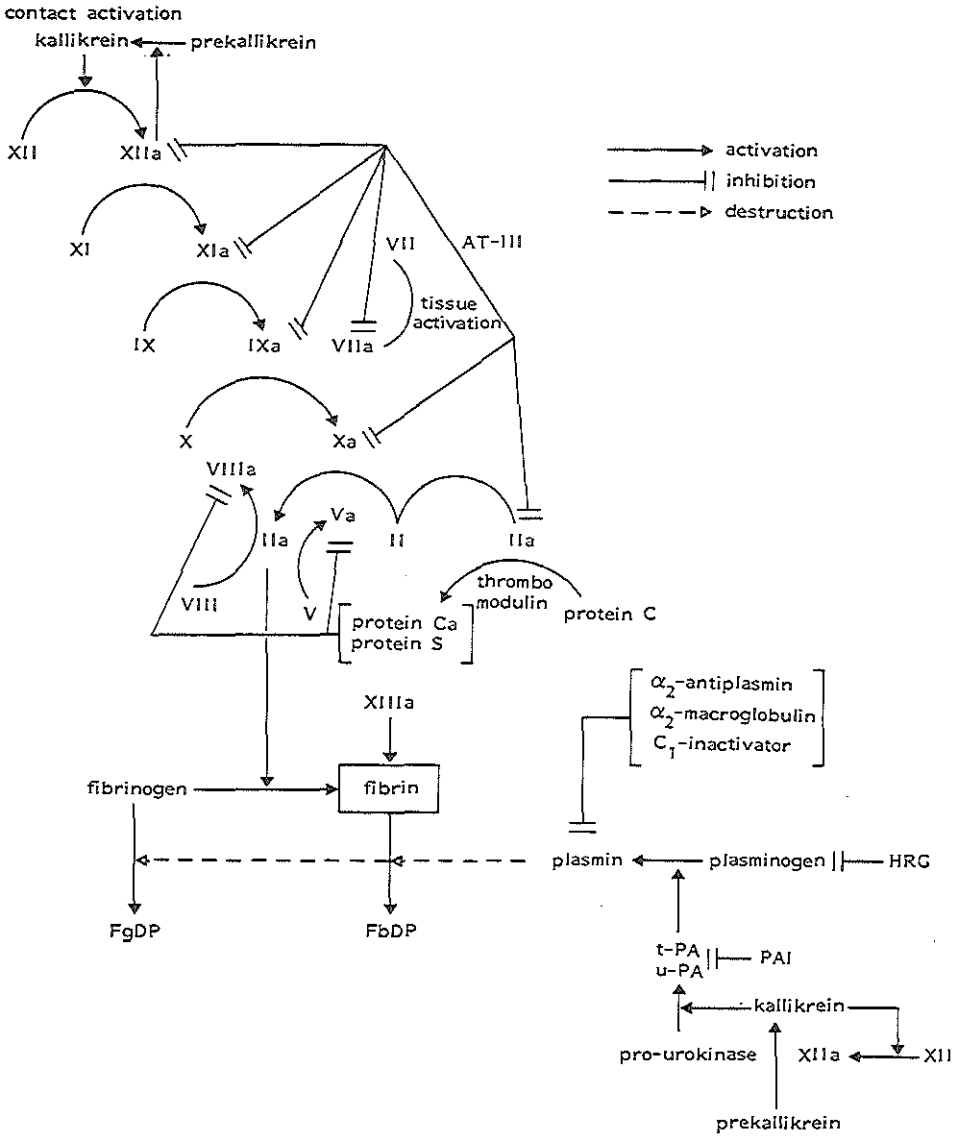

Figure 1. Schematic presentation of the coagulation cascade and the fibrinolytic system. AT-III = antithrombin III; t-PA = tissue-type plasminogen activator; u-PA = urokinase-type plasminogen activator; PAI = plasminogen activator inhibitor; HRG = histidine-rich glycoprotein; FbDP = fibrin degradation products; FgDP = fibrinogen degradation products.

2. To evaluate the assays for D-dimer, FbDP, FgDP and TDP in healthy volunteers and patients with an activated state of coagulation.

3. To study the haemostatic balance in diseases or conditions associated with an activated state of coagulation:
   a) patients with a suspected or proven thrombotic process.
   b) patients with cirrhosis of the liver to see whether haemostatic imbalance is due to primary fibrinolysis or diffuse intravascular coagulation.
   c) patients with septic shock to see whether haemostatic complications are due to increased activation of coagulation or inhibition of fibrinolysis.
   d) patients with an increased tendency to develop thrombotic complications who are treated with heparin, using measurements of fibrin degradation products, in order to evaluate effectiveness of treatment.

4. To evaluate the diagnostic value of the D-dimer assay for deep venous thrombosis using receiver operating characteristic methodology and Baysian analysis.

Chapter 1

MONOCLONAL ANTIBODY-BASED PLASMA ASSAYS FOR FIBRIN(OGEN) AND DERIVATIVES, AND THEIR CLINICAL RELEVANCE

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ABSTRACT

The haemostatic balance can basically be described as the equilibrium between fibrin formation (coagulation) and fibrin lysis (fibrinolysis). Therefore the status of this balance may be reflected by the products of these two processes.

Until recently, the tests for assessment of fibrin(ogen) degradation products were performed in serum since they were based on polyclonal antibodies, which crossreact with fibrinogen. The use of serum introduces many artefacts and therefore the utility of these serum tests is limited.

New assays have now become available which can be divided in quantitative enzyme immunoassays (EIA's) and semiquantitative latex agglutination assays. The new assays can be carried out in plasma since they use highly specific monoclonal antibodies, the majority of which do not crossreact with fibrinogen. This makes it possible to avoid the serum artefacts. Furthermore, these plasma assays can discriminate between degradation products of fibrin and those of fibrinogen (FbDP and FgDP, respectively).

The possible clinical utility of the new assays is discussed on the basis of literature data on the following clinical states: deep venous thrombosis (DVT) and pulmonary embolism, liver disease and liver transplantation, sickle cell disease, renal diseases, pregnancy and preeclampsia, disseminated intravascular coagulation (DIC), malignancy, coronary artery disease and thrombolytic therapy. Fibrinolysis appears to be accompanied by fibrinogenolysis. Detection of fibrin(ogen) derivatives may be used to rule out DVT; to monitor efficacy of anticoagulant treatment for DVT or DIC, and reflects severity of renal disease but not renal function. High levels of FgDP's were found during orthotopic liver transplantation and thrombolytic therapy. Fibrin(ogen) degradation products can not be used to predict reperfusion following thrombolytic therapy. The fibrinolytic system remained active during normal and complicated pregnancy and in patients with malignancies.

The new assays provide valuable information on fibrin(ogen)olysis in several diseases. More information on the haemostatic balance may be obtained by using these new assays for fibrin(ogen)olysis products in combination with assays for coagulation products.

INTRODUCTION

The haemostatic balance can be described as the equilibrium between the two opposing processes coagulation and fibrinolysis. Coagulation will lead to fibrin formation, and the formed fibrin will be lysed by fibrinolysis. It is conceivable that disturbances in the haemostatic balance will be reflected by the concentrations and composition of the products of the two processes i.e. fibrin(ogen) derivatives which can be used as molecular markers of the status of the haemostatic balance.

The measurement of levels of fibrin(ogen) degradation products has been practiced for at least two decades using a variety of procedures.[1] Several clinical disorders have been described that are associated with abnormalities in the degradation of fibrin(ogen). However, since no clinically useful test was available that could measure the degradation products of fibrinogen specifically, it was not possible to make a distinction between the degradation products of fibrin and fibrinogen (FbDP's and FgDP's respectively).

Elevated levels of fibrin(ogen) degradation products (FDP's) have been detected in serum in the past and were found in venous thromboembolism and diffuse intravascular coagulation, but also following trauma, surgery, infection, malignancy and sickle cell anaemia.[2-9,12-14] It has been postulated that increased FDP levels may be of value in confirming the diagnosis of diseases characterized by an activated coagulation system and that normal FDP levels could exclude the diagnosis.[8,14] The observation of an initial rise and subsequent fall of levels of fibrin(ogen) degradation products following thrombolytic therapy in patients with acute myocardial infarction, suggested that measurement of fibrin(ogen) degradation products could also be used to monitor the effect of thrombolytic therapy.[15,16]

In patient management, a test for the detection of a disease characterized by an activated coagulation system is clinically useful when it can rule out (suspected) patients who do not have the disease (specificity= true negative rate= number of patients without the disease with a normal test divided by the number of people without the disease) and does confirm diagnosis in case the disease is present (sensitivity= true positive rate= number of patients with the disease with an abnormal test divided by the

number of patients with the disease). However, due to a lack of specificity and accuracy of the serum assays for FDP assessment, the issue of measuring FDP levels as markers for pathologic conditions has been confounded. Most of these assays could only be performed in serum samples since they were based on polyclonal antibodies to fibrin(ogen) and derivatives. As will be pointed out below, the use of serum samples is unreliable since it introduces many artefacts. Also, polyclonal antibody-based serum assays could not discriminate between degradation products of fibrinogen and those of fibrin. Data based on such serum assays should therefore be interpreted with caution. New techniques have recently become available for the quantitative determination of fibrin (FbDP) - as well of fibrinogen degradation products (FgDP). These new assays are performed on plasma samples (thus avoiding the well known serum artefacts) and are based on monoclonal antibodies. Since a specific test has now become available to determine degradation products of fibrinogen, these assays permit a separate quantification of the extent lysis of fibrin (secondary fibrinolysis) and that of fibrinogen (primary fibrinolysis or fibrinogenolysis).

Application of the new assays as well their possibility to discriminate between fibrinogen-and fibrin degradation products may be of value in the diagnosis of a disease and the recognition of an altered state of haemostatic function, e.g. a state of strong fibrinogenolysis that may be associated with an increased risk of bleeding. This may have implications for the subsequent therapeutical approach in the clinical condition under study.

The clinical experience with the new assays is steadily growing, and it is becoming more and more clear that the current diagnostic concepts may have to be adapted, since they are based on the questionable serum assays. This review article describes the formation of fibrin(ogen) and their derivatives, the available plasma assays for their assessment and evaluates the clinical utility of the new assays in studies described until now.

FORMATION OF FIBRIN(OGEN) DERIVATIVES

a. Fibrin formation

Fibrinogen is a symmetrical glycoprotein with a molecular weight of 340,000 daltons. It is composed of six polypeptide chains: 2Aα-, 2 Bß- and 2 y-chains, which are kept together by disulphide bonds. In electron microscopic images of fibrinogen a central nodule and two larger outer nodules are observed.[17] The central nodule contains the amino-terminal ends of all six polypeptide chains and is designated as the E-domain. The more distal nodules are designated as the D-domains and contain the middle and carboxyl-terminal parts of the Aα-, Bß and y-chains. The D-domains are connected to the E-domain by relatively thin connecting structures. When the coagulation system is activated, thrombin is formed. Thrombin will convert the soluble fibrinogen to the insoluble fibrin through a series of intermediate products.[20] This process is initiated by the cleavage of the fibrinopeptides A (FpA) from the amino-terminal ends of the Aα-chains (in the central E-domain). The formed product is fibrin I (or desAA fibrin). The Aα-chains after release of FpA, are designated as α-chains. The new amino-terminal ends of the α-chains are able to interact with complementary sites in fibrinogen and in other fibrin I molecules. At low concentrations fibrin I will be kept in solution by complexation with fibrinogen. This is known as soluble fibrin.[18,31] At higher concentrations the fibrin I molecules will polymerise to a macroscopic gel [32], in which the subunits will be crosslinked by coagulation factor XIII in the presence of Ca^{2+}.[20] Simultaneously, but more slowly, thrombin can also release the fibrinopeptides B (FpB) from the Bß-chains (in the central E-domain), which results in fibrin II (desAABB-fibrin) formation. Like fibrin I, fibrin II can aggregate to form a gel, in which the subunits are crosslinked by factor XIII in the presence of Ca^{2+}.

The individual steps are summarized in Figure 1.

It is conceivable that fibrin formation, which is not counterbalanced by fibrin lysis, will result in increased blood levels of soluble fibrin. Soluble fibrin could thus be an indicator of an impending thrombotic event.

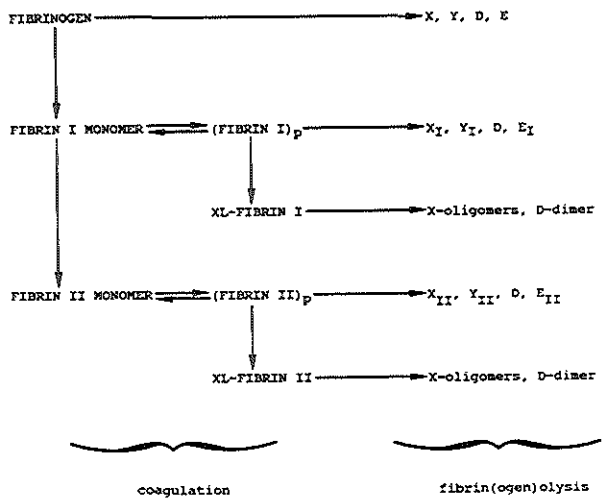

Figure 1. Possible pathways of the formation of fibrin and fibrinogen derivatives.

b. Formation of fibrinogen degradation products (fibrinogenolysis)

Activation of the fibrinolytic system will result in plasmin formation. Plasmin is not a fibrin-specific protease, however, and it may also degrade fibrinogen.

The carboxyl-terminal ends of the fibrinogen Aα-chains (in the distal D-domains) are highly sensitive to proteolytic activity. In normal healthy individuals, fibrinogen occurs in three main molecular forms: A high molecular weight form (HMW) in which both Aα chains are intact; a low molecular weight form (LMW) with one slightly shortened Aα-chain and a form with both Aα-chains partly proteolysed (LMW').[33-35] All three forms are clottable. The LMW'-form comprises only 4% of all clottable fibrinogen in a normal individual. The LMW and LMW' forms possibly result from plasmin activity. It is conceivable that changes in the blood proteolytic activity will first be reflected in an increase in the LMW'/HMW ratio.

When the plasmin digestion of fibrinogen proceeds further, a product is formed, in which only about 30% of the length of both Aα-chains is left. These products still contain the FpA, and are called fragments X. Subsequently fragment X is cleaved into one fragment Y and one D-fragment. Fragment Y is composed of the central E-domain of fibrinogen (with the FpA) and one D-domain. Eventually, fragment Y will be cleaved in one E- and one D-fragment. Thus one fibrinogen molecule will eventually yield one fragment E, and two fragments D, in full agreement with the three nodules seen in the electron microscopic images.

c. Formation of fibrin degradation products

The degradation by plasmin of the different forms of fibrin mentioned above, proceeds through intermediate products analogous to those described for fibrinogen. The reason for this appears to be that plasmin follows the same interdomainal cleavage pattern in fibrin as in fibrinogen.

Non-crosslinked fibrin I will be cleaved subsequently into fragments X_I, Y_I, D and E_I; non-crosslinked fibrin II will yield X_{II}, Y_{II}, D and E_{II}. The suffixes I and II denote the absence of FpA, and of FpA and FpB, respectively.

The crosslinked fibrins I and II consist of very long polymers of fibrin I and II, in which the subunits are crosslinked by isopeptide bonds. The subunits in these polymers will be proteolysed by plasmin in a random fashion. This will result in smaller (soluble) fragments of the original polymers, with a range of molecular weights.[20,36] These are collectively designated as X-oligomers.[10,11,36] Upon prolonged digestion by plasmin these X-oligomers will be converted to fragments D-dimer i.e. two covalently bound D-domains [20,36] and fragments E.

d. Molecular features of different fibrin(ogen) derivatives

On the basis of the brief description of the various fibrin(ogen) derivatives in the preceding paragraphs, several molecular features can be recognized, which discriminate one (group of) fibrin(ogen) derivative(s) from another. These are summarized in Table 1.

Table 1 Molecular characteristics of fibrinogen and some fibrin(ogen) derivatives.

fibrin(ogen) derivative	molecular characteristics
HMW-fibrinogen	carboxyl-terminals Aα-chains intact; contains FpA; no crosslinks
soluble fibrin/non-crosslinked	contains no FpA or crosslinks; new amino-terminus in α-chains
crosslinked fibrin	contains no FpA; contains cross-links; new α-chains amino-terminus
fibrinogen degradation products (FgDP)	contains FpA; altered conformation as compared with fibrinogen (neo-epitopes)
non-crosslinked fibrin degradation products (FbDP)	contain no FpA; altered conformation as compared with fibrin (neo-epitopes)
crosslinked fibrin degradation products (XL-FbDP)	contain no FpA; altered conformation as compared with fibrin (neo-epitopes); contain crosslinks.

Table 1 Molecular characterisctics of fibrinogen and some fibrin(ogen) derivatives

As will be shown below new monoclonal antibody-based assays are mostly based on the specific recognition of these molecular features.

ASSAYS FOR FIBRINOGEN AND ITS DERIVATIVES

Serum samples should not be used

Even in case of a disease, the blood concentrations of fibrin(ogen) derivatives will be low, as compared with the fibrinogen concentration i.e. in the microgram/ml range. For that reason sensitive immunological methods such as enzyme immunoassays (EIA) or latex agglutination assays are required. Until relatively recently, only serum could be used as a sample since the available polyclonal antibodies crossreact virtually completely with fibrinogen. The latter had to be removed e.g. by serum preparation. Well-known serum assays are the Thrombo-Wellco assay and the tanned red-cell hemagglutination inhibition assay.[37] Serum, however, is a notorious source of artefactual results:

* Incomplete clotting of the cross-reacting fibrinogen may occur in cases of dysfibrinogenemia; when a patient has been exposed to heparin [38] or when anticoagulant fibrinogen degradation products are present.[39-41] In polyclonal antibody-based serum assays this will inevitably lead to too high or false-positive results. Those can also result from partial lysis of the clot during serum preparation, not only in hyperfibrinolytic patients, but even in normal individuals.[19]
* Some degradation products will coagulate [40,42] or become adsorbed to the clot.[19,43,44] For that reason they will not be recovered in the serum and false-negative or too low results may be found.
* During serum preparation fibrinogen degradation products will loose their FpA. As a result they can no longer be discriminated from non-crosslinked fibrin degradation products, and primary fibrinogenolysis can not be detected.
* Serum can obviously not be used for the assessment of the products of ongoing coagulation in a patient.

These problems inherent in the use of serum could be avoided by the use of plasma. Only relatively recently, with the advent of the monoclonal antibody technology, it has

become possible to develop assays which can be performed with plasma samples and thus avoid the serum artefacts.

The new assays

In the following paragraph a brief outline will be given of the currently available assays based on monoclonal antibodies, which can be performed with plasma samples. The fundamental change as compared with the old technology is that monoclonal antibodies are used, which do not react with fibrinogen (except in the assay for intact fibrinogen, which will be described). This allows the assessment of coagulation and fibrin(ogen)olysis products in the same individual sample.

Available monoclonal antibody based plasma assays

Enzyme immunoassays (EIA)

All the available enzyme immunoassays are of the sandwich-type. They are based upon a specific monoclonal antibody absorbed to the wells of a microtitration plate. This is the solid phase, or capture antibody. Usually the capture antibody is directed against one of the molecular characteristics of a fibrin(ogen) derivative, summarized in Table 1. In the second step, the captured fibrin(ogen) derivative is tagged with an enzyme-labeled panspecific monoclonal antibody (or polyclonal antiserum), or with another enzyme-labeled monoclonal antibody with a defined specificity. The enzyme is usually horseradish peroxidase (HRP).

The most relevant EIA's described today are summarized in Table 2. As can be seen plasma assays are now available (or will be available soon) for intact fibrinogen; soluble fibrin; the total of degradation products derived from fibrinogen and fibrin; for

Table 2 Currently described EIA's for fibrinogen and its derivates

EIA specific for	specificity of: Capture antibody	specificity of: tagging antibody	remarks	references
intact fibrinogen	intact carboxyl-terminus of Aα-chains (G8)	amino-terminus of Aα-chain, incl. FpA (Y18)	Commercially available soon[1]. Duration of assay only 45 minutes	21,22
soluble fibrin	new amino-terminus of fibrin α-chain (anti-fbn-17)	non-specific (polyclonal)	-	23,24,25
fibrinogen degradation products (FgDP)	altered conformation in fibrin(ogen) degradation products (FDP-14)	amino-terminus of Aα-chain (incl. FpA) (Y18)	Commercially available[2]. Duration of assay only 45 minutes	26,27
X-oligomers	X-oligomers (NiBn 52)	X-oligomers (NiBn 178)	-	10,11
D-dimer	crosslinks in D-dimer (DD3B6)	panspecific anti-D monoclonal antibody	Commercially available[3].	28,29
D-dimer	mainly for D-dimer	panspecific antibody	Commercially available[4].	
fibrin degradation products (FbDP)	altered conformation in fibrin(ogen) degradation products (FDP-14)	monoclonal antibody against D of non-crosslinked fibrin and D-dimer (DD-13)	Commercially available[5]. Duration of assay only 45 minutes	30,27
total of degradation products (TDP) of fibrin (FbDP) plus those of fibrinogen (FgDP)	altered conformation in fibrin(ogen) degradation products (FDP-14)	mixture of two monoclonals (Y18 + DD-13) (see above)	Commercially available[6]. Duration of assay only 45 minutes	19

[1,2,5,6] from Organon Teknika, Turnhout, Belgium
[3] Mabco, Brisbane, Australia, and Americal Diagnostica, Greenwich, USA.
[4] Diagnostica Stago, Asnières, France; Boehringer Mannheim, Mannheim, FRG

early fibrin degradation products (X-oligomers); for late fibrin degradation products, and for fibrinogen degradation products. With this panel of EIA's it is possible to draw conclusions about the status of the haemostatic balance in much detail.[45]

Agglutination assays

Apart from the quantitative EIA's described above, also semiquantitative latex agglutination assays are available for use in plasma. These are:

* An assay for D-dimer, based on DD3B6 [28,29] monoclonal antibody (see table) and available form MabCo, Brisbane, Australia; from American Diagnostica, Greenwich, USA and from Ortho Diagnostics, Beerse, Belgium.
* A D-dimer test from Diagnostica Stago, Asnières, France; from Boehringer Mannheim, Mannheim, FRG and from Organon Teknika, Turnhout, Belgium.
* A D-dimer test reported by Soria [46], which is not commercially available.

CLINICAL STUDIES WITH THE NEW ASSAYS

The clinical experience of the new assays is rapidly growing and degradation products of fibrin and fibrinogen were measured in plasma samples of patients with several different diseases. Theoretically, clinical disorders associated with elevated fibrin(ogen) degradation products, as assessed by the new assays, can be divided in three different categories:

A) diseases in which the process of primary fibrinolysis (fibrinogenolysis) dominates, resulting in elevated levels of FgDP's;
B) disorders with a pronounced increase of FbDP levels, indicative for the occurrence of secondary fibrinolysis; and
C) clinical states characterized by an enhancement of both fibrinogenolysis and fibrinolysis.

Most diseases with increased levels of fibrin(ogen) degradation products can be placed in this last category and we prefer to discuss the different diseases separately.

Deep venous thrombosis and pulmonary embolism

The diagnosis of deep venous thrombosis (DVT) based on clinical symptoms only, is missed in 50% of the cases [47,48] Therefore it should be confirmed by objective methods and of these, ascending venography is considered as a golden standard. There is a need for non-invasive tests like impedance plethysmography, echography and also laboratory tests. A simple screening test that selects patients for further diagnostic procedures can be clinically useful. Efficacy of heparin treatment for DVT is often evaluated by repeated venography. Since this procedure is time consuming, expensive and not without risks, a method is needed that could replace this invasive technique. Using the new assays, the levels of fibrin(ogen) degradations products levels were measured in plasma to determine their role in both diagnosis and evaluation of treatment for DVT. Using a semiquantitative latex assay for detection of plasma D-dimer it was possible to make a clear distinction between normal subjects and hospitalized patients with DVT confirmed by venography.[49] In that study, all patients with confirmed DVT had plasma D-dimer levels above the minimal detectable concentration of 250 ng/ml. None of the healthy controls reached these levels. However, another study showed that plasma D-dimer levels of hospitalized patients with DVT (the authors do not mention how the diagnosis was confirmed) showed some overlap (<5%) with those of healthy volunteers.[50] In both studies, the correlation between plasma D-dimer levels assayed by the semiquantitative latex assay and the quantitative EIA was highly significant ($r=0.83, P<0.001$ [49]; $r=0.82$, $P<0.0001$ [50]) The plasma D-dimer latex assay was also applied to a population consisting of hospitalized-and outpatients suspected for DVT.[51] In this study the sensitivity and specificity of the latex assay (cut-off value for test positivity: 200 ng/ml) for DVT were 73% and 69% respectively. The test results were not separately determined in the in- and outpatient groups. The diagnosis of DVT was made by ascending venography. To be useful as a screening test for DVT, the latex D-dimer assay must allow to discriminate in the group of patients clinically suspected for DVT those who have DVT from those who don't. This can be determined by calculating the predictive value of a positive or negative test result, meaning the probability of having or not having the disease.

In that study [51] with a prevalence of DVT of 72%, the positive and negative predictive value were 63% and 78% respectively. In another study two different D-dimer latex assays were used in hospitalized -and outpatients suspected for DVT [52] The prevalence of DVT, as assessed by venography was 65%. One of the assays using a cut-off value of 500 ng/ml had a sensitivity of 76%, a specificity of 87% and the positive and negative predictive values were 80 % and 85% respectively. The other latex assay (cut-off value 200 ng/ml) had a sensitivity, specificity and a positive and negative predictive values of 60%, 97%, 92% and 80% respectively. The test results were calculated for the whole population and not for the subgroups of hospitalized patients and outpatients separately. Similar observations were reported by others.[53]

The above studies mentioned above clearly demonstrate that D-dimer levels, as determined by the latex agglutination assay, cannot be used to exclude the diagnosis DVT. Using this assay as screening test for DVT the diagnosis will be missed in 15%-22% of the patients [51,52] which is an unacceptably high percentage. Based on these grounds the conclusion is justified that the D-dimer latex assay is unsuitable as a screening test for DVT.

Using a D-dimer EIA, it was shown the level of plasma D-dimer was elevated in hospitalized patients with DVT, confirmed by venography.[54,55] No overlap was demonstrated with the D-dimer levels in normal healthy controls (cut-off value for test positivity:200 ng/ml).

In a population consisted of hospitalized patients suspected for DVT, the D-dimer EIA had a sensitivity and specificity for DVT of 100% and 23% respectively, as assessed by venography.[56] The used cut-off value for test positivity was 250 ng/ml. The prevalence for DVT was 52% and the negative predictive value was 100%. Similar observations were made in other studies using the D-dimer EIA's.[51,52] The studies mentioned above show that the diagnosis of DVT can be ruled out in hospitalized patients with clinically suspected DVT in case of normal D-dimer levels, as determined by the quantitative D-dimer EIA. As a consequence ascending venography could have been avoided in these patients.

The EIA for plasma D-dimer was also applied to an outpatient population with

clinically suspected DVT.[50] The sensitivity and specificity of the D-dimer EIA (cut-off value:200 ng/ml) for DVT were 100% and 61% respectively, as evidenced by venography. In that population with a prevalence for DVT of 35%, the predictive value of a positive test was 61% and the negative predictive value was 100%.[56] Similar results were found in other studies.[51,52] One of these studies showed that the negative predictive value declined from 100% to 93% when the cut-off value for test positivity was raised from 200 ng/ml to 500 ng/ml.[52] Another study reported a negative predictive value of 98% using a cut-off value of 200 ng/ml.[53] These studies, which were performed in outpatients, confirm the observations obtained on hospitalized patients, that normal D-dimer levels, determined by EIA, can be used to exclude the diagnosis in patients with suspected DVT. Based on these results it was suggested that the D-dimer EIA could be used as a screening test for DVT and that ascending venography can be avoided in patients with D-dimer levels below the cut-off point for test positivity.[53,54] In clinical practice this would be a great advantage. However, the predictive value of a test can dramatically be affected by the prevalence of the disease in the studied population i.e. the positive predictive value decreases with the prevalence, but the negative predictive value may rise simultaneously. It is not advisable to apply the predictive value of a test obtained in one population to another without knowing the prevalences in these populations. The utility of the EIA for D-dimer as a screening test for DVT in a certain population can be determined by studying the costs-effectiveness in relation to the prevalence of DVT in that population.

With a specific EIA for fibrin degradation products (FbDP's), increased plasma levels of FbDP's were demonstrated in patients with DVT relative to FbDP levels in plasma of healthy controls.[30,57-59] Patients with DVT had also increased levels of fibrinogen degradation products (FgDP), although the latter were less elevated than the FbDP's.[57] This suggests that secondary fibrinolysis was accompanied by fibrinogenolysis.

Applied to outpatients, clinically suspected for DVT, the EIA for FbDP (cut-off value 270 ng/ml) had a sensitivity of 92% and a specificity of 20% for the diagnosis of DVT, as determined by impedance plethysmography (IPG).[57] A second IPG examination was performed after one week when the first IPG examination was negative. In the same

study, the sensitivities of the EIA for FgDP (cut-off value: 250 ng/ml) and the EIA for TDP (cut-off value: 590 ng/ml) were 90% and 95% respectively. The specificities were 25% and 16% respectively. In this population with a prevalence of DVT of 25%, the negative predictive values of the EIA's for TDP, FbDP and FgDP were 90%, 87% and 88% respectively. The test characteristics of the EIA's were better, if compared to the results of the first IPG examination only, e.g. the sensitivity and the negative predictive value of the EIA for TDP were 98% and 97% respectively. The utility of these EIA's as screening test for DVT can be determined in the same way as described above.
Until now, no studies have been described that investigated the test characteristics of the EIA's for TDP, FbDP and FgDP in hospitalized patients suspected for DVT.
All patients with pulmonary embolism (PE), confirmed by lung perfusion scanning had increased plasma levels of D-dimer when compared to the levels of D-dimer in normal controls, as detected by EIA.[55] This finding was confirmed in another study where all patients with PE, as assessed by pulmonary angiography, had elevated levels of plasma D-dimer (above the normal value in healthy volunteers of 130 ± 65 ng/ml) in contrast to the normal level of D-dimer that was observed in one patient in whom PE could be excluded.[60] The same results were found using an EIA, specific for X-oligomers (cross-linked fibrin degradation products with a high molecular weight).[61]
With regard to daily clinical practice it is important to know whether the level of plasma D-dimer can predict the presence or absence of PE in a population of patients suspected for PE. By examination of patients suspected for PE using an EIA for D-dimer, it was demonstrated that a high suspicion of PE on the basis of a lung scan corresponded to D-dimer levels above 500 ng/ml in all patients, but in case the scan was inconclusive, a D-dimer value below 500 ng/ml could not exclude PE.[62]
Plasma of patients suspected for PE was also assayed with an EIA that recognizes cross-linked D-dimer fragments and related high molecular weight fibrin derivatives containing D-dimer (XDP's).[63] Using a cut-off value for test positivity of 145 ng/ml, the sensitivity and specificity of that EIA for the diagnosis of PE were 89% and 44% respectively, as evidenced by lung perfusion scanning. In that population with a DVT prevalence of 38%, the predictive value of a negative test was 92%. This study

demonstrates that the diagnosis PE can not be excluded in patients clinically suspected for PE with normal levels of XDP, as assessed by this XDP EIA. Since the diagnosis of this potentially life treathening disease will be underestimated in 8% of the cases [63] it can be concluded that the EIA for XDP assessment alone, can not be used as a screening test for PE in clinically suspected patients.

Similar observations were found with the semiquantitative latex agglutination assays for D-dimer. An increased plasma D-dimer level was demonstrated in 98% of patients with PE confirmed by lungscan but in none of the healthy controls (cut-off level for test positivity: 250 ng/ml).[49] The observed overlap between D-dimer levels in patients with PE and normal controls was confirmed by others using a cut-off level for test positivity of 320 ng/ml.[50] Another study showed that 54% of the patients with clinically suspected PE, who had a high probability on lungscan, had a negative latex agglutination assay (cut-off value: 500 ng/ml).[64] This supports the finding obtained by application of the D-dimer EIA's that it not possible to exclude the diagnosis PE in patients with clinically suspected PE on the basis of a normal D-dimer level alone. In these patients, further diagnostic procedures are indicated.

The possibility to use plasma levels of FbDP or D-dimer, as markers of secondary fibrinolysis to monitor thrombolytic and heparin therapy in patients with venous thromboembolism has been investigated using EIA's.[58,59,65] It was demonstrated that a change in plasma FbDP level was reflected in a change of thrombus size during heparin treatment. This was assessed by repeated venography based on Marder scores. A fall in FbDP level was accompanied by thrombus regression, but when the FbDP level remained constant during treatment, no reduction of thrombus size could be observed.[58] In that study a good correlation (r=0.79) was found between high initial plasma FbDP's and thrombus size. These results suggest that FbDP detection by EIA is useful in the monitoring of heparin treatment. More data are needed to confirm these findings.

Thrombolytic therapy with streptokinase (SK) or urokinase (UK) was followed by an increase of FbDP's during the first 12 hours of treatment.[59,65] Following SK or UK therapy it was demonstrated that low initial plasma FbDP level predicted a poor decrease in thrombus size, but the changes of FbDP levels were not correlated (r=0.21)

to the changes in thrombus size, as assessed by repeated venography.[59] This was confirmed by others.[65] However, an increase of FbDP levels is no evidence for lysis of the thrombus, since elevated levels of FbDP were also found in normal healthy volunteers following t-PA infusion.[66]

Liver disease and liver transplantation

Patients with chronic liver disease often develop localized bleeding episodes (ruptured esophageal varix, haemorrhagic gastritis [67]) that tend to massive haemorrhage and are an important cause of death in these patients.[68,69] The disturbance of blood coagulation is complex in patients with liver cirrhosis. It has been hypothesized that liver cirrhosis is frequently complicated by disseminated intravascular coagulation (DIC) and that consumption coagulopathy due to DIC plays a role in the pathogenesis of haemorrhage in patients with liver cirrhosis.[70-75] However, this view is not accepted by all.[72,76,77]

A number of studies reported that fibrinolysis is enhanced in patients with liver disease.[78-83] This was evidenced by the observation of increased plasma t-PA antigen levels presumably due to a diminished hepatic clearance of t-PA [81,84] and decreased levels of α_2-antiplasmin probably due to a decrease in synthesis capacity of the affected liver.[79,80]

The new plasma assays have been applied to patients with liver cirrhosis and patients undergoing liver transplantation. Using an EIA for intact fibrinogen (HMW + LMW) in patients with liver cirrhosis, classified according to the Child-Pugh classification, it was shown that the level of intact fibrinogen decreased with increasing severity of the disease, and was lowest in Child C.[85] The FgDP levels, as detected by EIA, were only slightly elevated compared to the level in normal controls and did not correlate to the severity of the disease.

This either suggests that extensive fibrinogenolysis is not a common feature in patients with liver cirrhosis and the decreased levels of intact fibrinogen can be explained by a lowered synthetic capacity of the diseased liver or that fibrinogen beyond the stage of LMW' formation does not occur (see above). Further it was shown in that study that both the FbDP and TDP levels were elevated and showed a positive correlation with

the severity of the disease. This indicates that the process of secondary fibrinolysis is more active in patients with an advanced stage of liver cirrhosis.

The observation of increased FbDP levels, as detected by EIA, in patients with liver cirrhosis was confirmed in another study.[86] These findings may be indicative of the presence of a hypercoagulable state, although a failure of the clearance of fibrin degradation products by the damaged liver can not be excluded. Further studies are needed to answer this clearance question and to determine whether increased FbDP levels are correlated with organ failure due to fibrin deposition.

Using a semiquantitative D-dimer latex assay in patients with liver cirrhosis classified according to Child and Pugh it was shown that the level of D-dimer was elevated ($p < 0.001$) in patients with Child C classification as compared to the D-dimer levels in patients with Child A and B.[87] In that study, no significant difference was observed between the D-dimer level in patients with Child A and B classification. Elevated plasma levels of D-dimer, detected by latex assay, were also demonstrated in patients with liver disease (the authors did not specify the disease) and increased when DIC was superimposed.[88] These latter studies, using semiquantitative assays, support the previous conclusion based on EIA's that secondary fibrinolysis is more pronounced in patients with advanced liver cirrhosis.

Orthotopic liver transplantation (OLT) is accompanied by serious bleeding complications [89,90] presumably caused by a hyperfibrinolytic state [91,92] or secondary to diffuse intravascular coagulation (DIC).[93-95] Measurement of plasma levels of fibrin(ogen) degradation products by EIA's, showed that the levels of FbDP's and FgDP's increased significantly relative to pre-operative values, at the end of the anhepatic phase during OLT. The rise of FgDP levels was, however, less than the rise of FbDP levels.[96] In that study, FgDP levels reached a maximum shortly after recirculation of the graft and just before the FbDP peak. Similar findings were reported by others.[86,97] Using a D-dimer EIA, it was demonstrated that plasma D-dimer level also rose in the anhepatic phase and following hepatic revascularization during OLT.[98-100]

In conclusion, the rise in FgDP level clearly demonstrates the occurrence of fibrinogenolysis during OLT, which may considerably contribute to the disturbance of

the haemostatic function. It can be concluded that OLT is accompanied by both hyperfibrinolysis and an activated state of coagulation. The observed peaks of the FbDP and FgDP levels in the early post anhepatic phase correspond to the clinical picture of increased bleeding tendency during that period. Whether the increase in the levels of fibrin(ogen) degradation products are indicative for the intra-operative blood loss and can be used to identify patients with active fibrinolysis with an increased risk of life treathening haemorrhage, remains to be determined.

During auxiliary partial liver transplantation (APLT) EIA's were also used to measure fibrin(ogen) degradation products. It was shown that FgDP, as detected by an EIA, was elevated during the operation (maximum level: 1.0 ug/ml) but was no longer detectable during the first day after the operation, whereas TDP and FbDP showed no abrupt changes during the operation. FbDP, however, remained detectable until 25 days after transplantation.[101] These data are based on measurements in one patient and more studies are needed to confirm these findings.

Sickle cell disease

The primary biochemical lesion in sickle cell disease is an alteration of the structure in the ß chain of the haemoglobin resulting in the so called haemoglobin S. Red cells containing haemoglobin S at a high concentration become eventually irreversibly sickled, and this has two main effects. Firstly it shortens the survival of the erythrocytes and secondly the viscosity of the blood increases because of the formation of aggregates of the sickled cells which may result in stasis and hence occlusion of the microvasculature, which is experienced as painful by many patients.

It is not clear whether activation of the coagulation system contributes to the vaso-occlusive crisis in patients with sickle cell disease. Several observations suggest that enhancement of coagulation with formation of fibrin may play a role.[9,102] Fibrin has been detected in vessels occluded by sickle cells.[103] During steady state, the fibrin(ogen) fragment E level was elevated.[9] Fibrinogen survival is shorter than normal in the steady state of the disease, and is further shortened during painful crisis.[104] Many coagulation laboratory tests show abnormalities during painful crisis [105,106] but no test is available

that clearly detects the occurrence of vaso-occlusive crisis. Fibrin degradation products were measured in plasma of patients with sickle cell disease during steady state and painful crisis as markers of fibrinolytic activity.

Using a semiquantitative D-dimer latex assay with a minimum detectable concentration 1 ug/ml, it was demonstrated, that 10% of the patients with sickle cell disease who were asymptomatic, had elevated D-dimer levels as compared to normal healthy controls.[107] The same study also showed that 97% of the hospitalized symptomatic patients during painful crisis had elevated D-dimer levels, but D-dimer was not detectable in plasma samples of hospitalized patients that had become asymptomatic following a painful crisis. With respect to the clinical status of the patients (assessed by history and physical examination) a significant correlation ($p < 0.001$) between disease activity and the presence of D-dimer plasma level was noticed. D-dimer was detectable in only 10% of the steady state (asymptomatic) patients, indicating that the fibrinolytic activity is more pronounced during painful crisis.

Using a D-dimer EIA, the observation of elevated D-dimer levels during painful crisis was confirmed in another study.[108] In that study, significant differences ($p<0.001$) in D-Dimer levels were noticed between healthy controls, asymptomatic patients with sickle cell disease during steady state and symptomatic patients during painful crisis. In 93% of the cases, D-dimer plasma levels in the asymptomatic patients during steady state were above the upper limit of the level of healthy individuals (129 ng/ml). This is higher than the 10% in the previously mentioned study [107] and can be explained by the fact that the EIA is more sensitive for the detection of low grade activation of coagulation and reactive fibrinolysis than the latex agglutination assay.

Plasma of patients with sickle cell disease has not yet been assayed by EIA's for TDP, FbDP and FgDP.

Based on the aforementioned data, it can be concluded that application of the new assays has demonstrated that fibrin formation and subsequent lysis is not an exclusive feature of the vaso-occlusive crisis, but is also present during steady state. Whether measurements of plasma levels of fibrin(ogen) degradation products are useful to detect the occurrence of vaso-occlusive crises or other complications in patients with sickle

cell disease may be assessed by studying the predictive value of the assays in the clinically relevant population.

Pregnancy and (pre)eclampsia

During preeclampsia, a decrease in platelet count, anti-thrombin III (AT-III), plasminogen and α_2-antiplasmin is observed.[109-111] However, none of these laboratory data reflects coagulation abnormalities accompanying preeclampsia satisfactorily.[112,113] Therefore these tests are not useful to monitor the occurrence of preeclampsia in high risk groups. It has been suggested that measurements of levels of fibrin(ogen) degradation products may be of value to diagnose (pre)eclampsia since it was observed that toxemia of pregnancy may be developing when these levels increase.[114-116] However, the assays were performed in serum.

Plasma of women with normal, uncomplicated pregnancy and of women with pregnancy complicated by preeclampsia was assayed for D-dimer and fibrin(ogen) degradation products, using semiquantitative latex agglutination tests.[117-119] Plasma samples of women with normal pregnancy were negative for fibrin(ogen) degradation products in 98%-100% of the cases [117,118] and only 5% of the women showed detectable levels of plasma D-dimer (cut-off value: 200 ng/ml).[118] In that study the increase of D-dimer showed a positive correlation (r=0.64) with the fibrin(ogen) degradation products levels.

During the preeclamptic state, elevated plasma levels of fibrin(ogen) degradation products have been observed.[117,118] In 38,7% of the preeclamptic women elevated Plasma D-dimer levels were observed, in contrast to the healthy pregnant women in whom no elevations were observed (detection limit 2 µg/ml).[117] The D-dimer positive women had a higher blood pressure (systolic and diastolic), more proteinuria and abnormal liver function tests, a higher serum creatin and blood urea nitrogen than preeclamptic women with normal D-dimer levels. In addition the D-dimer positive women showed a higher risk for prematurity and low birth weight. It was concluded that the semiquantitative latex agglutination assay for D-dimer may be a useful test to screen preeclamptic women in order to define a subgroup with a high risk for

coagulation abnormalities.

Also quantitative EIA's were used to study plasma levels of fibrin(ogen) degradation products in women with normal and preeclamptic pregnancy.[61,119-122] In women with normal pregnancy the plasma levels of TDP, FbDP, FgDP and D-Dimer were elevated as compared with a control group of healthy non-pregnant women, and especially increased during the last trimester of pregnancy.[119-123]

This is in contrast with the findings obtained with the Thrombo-Wellco serum test that detects FDP. At no time during normal pregnancy significant increases of the FDP levels could be demonstrated.[120] This may be explained by the fact that the serum assay is less sensitive than the EIA, or be an artefact due to serum preparation (see above).

Using an EIA for detection of cross-linked fibrin degradation products (X-oligomers), it was found that all women with clinically diagnosed preeclampsia had elevated plasma levels of X-oligomers, and showed no overlap with those of women with uncomplicated pregnancy.[61]

Despite a number of studies that reports a depressed fibrinolytic activity during pregnancy [124-126], the studies mentioned above using EIA's, demonstrate an increase of plasma levels of fibrin degradation products during normal and complicated pregnancy. This suggests that the fibrinolytic activity is not necessarily impaired during pregnancy. Detection of plasma fibrin(ogen) degradation products by EIA's are a useful aid in the early diagnosis of preeclampsia and may help to formulate therapeutic action to prevent a full blown expression of this condition.

Renal diseases

The role of hemostatic disorders in the pathogenesis of renal vascular and glomerular diseases is not fully understood.

Glomerular fibrin deposition is observed in many cases of glomerulonephritis and it is assumed to play a role in glomerular degeneration.[127,128] The presence of fibrin and fibrinogen (degradation products), was demonstrated immunochemically within the mesangium, in vascular cells and along the basement membrane in acute homograft rejection, postpartum renal failure and rapidly progressive glomerular nephritis.[129-131]

It has been shown that FDP, as detected by serum assays, are elevated in several renal diseases.[132]

Since extra-renal sites of fibrin(ogen)olysis may also be responsible for FDP elevation, it was suggested and confirmed that FDP levels measured in the urine would reflect disease activity better than FDP levels detected in serum.[133-136]

An EIA was used to detect urinary fibrin(ogen) degradation products of patients with various types of primary and secondary chronic nephrititis.[137] In that study, urinary Fb(g)DP levels were significantly higher ($p<0.05$) in patients with severe types of proliferative, membranous glomerulonephritis and in the active phase of SLE as compared to the urinary levels of normal volunteers. No correlation (the correlation coefficient was not given) was found between the levels of urinary Fb(g)DP and that of proteinuria.

Urine of patients with glomerulonephritis characterized by active cellular crescents was examined using an EIA or a radioimmunoassay (RIA) specific for cross-linked fibrin degradation products (XLFDP's).[138,139] The urinary level of XLFDPs correlated weakly ($r=0.52$, $p<0.01$) with the percentage of glomeruli containing cellular crescents. No significant correlation ($r=0.61$,p=ns) was found between the levels of urinary XLFDP's and proteinuria.[138] This may suggest lysis of fibrin(oid) material within the crescents or Bowman's space that was demonstrated by percutaneous renal biopsy in a majority of these patients.[138]

In patients with focal segmental sclerosis (FSS) or membranous nephropathy (MN), the urinary levels of XLFDPs correlated ($r=0.73$ and 0.70 for FSS and MN respectively) with the level of proteinuria.[139] The ratio urinary XLFDP/proteinuria was significantly higher ($p < 0.05$) in patients with glomerulonephritis (GN) with crescents than in patients with FFS and MN. The ratio urinary XLFDP/proteinuria was used to exclude that portion of urinary XLFDP that was filtered from the blood, but whether the detected high ratio in patients with crescents GN reflects intraglomerular fibrinolysis remains to be determined.

In children with several kinds of proliferative glomerulonephritis, the demonstrated increased urinary levels of XLFDPs and D-dimer, as assessed by EIA's, showed no

correlation with the histologically detected mesangial proliferation or with the selectivity of proteinuria.[140] This may suggest that lysis of intraglomerular fibrin is not the only source of urinary cross-linked degradation products.

Recently it has been shown that D-dimer levels in plasma, as detected by EIA, were significantly elevated in non-diabetic chronic renal failure ($p < 0.05$), diabetic nephropathy ($p < 0.01$) and acute renal failure ($p < 0.01$) when compared to the D-dimer plasma levels of healthy controls (< 220 ng/ml).[141] In that study, no correlation ($r = -0.14$) was found between plasma D-dimer and creatinin clearance. This suggests that detection of D-dimer in plasma by EIA can be used a marker of fibrin breakdown in several renal diseases and reflects disease activity. More studies are needed to confirm these findings. We are not aware of studies that used semiquantitative D-dimer assays or EIA's for TDP, FbDP or FgDP assessment in renal disease.

It can be concluded that EIA's specific for the detection of urinary cross-linked fibrin degradation products can be used to assess activity of several renal diseases. The EIA's are not suitable to assess renal function itself, since none of these assays showed significant correlations between fibrin degradation products and creatinin clearance. The origin of the observed increased levels of urinary cross-linked fibrin degradation products in patients with several renal diseases is not fully clear: it may result from filtration from the blood (because of an increased permeability of the damaged basement membrane), or from lysis of fibrin in the lower urinary tract or the kidney. In the future, the new assays performed in plasma may be applied to patients with homograft transplants, since in the past it has been shown that a rise in serum FDP is associated with impending rejection of the graft.[142]

Disseminated intravascular coagulation (DIC)

DIC may lead to a dramatic and generalized haemostatic failure with a wide spectrum of clinical symptoms and is often associated with a variety of other pathological conditions.[12,143-148] In case of a dominant intravascular clotting process and minimal secondary fibrin(ogeno)lysis, DIC may present primarily as diffuse thrombosis. When the process of secondary fibrinolysis dominates, the clinical manifestation shifts towards

haemorrhage. Patients suspected of having DIC often demonstrate both clinical conditions simultaneously, and a laboratory test capable of discriminating between these two situations is needed since both clinical conditions require a different therapeutic approach.

However, in patients with a clinical condition indicative of DIC, the currently used haemostasis parameters like prothrombine time, thrombin time, activated partial thromboplastin time, factor V, fibrinogen, platelet count and antithrombin III become difficult to interpret and none of these tests is specific for the diagnosis or the monitoring of therapy in case of DIC.[149-152]

Plasma of patients suspected of having DIC was examined by using latex assay's for Fibrin(ogen) degradation products.[49,50,87,147,153-155]

It was shown that all patients with DIC had elevated levels of plasma D-dimer as compared to D-dimer levels in normal healthy controls.[87] The minimal concentration detectable of the used latex assay was 200 ng/ml. In that study, the diagnosis of DIC was based on a prolonged thrombin time, hypofibrinogenaemia, thrombocytopenia and elevated serum FDP's (>8 μg/ml) assessed by the Thrombo-Wellcotest in combination with clinical conditions known to be associated with DIC. This finding was confirmed in another study using a latex assay with a minimal detectable concentration of 250 ng/ml.[49]

The latex assay was also used to measure plasma D-dimer levels in patients clinically suspected of having DIC.[154] In that study, the presence of fragment D-dimer, as demonstrated by immunoblotting, was used as a criterion for the diagnosis of DIC and all patients who had fragment D-dimer present showed clinical conditions consistent with the diagnosis of DIC. The sensitivity and specificity of the D-dimer latex assay for the diagnosis of DIC, as determined by immunoblotting, was 85% and 97% respectively. The prevalence was 51% and the positive and negative predictive value was 97% and 86% respectively. Although varying of the cut-off point may change the test results, the aforementioned study demonstrates, that the negative D-dimer latex assay alone can not be used to exclude the diagnosis of DIC in patients suspected of having DIC. More studies are needed to confirm the test characteristics of the D-dimer

latex assay obtained in this study. D-dimer measurements by latex assay may be valuable in addition to the currently used laboratory tests in patients suspected of having DIC.

Plasma of patients with DIC was also assayed by quantitative EIA's to demonstrate elevated levels of fibrin(ogen) degradations products.[26,30,50,55,61,122,156-160] using an EIA specific for cross-linked fibrin degradation products (XLFDP's) it was shown that all patients with DIC, confirmed by laboratory evidence of consumption coagulopathy and clinical conditions characteristically associated with DIC, had levels of XLFDP's above the upper limit of 200 ng/ml that was observed in healthy controls.[55] However, others detected some overlap in XLFDP levels between patients with DIC and normal healthy controls.[50] Similar observations were reported using an EIA specific for X-oligomers (the earliest fragment released from cross-linked fibrin): patients with DIC had elevated levels of X-oligomers and showed no overlap with the levels of X-oligomers in normal healthy controls.[61,160] In these studies, the clinical diagnosis of DIC was confirmed by a low fibrinogen, elevated FDP in serum by latex assay, low platelet count ($<10^5$/ml) and a positive ethanol gelation test.

Using the D-dimer assay it was shown that all patients with DIC had elevated levels of D-dimer (>200 ng/ml) and no overlap was shown with those in normal healthy controls.[87] However, 10% of the hospitalized control group had elevated D-dimer levels.[87] Plasma of patients was examined one week before the onset of DIC (scored according to Maegawa's criteria of DIC) by D-dimer EIA.[159] Of the used laboratory parameters (serum FDP, D-dimer, prothrombin time, fibrinogen and platelet count), plasma D-dimer was the only parameter that was significantly elevated (no p value is given) before the onset of DIC, while the other coagulation parameters became significantly (p value unknown) abnormal only after the onset of DIC. This suggests the possibility to identify an early state of DIC by D-dimer measurements in which treatment may be started, and requires further confirmation.

Plasmas of patients with DIC were also assayed by EIA's specific for FbDP's, FgDP's or TDP's.[19,26,30,122,156-159]

Patients with a high grade DIC had increased levels of FbDP's, TDP's and FgDP's as

compared to the levels in healthy controls.[122] In these patients, the observed level of FbDP was higher than the level of FgDP, suggesting that the process secondary fibrinolysis is dominant and is accompanied by fibrinogenolysis.

Monitoring time dependant change of FbDP and TDP levels in plasma of patients with DIC showed the possibility to detect the onset of primary fibrinolysis.[30] In that study, the FbDP and TDP levels were approximately equal during the first four days, suggesting that nearly all degradation products are derived from fibrin. During the next four days the levels of TDP exceeded the Fbdp levels indicating the occurrence of fibrinogenolysis. The justification of this conclusion may follow from another study where a good correlation (r=0.98) was observed between the values obtained by adding the levels of FbDP and FgDP, and the actually measured TDP levels in patients with DIC.[156]

The possibility to use TDP, FbDP and FgDP levels, as assessed by EIA's, for the monitoring of anticoagulant therapy (heparin and coumarin) and follow-up in patients with chronic DIC was demonstrated in another study.[158] In that study, the diagnosis of DIC was based on the clinical picture and abnormalities in the following laboratory parameters: platelet count, (pro)thrombin time, factor V, fibrinogen (Clauss), FM, antithrombin-III, α_2-antiplasmin and FDP in serum (Thrombo-Wellcotest). The levels of FbDP and FgDP changed concomitantly, FbDP being higher than FgDP. This indicates that both fibrinolysis and fibrinogenolysis occur. It was concluded that, of all used laboratory parameters, soluble fibrin, TDP, FbDP and FgDP were the most sensitive markers for active DIC and the effect of anticoagulant treatment. The generally accepted view that an increased level of serum FDP's is required to permit the diagnosis of DIC [12,36,146] has to be revised.

Application of the EIA's showed that the secondary fibrinolysis in DIC is accompanied by fibrinogenolysis. More studies are needed to determine the role of fibrin(ogen) measurements by EIA's for the diagnosis of DIC. The EIA's for fibrin(ogen) degradation products have not been applied to a population suspected of having DIC to determine the sensitivity and specificity of these assays for the diagnosis of DIC. Further studies are needed to determine these test characteristics and the predictive

values of the assays.

The recognition of the activity of each the two opposing processes coagulation and fibrinolysis is important since it may have implications for a subsequent therapeutical approach. Changes of plasma levels of fibrin(ogen) degradation products and soluble fibrin, as detected by EIA's, were longitudinally measured in a patient with acute promyelocytic leukemia, a condition known to be associated with DIC.[45] The levels of several fibrin degradation products, such as of D-dimer, FbDP's and X-oligomers followed the same pattern in time. Surprisingly, an increase of these levels coincided with decreased levels of soluble fibrin and vice versa. This suggest that valuable information on the status of the haemostatic balance can be obtained by using one of the EIA's for FbDP, D-dimer or X-oligomers (as markers of fibrinolysis) in tandem with the soluble fibrin EIA (as a marker of coagulation). This should be confirmed in other studies.

Coronary artery disease (CAD) and thrombolytic therapy

It is well known that thrombotic occlusion of a coronary artery is frequently associated with acute myocardial infarction (AMI).[161,162] Early recanalization of these arteries in selected patients with AMI by fibrinolytic agents reduces short and long term mortality and improves left ventricular function.[163-165] The clinical use of thrombolytic agents is rapidly growing and a number of treatment strategies has been used.[166] The success of this therapy is probably based on the induction of an extensive fibrinolytic state. The recognition of an increased fibrinogenolytic state is important since it is associated with severe bleeding complications.[167] But unfortunately, this cannot be detected by the standard haemostatic screening tests like the PT, the APTT or the bleeding time.

The quantitative plasma assays for fibrin(ogen) degradation products were applied to patients with AMI in order to assess the intensity of fibrin(ogen)olysis induced by thrombolytic therapy and to determine the role of fibrin(ogen) degradation products as markers of this therapy. Increased levels of cross-linked fibrin degradation products (XLFDP's), detected by EIA, have been detected in patients with AMI.[168] Elevations

of plasma levels of XLFDP's above 1000 ng/ml occurred within one hour in all patients receiving tissue-type plasminogen activator (t-PA) and in 83% of the patients treated with streptokinase (SK).[169] Seven hours after treatment, the XLFDP levels were significantly elevated ($p<0.01$) in the t-PA treated group when compared to the (declining) values that were observed in the SK treated group. This indicates that the persistence of lysis of fibrin, as assessed by the EIA for XLFDP's, is of longer duration by administration of t-PA than following SK. The response of XLFDP to SK was similar in the groups of patients with and without reperfusion, although in late samples (> 7 hours) the elevations of XLFDP tended to be greater in the reperfusion group. The difference, however, was statistically not significant.

Another study showed that plasma XLFDP levels increased 6 hours after t-PA infusion as compared to pre-treatment values and no significant difference was observed between the groups that received a dosages of 150 mg and 100 mg respectively. This could indicate that the extents of lysis of fibrin are comparable in both groups.[170] In the same study, the extent of fibrinogenolysis was assessed by using an EIA for Bß1-42, a peptide that is released when fibrinogen is degraded to fragment X.[171] In contrast to the levels of XLFDP's, the levels of plasma Bß1-42 levels were significantly elevated ($p<0.01$) in the group receiving 150 mg t-PA as compared to values in the group given 100 mg. This indicates a more intense fibrinogenolysis following administration of the higher dose. It might be concluded that increasing the dose of t-PA from 100 mg to 150 mg results in an enhanced fibrinogenolysis, but has no influence on the extent of secondary fibrinolysis. This finding was confirmed in another study [66] following rt-PA infusion in normal healthy volunteers, and may be used to define dose regimen since it was demonstrated that a state of strong fibrinogenolysis is associated with bleeding complications.[167] The study [66] demonstrates, that an increase of fibrin degradation products, as assessed by the EIA for FbDP, after administration of rt-PA is no evidence for lysis of the thrombus, but merely for lysis of a body-pool of fibrin.

Systemic effects of thrombolytic therapy were also assessed by measuring plasma D-dimer levels by EIA. D-dimer levels were in the upper limit of the normal range (0.4 ug/ml) at the time of diagnosis for AMI before treatment and increased significantly

($p<0.001$) to 2.7ug/ml during rt-PA infusion.[172] However, elevated of plasma D-dimer (of 980 ng/ml) were also observed in normal volunteers after t-PA infusion [173], suggesting that another fibrin pool is lysed in addition to the coronary thrombus (e.g. circulating soluble cross-linked fibrin polymers). As a consequence, an increase of D-dimer levels following t-PA administration is no evidence for lysis of the thrombus. This view is supported by the observation that there is a discrepancy between thrombolysis (i.e. reperfusion) and the elevation of plasma D-dimer levels.[174]

XLFDP levels were measured by EIA in patients with AMI presenting with and without complications.[175] The complications were ventricular tachycardia or fibrillation requiring electrical countershock, mural thrombus detected by echocardiography, severe congestive heart failure defined as Class IV by MIRU criteria or death within 72 hours. The used cut-off value for test positivity was 300 ng/ml. The sensitivity and specificity of the EIA for the detection of complications in patients presenting early after onset of symptoms (<8 hours) were 80% and 81% respectively. The prevalence of the complications was 17% and the positive and negative predictive values were 47% and 95% respectively. In patients presenting late after (>8 hours) onset of symptoms the sensitivity, specificity, positive and negative predictive values were 63%, 67%, 56% and 73% respectively, at a prevalence of 40%. It can be concluded that normal levels of XLFDP alone in patients with AMI can not exclude the presence of complications. No correlation was observed between the size of the infarction and the levels of XLFDP. The normal levels of XLFDP found in patients with uncomplicated AMI's after early onset of symptoms, may be indicative for impaired fibrinolysis, as has been suggested before.[176-178] It was demonstrated that the plasma levels of XLFDP's in man 20 days (mean) after discharge from hospital following a myocardial infarction (MI) were higher ($p<0.001$) than the XLFDP levels in the age matched controls selected from a general practice list, suggesting an increased thrombogenesis, fibrinolysis or both in persons who suffered from MI.[179]

Using an EIA for FbDP, it was shown that patients with unstable angina had elevated plasma levels of FbDP ($p<0.001$) when compared to normal controls or patients with acute AMI.[180] The FbDP levels in patients with stable angina was only slightly elevated

when compared to control levels ($0.01<p<0,02$), but no difference was observed with the levels in patients with uncomplicated AMI ($p<0.001$). The level of FbDP's remained high in patients with recurrent episodes of chest pain, but declined in patients who had only one episode of chest pain. It was suggested that significantly increased FbDP levels in patients with unstable angina may be indicative for an ongoing thrombolytic process, while the absence of elevated FbDP levels in patients with AMI may suggest that clot dissolution is not taking place. However, this could not be confirmed in another study by measuring plasma D-dimer levels by EIA.[181]

Both patients with AMI and patients with unstable angina had moderate elevations of plasma D-dimer as compared to patients with stable angina.[181] The sum of the FbDP and FgDP levels, as measured by EIA, correlated ($r=0.98$) with the level of TDP in patients with AMI following rt-PA treatment.[156] Normal values for FgDP and TDP in plasma are below 0.25 ug/ml.[19,26,182] A rise of FgDP and TDP was observed in patients with AMI two hours after infusion of SK.[19,26,182] The amount of FgDP was slightly less than the amount of TDP, suggesting that nearly all circulating degradation products were derived from fibrinogen.[26,182] The clearance of FgDP paralleled that of TDP and a half-life time in the circulation of 5 hours was found.[182]

T-PA infusion in patients with AMI resulted in elevated levels of FbDP, as detected by EIA (mean: 2,7 ug/ml).[183] As mentioned, increased levels of FbDP were also found in normal healthy volunteers following rt-PA infusion (mean:0.8 ug/ml).[66] In that study, no significant differences in FbDP levels were found between volunteers who received a rt-PA dose of 0.25mg/kg or 50mg/kg, suggesting that the extent of lysis is comparable in the two groups. It was concluded that an increase of FbDP's is not a specific marker of intracoronary lysis, but also reflects systemic lysis of fibrin. This view is supported by another study.[184]

In summary, application of the EIA for XLFDP's demonstrated that the lysis of fibrin induced by administration of t-PA persists longer than by SK. The occurrence of fibrinogenolysis, as assessed by an EIA for FgDP, has been demonstrated during rt-PA and SK therapy and possibly contributes to an increased bleeding risk.[167] Detection of cross-linked degradation products by EIA can not be used to predict recanalization in

patients with AMI and the levels of XLFDP do not correlate to the size of an infarction. Elevated plasma levels of fibrin degradation products are not specific markers for the extent of fibrinolysis induced by thrombolytic therapy. The increase of FbDP levels during thrombolytic therapy is probably caused by lysis of an other body pool of fibrin outside the thrombus. Further studies are needed to determine the origin of the fibrin pool that is lysed in addition to the coronary thrombus.

Malignancies

It has been shown that the coagulation system is activated in case of leukemia or solid malignant tumors.[147,185-189]

Tumor associated fibrinolysis is supposed to play a role in the local invasion or metastatic dissemination of certain malignancies. Some studies demonstrated increased levels of plasminogen activators in the blood of patients with carcinomas which may contribute to an enhanced fibrinolysis.[185,190-192] However, others reported a reduced fibrinolytic activity in cancer patients.[193,194]

The new assays were applied to measure the level of fibrin(ogen) degradation products in patients with various malignant tumors.

With EIA's for FgDP and D-dimer it was shown that patients with malignancies had significantly higher plasma levels of FgDP ($p<0.01$) and D-dimer ($p < 0.05$) as compared to the levels in age-sex matched healthy controls.[185] This suggests that fibrinolysis is accompanied by fibrinogenolysis in patients with malignancies.

In tissue specimen of patients with ovarian cancer (Figo stage III and IV) or breast cancer (T_1-T_4/N_0-N_2) elevated levels of D-dimer, as assessed by EIA, were found relative to D-dimer levels in tissue specimen of benign controls.[195] It was shown that the D-dimer levels in ovarium cancer tissue were higher than in tissue of patients with breast cancer. This was confirmed in another study.[196]

Using an EIA for D-Dimer measurements in plasma it was shown that patients with ovarian cancer or cervical carcinoma had significantly ($p < 0.01$) elevated levels of plasma D-dimer when compared to D-dimer levels in normal healthy controls.[197]

These findings suggest that fibrinolysis is a common feature in patients with

malignancies. With an EIA for the assessment of TDP's, it was shown that 74% of patients with ovarium cancer treated by chemotherapy had increased levels of TDP's as compared to normal values (<110 ng/ml).[19] In a majority of these patients (no percentage is mentioned) a decrease of the tumor mass (serially assessed by laparoscopy, CT scanning or second look operations) was accompanied by a decline in plasma TDP levels. Since it is possible to detect low levels of TDP (i.e. patients in partial remission) with this EIA, it may be particularly valuable in combination with the tumor marker CA 125. The latter has been shown to lack sensitivity in detecting small tumor masses, which are observed by second look operations.[198] More studies are needed to confirm these findings.

By EIA's, elevated plasma levels of fibrin(ogen) degradation products were also found in patients with carcinoma of the liver (FbDP,FgDP and TDP [122]), lung cancer (D-Dimer [200]) and acute promyelocytic or myeloid leukemia (D-Dimer [87,199]; XDP [157]; FbDP, FgDP, TDP and X-oligomer [199]).

The studies mentioned above, using the new plasma assays, demonstrate that the levels of fibrin(ogen) degradation products are elevated in patients with malignancies, which may indicate that the fibrinolytic system remains active in these patients.

Conclusions

In this paper we discussed the possible clinical utility of assays for determination of degradation products of fibrin and fibrinogen. We pointed out that assays based on polyclonal antibodies and performed in serum are unreliable and therefore their clinical use is limited. Until recently it was not possible to determine the extent of primary activation of the fibrinolytic system specifically. The new assays are performed on plasma and make it possible to quantify the extent of fibrin(ogen)olysis reliably. Furthermore they allow discrimination between the degradation products of fibrin and of fibrinogen, since a specific assay has been developed for separate and specific determination of fibrinogen degradation products.

Fibrinolysis appears to be associated with (low grades of) fibrinogenolysis. The process of secondary fibrinolysis, as derived from FbDP and D-dimer levels, appears to

predominate in deep venous thrombosis, pulmonary embolism, sickle cell disease, myocardial infarction and unstable angina. It was shown that thrombolytic therapy results in a pronounced fibrinogenolysis (primary fibrinolysis), as assessed by the levels of FgDP. The occurrence of an extensive fibrinogenolysis was also observed during orthotopic liver transplantation. Several diseases, such as DIC, malignancies, liver disease and renal diseases, exhibit both fibrinogenolysis and fibrinolysis secondary to an activated state of coagulation.

The EIA for plasma D-dimer can be used to exclude the diagnosis in patients clinically suspected for DVT and ascending venography is not required. The semiquantitative latex assays for D-dimer can not be used for this purpose. Plasma FbDP levels, as detected by EIA, reflect decrease in thrombus size and may be used to monitor efficacy of heparin treatment. Low initial values of FbDP are predictive of poor thrombus dissolution in patients with DVT following SK or UK therapy. Elevated plasma levels of cross-linked fibrin degradation products have been measured during thrombolytic therapy of AMI. They are derived mainly from extracoronary sites and are non-predictive of recanalization following thrombolytic therapy.

Plasma Fibrin degradation products levels are related to severity of liver cirrhosis and renal disease, but can not be used as markers of renal function. The high sensitivity of the EIA's made it possible to detect increased levels of fibrin degradation products during steady state in patients with sickle cell disease. The observed increased fibrin(ogen) levels during normal and complicated pregnancy suggest that fibrinolysis is not necessarily depressed. The fibrinolytic system remains active in patients with malignancies, as demonstrated by elevated levels of D-dimers and FbDP's.

The following guidelines for future studies are suggested. As mentioned in the introduction, a disturbance in the haemostatic balance will presumably be reflected in the products of both coagulation and fibrinolysis. In this article we focussed on fibrin(ogen)olysis only. Detection of fibrin(ogen) derivatives by the monoclonal antibody based assays provides valuable information on haemostasis in several diseases. However, it is conceivable that more information can be obtained when the products of the two opposing processes coagulation and fibrinolysis are simultaneously

measured. This possibility is available. Soluble fibrin, the product of an activated coagulation system, can also be assessed by EIA in plasma.[23-25] In the future, studies may be performed using a combination of an EIA for a coagulation product (i.e. soluble fibrin) and an EIA for a fibrinolysis product (i.e. FbDP or D-dimer).

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Chapter 2

CORRELATIONS BETWEEN PLASMA LEVELS OF FIBRIN(OGEN) DERIVATIVES AS QUANTIFIED BY DIFFERENT ASSAYS BASED ON MONOCLONAL ANTIBODIES

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ABSTRACT

New plasma assays for fibrin(ogen) degradation products have become available which are based upon monoclonal antibodies and can be performed in plasma. In this study we have evaluated four of such specific enzyme immuno assays i.e.: for the total of degradation products of fibrin and of fibrinogen (TDP), fibrin degradation products (D-dimer and FbDP) and fibrinogen degradation products (FgDP) in patients suspected of having deep venous thrombosis of the leg (DVT) and patients with cirrhosis of the liver. In each of the (sub)groups of patients, a very good correlation ($0.90 < r < 0.98$) was observed between the actually measured TDP values and the calculated sum of the separately measured FbDP and FgDP levels. Only 2% (5 patients) of the cases showed a discrepancy of more than a factor two between the found TDP values and the calculated sum of the measured FbDP and FgDP levels.

About 90% of the fibrin degradation products were crosslinked. FbDP levels correlated well with the FgDP levels ($0.72 < r < 0.94$) and D-dimer levels ($0.82 < r < 0.91$) in both patients with DVT and cirrhotics. In those patients also a good correlation ($0.67 < r < 0.83$) was observed between FgDP and D-dimer levels, but not in patients suspected of having DVT but with a normal IPG test result. Secondary fibrinolysis appeared to be accompanied by fibrinogenolysis.

INTRODUCTION

For at least two decades, the measurement of the products of plasmin digestion of fibrin and fibrinogen has been used to assess the extent of fibrino(geno)lysis in several clinical conditions (1-11). The levels of FDP's were mostly measured in serum using a variety of procedures (13). The Thrombo-Wellco test and the tanned red-cell hemagglutination inhibition assay are well known examples of such serum-based assays (13). These assays have been evaluated in order to determine their potential both in the diagnosis and in the monitoring of the effect of treatment of different diseases (1-7). Since most of these classical assays for FDP assessment are based upon polyclonal antibodies which cross-react with fibrinogen, their use is limited to serum samples. Serum, however, is known to be a notorious source of artifacts and it has been demonstrated that, in many cases, the currently used assays for FDP assessment in serum do not reflect the true level of FDP in plasma (14). Falsely positive results can be expected if residual fibrin or fibrinogen in serum reacts with the antiserum used to detect FDP (14) or if lysis occurs during serum preparation. Too low or falsely negative results may be obtained during serum preparation if certain degradation products, e.g. fragment X, coagulate (15) or become adsorbed to the clot (14,16). As a consequence, data based on these serum assays should be interpreted with caution.

Since a few years, new assays have become available for the quantitative determination of fibrin(ogen) degradation products. These enzyme immunoassays (EIA's) are based upon specific monoclonal antibodies each of which is directed against specific epitopes of a certain fibrin derivative. They can be performed on plasma samples, thus avoiding the serum artifacts. The clinical utility of these assays in several states of diseases has been reviewed (21).

The currently available EIA's allow the specific determination of degradation products of fibrinogen (FgDP) (22), the total of degradation products derived from fibrin and fibrinogen (TDP) (23) and fibrin degradation products, FbDP (19) and D-dimer (17, 18, 28). Using a combination of these assays it is possible to discriminate between lysis of fibrinogen and lysis of fibrin (primary and secondary fibrinolysis respectively). It has been suggested that secondary fibrinolysis is associated with low levels of

fibrinogenolysis (30).

Theoretically, it may be expected that the calculated sum of the FbDP and the FgDP plasma levels will approximate the actually measured TDP values. Such an observation has been made in patients with ischemic heart disease and patients with disseminated intravascular coagulation (DIC) (29). Secondary or reactive fibrinolysis is reflected by thelevels of fibrin degradation products (FbDP), which can be determined by the EIA for FbDP (which measures both crosslinked and non-crosslinked degradation products) or (when they are crosslinked by factor XIIIa) the D-dimer EIA.

In this study we evaluated these plasma based assays in groups of patients with an activated state of coagulation. The aims of our study were: 1. To examine whether the measured levels of the total of degradation products of fibrin and fibrinogen (TDP) correlate with the levels obtained by calculation of the sum of the separately measured FbDP and FgDP levels. 2. To compare the levels of fibrin degradation products, assessed by the D-dimer EIA, with the levels of fibrin degradation products, as determined by the EIA for FbDP. 3. To see whether secondary fibrinolysis is associated with fibrinogenolysis by investigating the relationship between FgDP levels and the levels of fibrin degradation products, as detected by the EIA's for FbDP and D-dimer.

PATIENTS AND METHODS

The following groups of subjects were studied:

- group 1 consisted of 232 consecutive outpatients clinically suspected of having deep venous thrombosis (DVT), which have been described before (24). They were referred to the Thrombosis Service Center by their general practitioner. The male: female ratio was 63:169, the mean age 67 (range:17-94) and the mean duration of symptoms 9.5 days. DVT was assessed by impedance plethysmography (IPG) and the results were interpreted according to Hull's criteria (25). The prevalence of DVT in this outpatients population was 25% (59 out of 232 patients).
- group 2 consisted of 31 hospitalized patients with cirrhosis of the liver as confirmed by liver biopsy. All patients were admitted to the hospital for analysis of the severity of their liver disease. Based on the Child-Pugh classification (26), the patients were

divided in 13 patients with Child A, 9 patients with Child B and 9 patients with Child C. The mean age was 49 (range:31-70) and the male:female ratio was 13:18.
- group 3: The reference group consisted of 43 apparently healthy hospital employees (age range: 22-45).
In all cases, informed consent was obtained and the research was carried out according to the principles of the Declaration of Helsinki.

Blood samples

Venous blood samples were collected by venepuncture into vacuum tubes containing cold trisodiumcitrate 0.11 mol/l (1 volume per 9 volumes blood). Plasma was obtained by centrifugation for 30 minutes at 2000 x g at 4° C. The plasma samples were stored frozen in small aliquots at -70°C until tested and carefully thawed at 37°C prior to the start of the assay.

Assays

All assays were performed on plasma samples in duplicate. The three used assays for the specific determination of the total of degradation products of fibrin and fibrinogen (TDP; Fibrinostika TDP), fibrin degradation products (FbDP; Fibrinostika FbDP) and fibrinogen degradation products (FgDP; Fibrinostika FgDP) were enzyme immunoassays of the sandwich type (Organon Teknika, Turnhout, Belgium). All three test kits are based upon the same specific monoclonal antibody as capture antibody (FDP-14). FDP-14 has its epitope in the E-domain of the fibrinogen molecule i.e. on the Bß-chain between amino-acids 54-118. It reacts both with degradation products of fibrin and of fibrinogen, but not with the intact parent molecules (27). For the specific separate determination of fibrinogen degradation products (FgDP's) in the EIA for FgDP, a horse-radish peroxidase (HRP) conjugated monoclonal antibody (Y-18) is used as tagging antibody which reacts with fibrinopeptide A-comprising fibrinogen fragments (22). The tagging antibody for the specific FbDP assay is a HRP conjugated monoclonal antibody (DD-13) against fibrin degradation products (crosslinked and non-crosslinked) (19). In the TDP assay kit for determination of degradation products of

fibrin and fibrinogen, a mixture of the two monoclonals Y-18 (22) and DD-13 (19) is used as tagging antibody (27). D-dimer was determined by an enzyme-linked immunosorbent assay (Boehringer Mannheim, West-Germany) using a monoclonal anti-FDP-D.D. antibody as capture antibody, which strongly reacts with a neoantigenic determinant in D-dimer and has some cross reactivity with fragment D (28). The tagging antibody is a panspecific anti-FDP-D peroxidase-conjugated antibody.

Statistical analysis

The difference between the means of the test results in the different groups of patients was determined using the Wilcoxon ranksum test (two tailed). Pearson's correlation coefficient was calculated to determine the relationship between the plasma levels of fibrin(ogen) degradation products by using linear regression analysis.

RESULTS AND DISCUSSION

The median and range of the plasma levels (in ug/ml) of TDP, FbDP, FgDP and D-dimer in the different groups of subjects are shown in table 1.

	group 1 (n=232)	group 2 (n=31)	group 3 (n=43)
FbDP	0.55 (0.17-14.6)	0.61 (0.21-8.2)	0.19 (0.17-0.45)
FgDP	0.36 (0.17-9.6)	0.34 (0.23-1.27)	0.19 (0.17-0.29)
TDP	0.88 (0.21-23.1)	0.97 (0.29-10.3)	0.42 (0.21-0.65)
D-dimer	0.66 (0.08-13.7)	1.1 (0.12-8.0)	0.14 (0.05-0.33)

Table 1. The median and range of the plasma concentrations (in ug/ml) of FbDP, FgDP, TDP and D-dimer in patients suspected for DVT (group 1),patients with cirrhosis of the liver (group 2) and normal healthy controls (group 3).

In both groups of patients, those suspected of having DVT and in the group of patients with cirrhosis of the liver, the plasma levels of FbDP, FgDP, TDP and D-dimer were higher than the corresponding plasma levels in normal healthy controls (in all cases P<0.0001). Among the group of patients suspected of having DVT, those with confirmed DVT had elevated plasma levels (p<0.0001) of fibrin(ogen) degradation products when compared to the levels in patients with a normal IPG test result.
In this study we have evaluated the correlation between the plasma levels of fibrin(ogen) derivatives, as determined by EIA's for FbDP, D-dimer, FgDP and TDP in patients suspected of having DVT and cirrhotic patients. The correlation coefficients of the fibrin(ogen) degradation products are shown in table 2.

	suspected DVT (n=232)	confirmed DVT (n=59)	noDVT (n=173)	cirrhosis (n=31)	normals (n=43)
TDP versus FgDP + FbDP	0.94	0.92	0.90	0.98	0.40[1)]
FbDP versus D-dimer	0.81	0.82	0.35	0.91	0.35[1)]
FgDP versus FbDP	0.93	0.94	0.45	0.72	0.65
FgDP versus D-dimer	0.81	0.83	0.01[2)]	0.67	0.39[1)]

All P values were < 0.0001, except: 1) $p < 0.05$; 2) p = ns

Table 2. Correlation coefficients of fibrin(ogen) degradation products levels in different (sub)groups of patients.

In the patients suspected of having DVT (fig 1) and patients with cirrhosis of the liver (fig 2), the actually measured plasma levels of the total of fibrin and fibrinogen degradation products (EIA for TDP) showed a very good correlation ($0.90 < r < 0.98$, $p<0.0001$) with the values calculated by adding the measured levels of FbDP to the

levels of FgDP. This observation is in accordance with observations by others in patients with ischemic heart disease and DIC (29). The correlation between TDP and the sum of FbDP and FgDP did not differ much in the subgroup of patients with confirmed DVT (r=0.92) and patients with a normal IPG test result (r=0.90). The correlation between the measured TDP values and the calculated sum of the measured FbDP and FgDP levels in the whole group of patients suspected of having DVT is shown in figure 1.

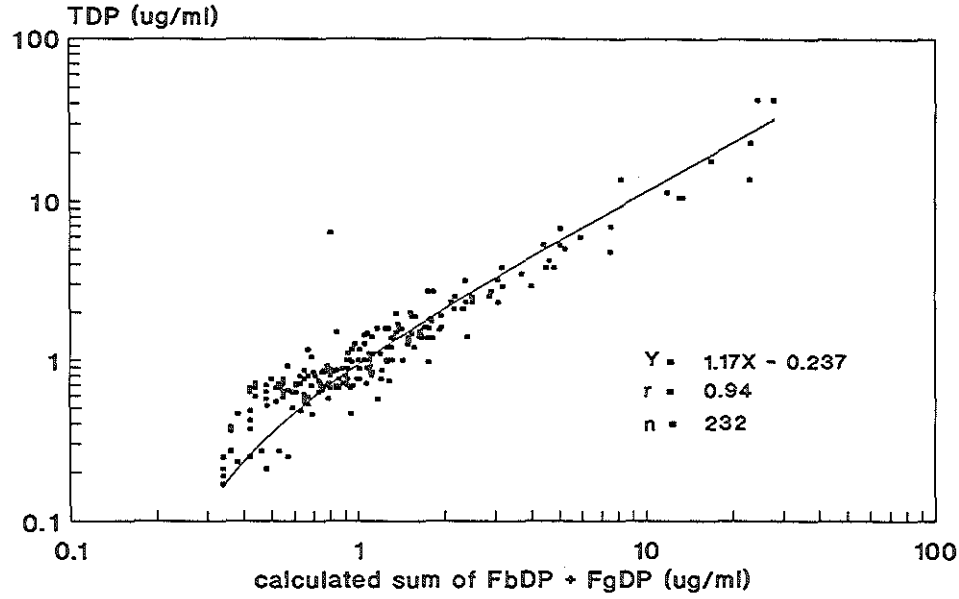

Fig 1. Correlation between the measured TDP values and the calculated sum of the FbDP and the FgDP levels (in ug/ml) in patients clinically suspected of having DVT (n=232).

In five of the 232 patients suspected of having DVT, we found a discrepancy of more than a factor two between the measured TDP levels and the calculated sum of the FbDP and FgDP levels . The ratio (FbDP+FgDP)/TDP was higher than 2 in four subjects who happened to have an IPG examination with a normal test result. In one

patient with an abnormal IPG test result, the ratio was only 0.13. In this latter case, it means that the plasma levels of fibrin(ogen) degradation products obtained by the EIA for TDP was 7.88 times higher than the values obtained by calculating the sum of the FbDP and the FgDP levels. The reason for this is not clear. None of the patients with cirrhosis of the liver nor the healthy controls had (FgDP+FbDP)/TDP ratios above 2.0 or below 0.5.

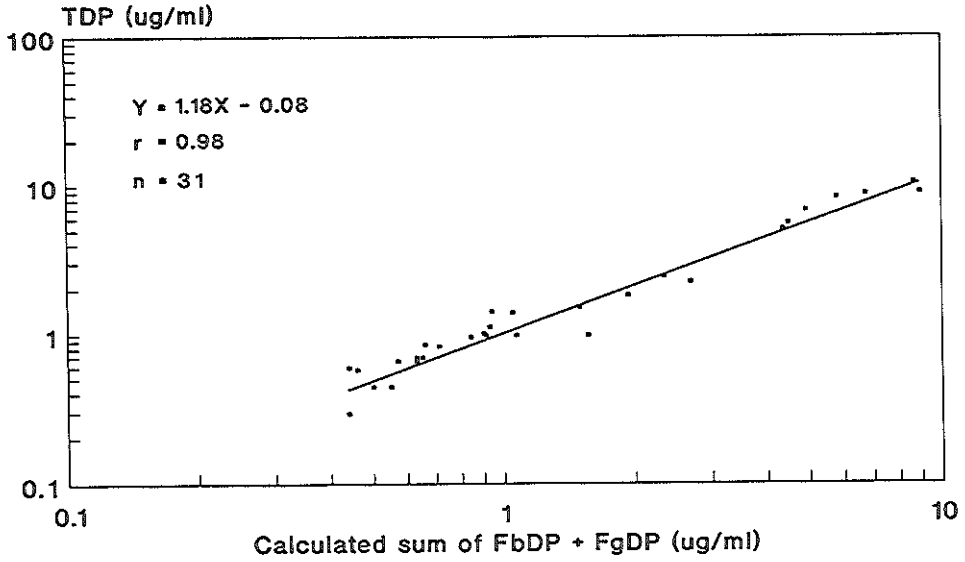

Fig 2. Correlation between the measured TDP values and the calculated sum of the FbDP and the FgDP levels (in ug/ml) in patients with cirrhosis of the liver (n=31).

Secondly, we investigated the correlation between the plasma levels of fibrin degradation products as measured by the D-dimer EIA and the EIA for FbDP. A good correlation was observed between D-dimer values and FbDP values (both of which reflect secondary fibrinolysis) in patients with confirmed DVT and patients with cirrhosis of the liver ($0.82 < r < 0.92$, $p<0.0001$).

The correlation coefficient for the FbDP and D-dimer values was $r = 0.81$ ($p<0.0001$)

in all patients suspected of having DVT (see figure 3). This correlation was the about same in the subgroup of patients with confirmed DVT ($r=0.82$; $p<0.0001$) and poor in the subpopulation of patients with a negative IPG test result ($r=0.35$; $p<0.0001$).

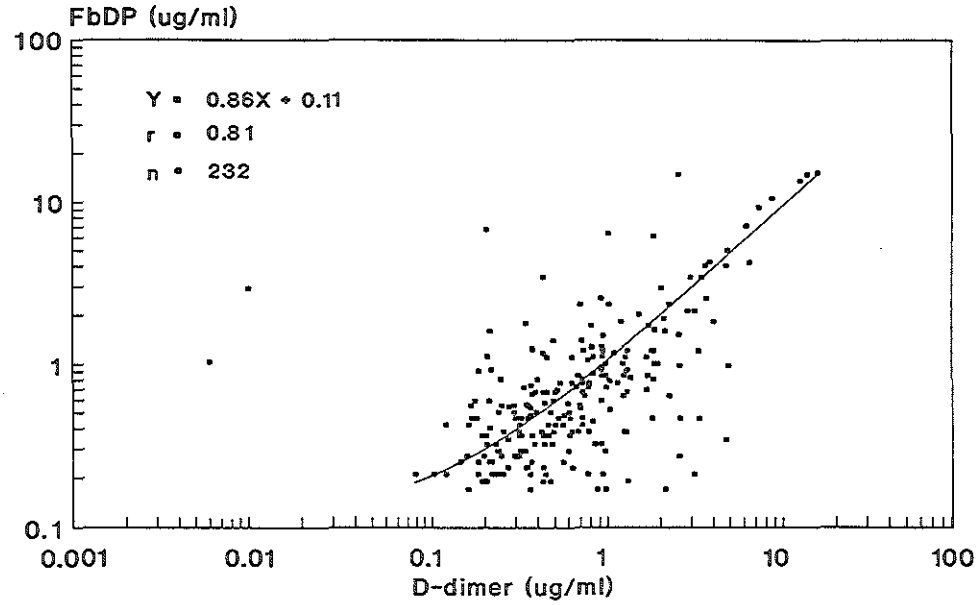

Fig 3. Correlation between plasma levels of FbDP and D-dimer (in ug/ml) in patients suspected of having DVT.

The correlation between FbDP and D-dimer in patients with cirrhosis of the liver is shown in figure 4.

In contrast to the EIA for D-dimer, which only detects crosslinked fibrin degradation products, the FbDP EIA measures both crosslinked and non-crosslinked fibrin derivatives. When one assumes that the calibration materials in the FbDP and the D-dimer EIA are comparable, one can conclude from the equations of the regression lines in figure 3 and 4, that about 90% of the measured FbDP levels consisted of D-dimer. This suggests that nearly all of the measured fibrin degradation products are crosslinked in those patients. We are aware of only one study that has described a correlation

(r=0.72, p<0.01) between D-dimer values and FbDP levels (30). However, this concerned a different

D-dimer EIA using another monoclonal antibody (DD-3B6/22).

The third purpose of our study was to examine whether secondary fibrinolysis is accompanied by fibrinogenolysis. In each of the (sub)groups of patients, the FgDP levels correlated less with D-dimer values than with FbDP levels (table 2). The observation of positive correlations between plasma levels of fibrin degradation products, as assessed by EIA's for FbDP and D-dimer, and the FgDP values confirms earlier observations that secondary fibrinolysis seems to be accompanied by lysis of fibrinogen (30). Interestingly, both the correlations between FgDP and FbDP and between FgDP and D-dimer levels were higher in the reference group (r=0.65, r=0.39 respectively) than in the group of patients with a negative IPG test (r=0.45, r=0.01 respectively). We have no explanation for the lack of correlation (r=0.01, p=ns) between D-dimer and FgDP levels in patients with a negative result of IPG examination. This observation, however, does not necessarily imply that there is no relationship, but rather that there is no linear relationship.

In general, the correlations between the fibrin(ogen) degradation products were lower in normals than in the different (sub)groups of patients. This may be explained by the fact that the fibrin(ogen) concentrations in normals are generally very low. Following the manufacturers instructions for dilutions of the samples, this means that the concentrations have to be read from the lower (nearly horizontal) part of the sigmoidal shaped calibration curves, and this yields inaccurate results. When this study was finished, we learned that lower dilution factors than 20-fold (as prescribed by the manufacturer) are allowed. This might improve the correlations. Summarizing, we found that: 1. The levels of the total degradation products (TDP EIA) show a very good correlation with the calculated sum of the measured FbDP and the FgDP levels. 2. There is a good correlation between plasma D-dimer and FbDP levels and most of the fibrin degradation products were crosslinked. 3. Secondary fibrinolysis appeared to be associated with fibrinogenolysis.

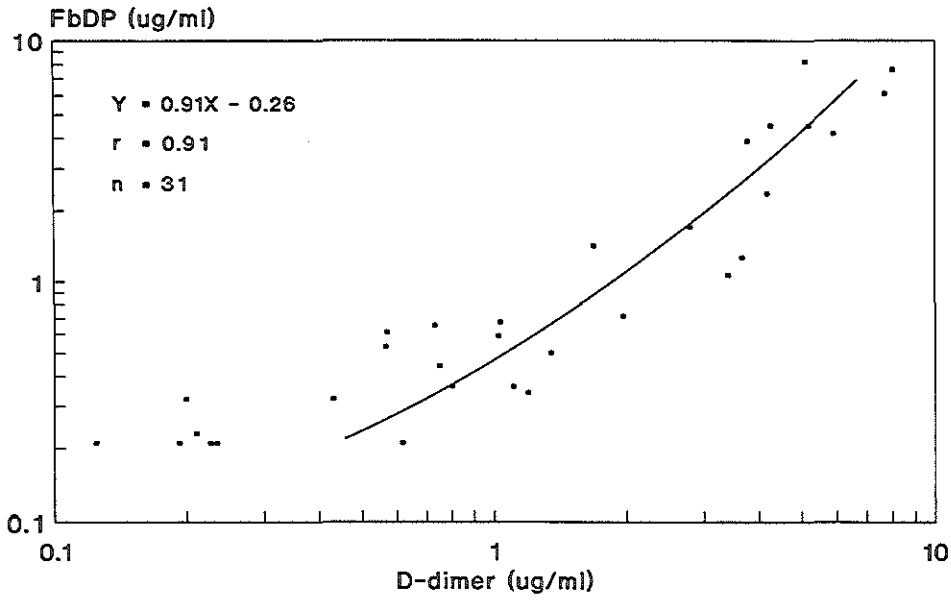

Fig 4. Correlation between FbDP and D-dimer plasma levels (ug/ml) in patients with cirrhosis of the liver (n=31).

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Chapter 3

DIAGNOSTIC VALUE OF D-DIMER FOR DEEP VENOUS THROMBOSIS IN OUTPATIENTS

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ABSTRACT

We have studied the diagnostic value for deep venous thrombosis (DVT) of an enzyme immunoassay (EIA) for the detection of D-dimer in plasma of 239 consecutive out-patients suspected of having DVT by their general practitioner. DVT was confirmed by impedance plethysmography in 60 patients. Using the 95th percentile range of 42 healthy volunteers, the sensitivity for the detection of DVT was 92%, with a specificity of 21%. In our population with a prevalence of 25%, the D-dimer EIA showed a negative predictive value of 88% and a positive predictive value of 28%. We conclude that this D-dimer ELISA has limited value, either to confirm or to exclude DVT in out-patients.

INTRODUCTION

The diagnosis of deep venous thrombosis (DVT) based upon clinical signs and symptoms only, is confirmed by objective testing in less than 50% of the cases [1-3]. In order to avoid unnecessary exposure to the risk of haemorrhagic complications due to anticoagulant treatment [4], the need for confirmative diagnosis by objective methods is widely accepted. The gold standard for the diagnosis of DVT, contrast venography [5], is an invasive, often painful and expensive technique that requires special expertise [6]. Therefore several non-invasive alternatives have been developed, of which impedance plethysmography (IPG) [7-10], ultrasonography [11, 12] and ^{125}I-fibrinogen leg scanning [13] have extensively been evaluated. IPG has shown to be a practical non-invasive alternative for the diagnosis of proximal DVT of the leg in outpatients [8-10].

In the past, laboratory assays have also been investigated to establish their potential role in the diagnosis of DVT [14-20]. Most of these assays were based upon the idea that the presence of products in the circulation, resulting from plasmin mediated lysis of fibrin(ogen), might be a useful marker of a thrombotic event. Because these assays employed polyclonal antibodies which crossreact with fibrinogen, their use is limited to serum samples. It has been demonstrated, however, that results obtained by these serum-based assays are unreliable [21]. Other laboratory assays focused upon the assessment of substances generated during thrombus formation, such as fibrinopeptide A and ß-thromboglobuline, but with disappointing results [22-24].

Assays have now become available for the detection of fibrin(ogen) degradation products in plasma [25-30]. They can be divided in (semi-quantitative) latex agglutination assays and (quantitative) enzyme immunoassays (EIA). These assays are based upon monoclonal antibodies with a well defined specificity directed against epitopes of a certain fibrin(ogen) derivative and can be performed in plasma samples. The D-dimer EIA is a well known representative of these plasma assays. Plasmin induced lysis of cross-linked fibrin produces derivatives containing D-dimer fragments, i.e. two covalently bound D-domains [31] and fragment E. Therefore the presence in blood of circulating fibrin derivatives containing D-dimer are specific markers of a

thrombotic process and its subsequent reactive fibrinolysis in vivo. The purpose of this study was to determine the test characteristics of the D-dimer EIA for the diagnosis of deep venous thrombosis in outpatients.

PATIENTS AND METHODS

The study comprised consecutive outpatients clinically suspected of having DVT who were routinely referred to the Thrombosis Service Center Rotterdam by their general practitioner. The same group of patients has been used to determine the test characteristics of the assays for Thrombin-antithrombin III (TAT) complexes, crosslinked and non-crosslinked fibrin degradation products (EIA for FbDP), fibrinogen degradation products (EIA for FgDP) and the total degradation products of fibrin and fibrinogen (EIA for TDP) [32]. Informed consent was obtained from each patient before IPG was performed and a venous blood sample was drawn. Blood samples were coded and handled independently from the IPG result. All IPG results were interpreted by one of the authors according to the criteria described by Hull [33].

The study was carried out according to the principles of the Declaration of Helsinki.

Venous blood samples were obtained from the antecubital vein into vacuum tubes containing trisodiumcitrate 0,11 mol/l (9:1). Within 2 hours after collection, blood was centrifuged at 1500 x g for 10 minutes at 10°C. Plasma was then collected and stored frozen in small aliquots at -70°C, and carefully thawed prior to the assay. Venous blood samples of forty-two ostensibly healthy hospital employees (age 22-35 years) were handled identically and tested for normal reference values.

D-dimer was determined in plasma (in duplicate) by a sandwich type EIA (Boehringer Mannheim, Mannheim, Germany), using a specific monoclonal antibody (anti-FDP-D.D.) as capture antibody, which strongly reacts with a neoantigenic determinant in D-dimer and to a lesser extent with fragment D [34]. The tagging antibody was a panspecific anti-FDP-D peroxidase conjugated antibody.

Statistical analysis

Differences in results of patient groups have been tested by means of the Wilcoxon

Rank Sum test (two tailed). Any probability less than 0.05 was considered to represent a significant difference between the studied samples.

RESULTS

A total of 239 consecutive outpatients were entered into the study. DVT was confirmed by IPG in 60 patients; in 10 of these patients the IPG converted to abnormal in the second examination, one week after the first examination. Seven patients were excluded because of improper handling of the plasma samples. The basic characteristics of the total of 232 patients that remained for analysis are shown in table I. Of these, 59 patients (25%) had an abnormal IPG examination of whom 27% (16 subjects) had a prior history of DVT. The median delay between onset of symptoms and the first IPG examination was 8 days in IPG positive patients and 10 days in patients with a normal IPG test result. This difference was statistically not significant (p=0.24). No relationship was found between D-dimer levels and duration of symptoms in IPG negative patients (p=0.03, p=0.67) or IPG postive patients (p=0.16, p=0.23).

Within patients that were clinically suspected for DVT, the median D-dimer plasma levels in patients with a normal IPG examination was 528 (range:10-5172) ng/ml, while those with a abnormal IPG examination had a median D-dimer level of 1236 (range:6-16000) ng/ml. The median D-dimer plasma levels in 42 healthy volunteers (the reference group) were 148 (range: 53-330) ng/ml (Table II). The cut off point for test positivity (268 ng/ml) of the D-dimer EIA was obtained by using the 95th percentile of the measured D-dimer plasma levels of the normal reference group.

Mean plasma D-dimer values were higher ($p<0.0001$) in patients with DVT, as confirmed by an abnormal IPG, when compared with the values in symptomatic non-DVT patients, as determined by a normal IPG examination. Furthermore, symptomatic non-DVT patients had elevated ($p<0.0001$) D-dimer levels in comparison to the reference group.

As can be seen in table III, 92% of the patients with DVT had D-dimer levels above the cut off point for test positivity, leaving 5 cases with a false negative result. Among the 173 patients, in who DVT could not be confirmed, there were 137 cases

Table I. Characteristics of consecutive outpatients with suspected deep venous thrombosis.

	IPG abnormal	IPG normal
N	59	173
Male/Female	23/36	40/133
Age*	74 (23-94)	64 (17-89)
Prior history of DVT	6 (27%)	34 (20%)
Duration of symptoms*(in days)	8 (1-122)	10 (1-700)

* Median and range

Table II. Test results* of plasma samples of consecutive outpatients with suspected DVT and of the reference group.

Tests	Abnormal IPG	Normal IPG	Reference group
D-dimer	1236 (6-16000)	528 (10-5172)	148 (53-330)

* median and range in ng/ml

Table III. Dichotome table showing the results of D-dimer Elisa versus results of IPG examination in consecutive outpatients with suspected DVT

	IPG			
	Abnormal	Normal		
D-dimer≥268 ng/ml	54	137	191	*PV +: 54/ 191=28%
D-dimer<268 ng/ml	5	36	41	*PV -: 36/ 41=88%
	59	173	232	
	Sensitivity	Specificity		
	54/59=92%	36/173=21%		

* PV+: predictive value of a positive test

PV-: predictive value of a negative test

with a false positive result. Therefore the sensitivity and specificity of the D-dimer EIA were 92% and 21% respectively. The prevalence of DVT was 25% and the predictive value of a positive and negative test result were 28% and 88% respectively.

DISCUSSION

The introduction of assays based on monoclonal antibodies, for the detection of intermediate or endproducts of fibrinolysis in plasma, has raised new interest to investigate the value of laboratory assays in the diagnosis of several diseases involving the haemostatic system.

In this study we have measured the test performance of the D-dimer EIA in outpatients who were clinically suspected of having DVT. The D-dimer assay had a sensitivity of 92%: five of all patients with DVT, confirmed by IPG, had D-dimer levels below the cut-off point for test positivity (false negative rate of 8%). The specificity was low (21%). The test characteristics (sensitivity and specificity) are considered to be independent of the prevalence of disease (or pretest probability). However, for application of the D-dimer assay in daily clinical practice, it is relevant to know whether a test result can predict the presence or absence of disease. Therefore it is important to be aware of the positive or negative predictive value (or post test probability) of a given test result.

In our population, the prevalence of DVT was 25%. A positive D-dimer test was followed by an abnormal IPG test in only 28% of the cases (i.e. the positive predictive value is 28%). As a consequence, the observation of elevated D-dimer levels alone in patients suspected of having DVT is not sufficient to indicate anticoagulant treatment. The predictive value of a negative test result was 88%. Based on a D-dimer test result only, the diagnosis DVT will be missed in 12% of the cases with D-dimer levels below the cut-off point for test positivity. This is an unacceptably high percentage.

The sensitivity of the D-dimer EIA in our study is comparable with results reported by others using ascending venography [35-38], but the specificity is lower. This latter may be explained by the fact that we used IPG to confirm DVT, which has a limited ability to detect calf vein thrombosis. The use of ascending venography may lead to a shift of

IPG negative patients with elevated D-dimer levels due to calf vein thrombosis to a DVT positive classification. which results in a higher specificity. It has been demonstrated, however, that safe management with the use of IPG is possible in symptomatic outpatients [9].

Application of a different cut-off point for test positivity may also influence the test results. In our study it was not possible to reach a 100% negative predictive value by variation of the cut-off point. Furthermore, a difference of prevalence of DVT may alter the predictive value of a test result: e.g. when the prevalence decreases, the positive predictive value will also fall, but the negative predictive value may rise.

Other recently developed laboratory tests, like the assays for thrombin-antithrombin (TAT) complexes [32,39] and the assays for cross-linked and non cross-linked fibrin degradation products (FbDP EIA), for the total of fibrin and fibrinogen degradation products (TDP EIA) and for fibrinogen degradation products (FgDP EIA) have also been evaluated in the diagnosis of DVT [32]. Neither of these assays reached a negative predictive value of 100%, although it should be noticed that the test characteristics of the EIA's for FbDP, TDP and FgDP has not yet been determined using ascending venography in patients suspected of having DVT.

Other studies, using a semiquantitative D-dimer latex agglutination assay, demonstrated that DVT could not be excluded if only based on D-dimer levels below the cut-off point for test positivity [35-37]. Formerly developed assays for determination of fibrinopeptide A (FPA) and betathromboglobulin (beta-TG) in plasma have also been evaluated in the diagnosis of DVT [22-24] and have shown to be of little value in the diagnosis of DVT.

Summarizing, the results of this study seem to indicate that the D-dimer EIA can not be used in our population of outpatients clinically suspected of having DVT to select patients for further diagnostic procedures (IPG).

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Chapter 4

FIBRIN AND FIBRINOGEN DEGRADATION PRODUCTS IN PLASMA DURING GRAM-NEGATIVE SEPTIC SHOCK

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ABSTRACT

Gram-negative septic shock is an increasingly common event associated with a high mortality. Complicating factors, such as multiple organ failure and adult respiratory distress syndrome are due to widespread fibrin depositions, and are considered to depend largely upon extreme activation of the coagulation pathway. It has recently been suggested, however, that impairment of fibrinolysis may also play a role. In this study we foccussed on the fibrinolytic system in 30 patients with septic shock, by measuring plasma levels of the total fibrin and fibrinogen degradation products (TDP) and fibrinogen degradation products (FgDP). Mean TDP and FgDP levels were elevated in patients with septic shock and showed a tendency toward normalization during follow-up. The mortality rate was 50%. No difference could be detected between plasma TDP and FgDP levels in survivors and nonsurvivors of septic shock. This study suggests that the fibrinolytic system is activated during septic shock. Further studies are needed to determine whether activation of the fibrinolytic system in patients with septic shock may improve the prognosis.

INTRODUCTION

Gram-negative sepsis is an increasingly common clinical event with a mortality rate between 20% and 50%.[1,2] In patients who develop septic shock, mortality exceeds 40% and may even approach 90% in patients with serious underlying disease.[3]

Endotoxins, lipopolysaccharide, and especially the lipid-A fraction of the gram-negative bacterial cell wall, trigger the pathophysiologic response to gram-negative septicemia.[4] They exert numerous biological effects, such as generation of vasoactive peptides,[5,6] and activation of coagulation[7] and complement pathways.[8] Endotoxin is also a potent stimulator for the release of tumor necrosis factor (TNFα) from macrophages and monocytes.[9] TNF activates factor X and thus the common coagulation pathway.[10]

Septicemia is frequently associated with disturbances of the hemostatic balance.[11] During septic shock, decreased levels have been detected of prekallikrein and coagulation factors XII, XI and VII, suggestive for consumption of these factors.[12,13] The activation of the coagulation system may cause disseminated intravascular coagulation (DIC) with widespread depositions of fibrin in the microvasculature and subsequent development of multiple organ failure.[14,15] The activation of kallikrein in turn may also cause the liberation of bradykinin and the activation of plasmin.

Bertozzi and coworkers recently demonstrated that inhibition of fibrinolytic activity due to an increase in urokinase inhibitors and antiplasmins contributes to the formation and persistence of hyaline membranes in the lung and thus to the development of adult respiratory distress syndrome, a common complication of septic shock.[16] Furthermore it has been demonstrated that endotoxin administration to normal subjects results in early activation of fibrinolysis through release of tissue plasminogen activator (t-PA), which is abruptly terminated by a rise of plasminogen activator inhibitor 1 (PAI-1) activity.[17] Extremely high PAI-1 levels have indeed been encountered in patients with septicaemia[12,18,19] suggesting that plasmin mediated lysis of fibrin and fibrinogen may be impaired during septic shock.

Fibrinolysis is an important mechanism in protecting the circulation from massive thrombosis and is reflected by the formation of degradation products of fibrin (FbDP) and fibrinogen (FgDP). Until recently, blood levels of fibrin and fibrinogen

degradation products could not be detected reliably, because they could only be assessed in serum. The use of serum samples leads to unreliable results.[20,21]

During the past few years, enzyme immuno assays (EIA's) have become available for quantitative assessment of fibrin and fibrinogen degradation products in plasma. The aim of our study was to investigate the fibrinolytic state in patients with septic shock by measuring degradation products of fibrinogen (FgDP) and the total of fibrin and fibrinogen degradation products (TDP) in plasma.

PATIENTS AND METHODS

This investigation was performed as an ancillary study to the so called Swiss-Dutch trial, which was designed to determine the efficacy of hyperimmune IgG against E-coli J5 in comparison to a normal IgG preparation in patients with gram-negative septic shock.[22] Blood samples were obtained from 30 consecutive patients in the Netherlands (participating centres and principal investigators: Sint Radbout Hospital Nijmegen (E. Kalter), University Hospital Utrecht (J. Schellekens and J. Verhoef), who fulfilled the inclusion criteria of the Swiss-Dutch trial: 1. The presence of a gram-negative bacteremia, a proven or firmly suspected gram-negative focal infection or a clinical condition predisposing to gram-negative infection (e.g. granulocytopenia, septic abdominal surgery, esophageal surgery or surgery for ruptured aortic aneurysma) and 2. the presence of septic shock based on clinical grounds and at least one of the following signs: oliguria (<30 ml/h), hypothermia (<36°C), hypoxemia (P_{o2}<30 mm Hg), respiratory alkalosis (pH >7.5 or P_{CO2}<30 mm Hg), metabolic acidosis (pH <7.3 or base excess <-10 mmol/l), coagulation abnormalities (plasma thromboplastin level of <50% of normal, APTT >40s, or the elevation of fibrin split products in serum) and thrombocytopenia (<100 000/mm^3 or a decrease of >50% of a previously normal value). All patients received standard therapy for septic shock (fluid resuscitation, vasopressors and antibiotic treatment) and support of the vital functions if required (Swan-Ganz catheterization, artificial ventilation and hemodialysis). In addition, the patients were randomized to receive a single intravenous dose of 200 mg/kg (max 12 g) of either a standard IgG preparation or a preparation of human IgG (J5) antibody to Escherichia

coli during one hour.

Blood samples were taken prior to infusion (t=0), 2 hours after infusion (t=1), 24 hours after infusion (t=2) and after 10 days (t=3). Plasma was obtained by centrifuging a mixture of nine volumes of blood and one volume of 0.11 M sodium citrate for 30 minutes at 2000 x g at 4° C. The plasma samples were stored frozen in small aliquots at -20°C until tested and carefully thawed at 37°C prior to assay. In order to be able to establish normal reference values, venous blood samples were obtained from 43 non-patient volunteers (male:female = 24:19; median age: 31 yrs, range: 22-45 yrs) and processed in an identical manner.

Assays

FgDP and TDP were determined in plasma using specific EIA's of the sandwich type based on monoclonal antibodies (Organon Teknika, Turnhout, Belgium). The capture antibody was FDP-14, the tagging antibody for detection in the FgDP EIA was FDP Y-18.[23] A combination of FDP Y-18 and FDP DD-13 was used as tagging antibody in the EIA for TDP assessment.[24]

Statistical analysis

Results are given as mean ± standard error of the mean (s.e.m.), unless reported otherwise. Differences between groups were tested by applying the Wilcoxon ranksum test for independent samples. Within group differences were determined by Wilcoxon matched-pairs signed rank test. Any P value <0.05 was considered to represent a significant difference. All reported significance levels were two-sided. Regression analysis was performed by method of least squares and the correlation coefficient was calculated.

RESULTS

The mortality rates were 50% in either of the treatment groups. We therefore analysed all data assuming that treatment difference did not exist.

Of the 30 patients with a gram-negative septic shock (median age:64, range 21-78, male/female ratio: 20:10), 15 died due to septic shock; 8 of whom died within 7 days. The median plasma values of the TDP and FgDP in normal healthy controls and

patients with septic shock are shown in table 1.

The mean plasma TDP concentration in the baseline plasma sample (t=0) of patients with septic shock (6.52 ug/ml ± 1.83) was markedly elevated ($p<0.0001$) as compared with that of healthy subjects (0.42 ug/ml ± 0.02). In addition, patients with septic shock had significantly higher plasma FgDP levels (2.59 ug/ml ± 0.93) than healthy subjects (0.20 ug/ml ± 0.004, $p<0.0001$).

Patients	TDP	FgDP
normals	0.42 (0.21-0.65)	0.19 (0.17-0.29)
sepsis	3.01 (0.25-42.9)	0.65 (0.25-19.9)
survivors	2.61 (0.25-42.9)	0.62 (0.25-19.4)
nonsurvivors	3.09 (0.25-36.5)	0.81 (0.25-19.9)
death <7 days	3.09 (0.25-36.5)	0.43 (0.25-19.9)
death >7 days	2.90 (2.29-14.4)	1.01 (0.25-5.50)

Table 1 Median and range of TDP and FgDP plasma levels (ug/ml) in patients with gram-negative septic shock and normal subjects.

The differences between survivors and nonsurvivors of septic shock at t=0 were not significant for mean TDP (6.65 ± 2.89 vs 6.38 ± 2.35 respectively, $p=0.53$) and mean FgDP (2.65 ± 1.37 vs 2.47 ± 1.29 respectively, $p=0.72$) plasma levels.

The mean ratio of FgDP/TDP in patients with septic shock (0.39 ± 0.05 ug/ml) in the first plasma sample was lower ($p<0.01$) than in healthy subjects (0.52 ± 0.02 ug/ml). The difference in mean FgDP/TDP ratios between survivors (0.46 ± 0.08 ug/ml) and nonsurvivors (0.34 ± 0.06 ug/ml) was not statistically significant ($p=0.12$).

Fig 1 shows the mean plasma levels of TDP, FgDP and the FgDP/TDP ratio of patients with septic shock in the first plasma sample, after 3 hours, after 24 hours and after 10 days. The variation in mean TDP and FgDP values was not significant ($0.32<p<0.86$

and 0.22 <p<0.76 respectively). The mean FgDP/TDP ratio remained unchanged (p=0.03) after 3 hours (0.44 ± 0.06 ug/ml), but declined significantly (p<0.005) to 0.29 ± 0.04 ug/ml after 24 hours. During follow-up, no significant differences were observed in mean TDP plasma levels (0.39<p<0.71) and FgDP values (0.35<p<0.72) between survivors and nonsurvivors.

FgDP values correlated extremely well with TDP values in the first plasma sample (r=0.97, p<0.0001, n=30) and after 3 hours (r=0.96, p<0.0001, n=29). However, this correlation disappeared after 24 hours (r=0.42, p<0.05, n=24) and after 10 days (r=0.33, p=0.18, n=18).

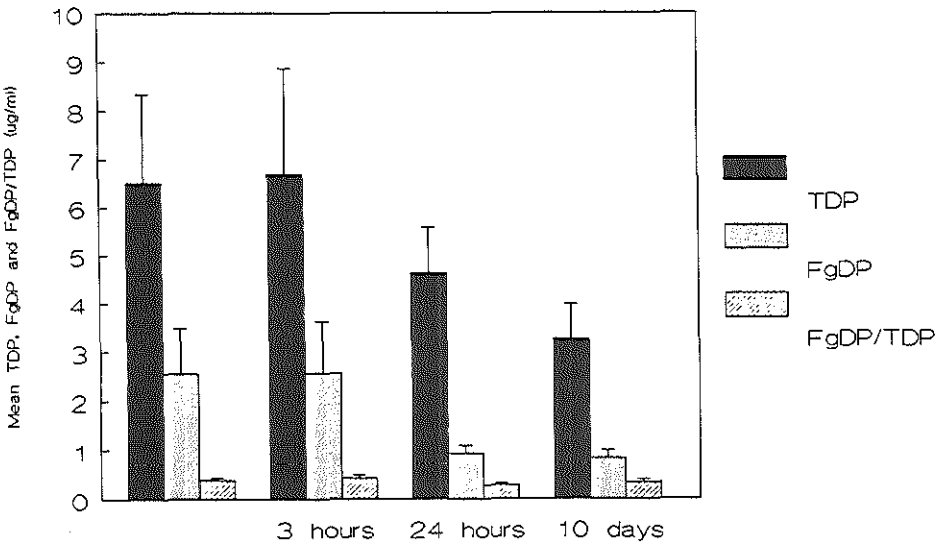

Figure 1. Mean levels of TDP and FgDP and the ratio of FgDP/TDP in patients with septic shock in the first plasma sample, after 3 hours, 24 hours and and 10 days.

DISCUSSION

In this study we investigated the fibrinolytic system in patients with septic shock by measuring FgDP and TDP plasma levels with new sensitive EIA's. One of the

pathophysiological hallmarks of septic shock are the DIC related events, such as fibrin deposition at the microvascular level.[1] Degradation of fibrin, deposited in the microvasculature, by a physiological fibrinolytic response is an important mechanism of the organism in the defence against massive thrombosis.

The fibrinolytic activity depends primarily on the balance between t-PA and PAI-1 activity. Recent studies suggest that the fibrinolytic system may be impaired in septicemia through an increased PAI-1 activity.[18,19] High PAI-1 levels appear to be associated with a poor prognosis of septic shock.[12,19] Although measurement of both t-PA and PAI-1 seems thus of importance in the prognosis of septic shock, circulating levels of fibrin and fibrinogen degradation products could be a more reliable measure of their resultant activity in blood. The most tangible evidence for ongoing fibrinolysis in blood is the presence of degradation products of fibrin and fibrinogen. Plasma levels of TDP and FgDP were markedly elevated in patients with septic shock as compared with healthy subjects. This is in agreement with findings of others who found elevated D-dimer levels during septic shock[12,25] or increased FgDP and TDP levels in patients with septicemia[26] and suggests that simultaneous activation of both the coagulation and the fibrinolytic system occur in these patients. Moreover, the elevated levels of FgDP indicate the occurrence of primary fibrinolysis (fibrinogenolysis), which has been shown to accompany fibrinolysis in many cases[27]. FgDP's possess anticoagulant properties[29-31] and their presence in plasma could theoretically helping preventing the formation of microthrombi. However, this study suggests that their contribution to the course of septic shock is limited as no difference could be detected between mean FgDP levels of survivors and nonsurvivors. It could well be that their contribution is of greater importance at an earlier stage.

No difference could be observed between survivors and nonsurvivors in mean TDP and FgDP plasma levels in the first plasma sample. Measurements during the follow-up period of the whole group showed that there was a tendency to normalization in plasma levels of degradation products (see Fig.1). This tendency was slightly more pronounced in survivors than in nonsurvivors, but the difference was not significant. Whether this latter is due to a type II error needs to be determined by studying larger groups. The

decline of the FgDP/TDP ratio in the sample after 24 hours may be explained by an increase of FbDP plasma levels, since TDP is the sum of FgDP and FbDP.[28]

In conclusion, this study shows that TDP and FgDP levels are significantly elevated during septic shock, indicating the process of ongoing fibrinolysis. No difference could observed between levels of degradation products of survivors and nonsurvivors, which suggest that the fibrinolytic activity was comparable in both groups. This study was not designed to determine whether the extent of fibrinolytic response is adequate for the degradation of fibrin deposits and microvascular thrombi during septic shock. Further studies are needed to see whether stimulation of fibrinolytic activity, e.g. by preventing the increase of PAI-1 or administration of t-PA, has a beneficial effect on the prognosis of septic shock. That this may be the case is suggested by experimental results of t-PA therapy on glomerular thrombi in the Shwartzman reaction in rabbits.[32]

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Chapter 5

PHARMACOKINETICS OF LOW MOLECULAR WEIGHT HEPARIN AND UNFRACTIONATED HEPARIN DURING ELECTIVE AORTOBIFEMORAL BYPASS GRAFTING

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ABSTRACT

Peroperative monitoring has demonstrated that administering of heparin on empirical basis is associated with a wide variation in patient response and elimination rate. This problem may be overcome by intervention on the basis of peroperative monitoring or by using heparins with different pharmacokinetic properties. When compared with unfractionated heparin (UFH), low molecular weight heparins (LMWH) have a higher bioavailability after subcutaneous administration, a linear clearance mechanism with a prolonged half life and are at least as effective in preventing postoperative vein thrombosis. Theoretically these characteristics of LMWH could lead to more predictable levels of heparin activity.

In this study we compared the pharmacokinetics of LMWH and UFH after an intravenous injection in patients undergoing aortic graft surgery. Heparin activity was measured before heparin administration and at 5, 20, 35, 50, 65, 80, 95 and 110 minutes after administration. The anti-Xa activity in the LMWH group showed less variation and was more sustained when compared to the UFH group. Fibrin degradation products were moderately correlated with the anti-factor Xa levels of the LMWH group, but no correlation was found in the UFH group. The anti-factor Xa activity of LMWH was, in contrast to that of UFH, not completely reversible by protamine administration. The blood loss was comparable in both groups. In contrast to what was expected, the pharmacokinetic profiles of LMWH and UFH showed a similarity after intravenous injection in patients undergoing aortobifemoral bypass grafting. Factors that could have influenced the pharmacokinetic behaviour of the heparins are discussed.

INTRODUCTION

The use of heparin in the surgical treatment of arteries has first been described in 1940 and it was shown that heparin had a favourable effect on vascular patency[1]. Gradually, the administration of heparin during reconstructive arterial surgery has become widely accepted for the prevention of intravascular peroperative thrombosis. At present, the majority of surgeons routinely anticoagulate their patients by giving a fixed dose heparin (mostly 4000-8000 IU) intravenously[2-5], although some prefer a bodyweight dependent dose of heparin i.v.[5-10]. Previously, we have studied heparin levels during abdominal aortic reconstructions[4]. In accordance with other studies[2,10-13], it was demonstrated that patients response to heparin showed a large interindividual variability, which makes it difficult to maintain predictable or adequate levels of anticoagulation for all patients. One way to attack this problem may be the close monitoring of heparin levels in each patient, another approach is to study the utility of heparins with different pharmacological properties.

The heterogeneous nature of unfractionated heparin (UFH), like its polydispersity of molecular weight and different chemical properties, may contribute to the variable response in patients. After intravenous administration, the disappearance curve of UFH anticoagulant activity, as measured with the APTT or with the anti-Xa assay, follows non-linear kinetics and is characterized by a rapid initial disappearance phase followed by a convex curve[14]. During aortobifemoral bypass grafting, the duration of the aortic clamping time is such, that the UFH elimination will mainly occur in this rapid disappearance phase.

In the search for improvement of heparin therapy, attempts have been made to develop heparin derivatives with modified pharmacological and biological properties. One group of heparin derivatives, the low molecular weight heparins (LMWH), have become of great clinical interest. Some LMWH preparations have already been approved and registered for clinical use. LMWH are derivatives of UFH characterized by a shorter oligosaccharide chain length and a prolonged half life time in comparison to the parent heparin. LMWH's exert anticoagulant activity in a different way than UFH. A minimum of 18 monosaccharide units is needed for the simultaneous binding of

thrombin and antithrombin on a heparin template, while the saccharide size needed for interaction with factor Xa is smaller[15]. Hence, because of their shorter chain length, LMWH's have a diminished anti-factor IIa activity, which is reflected by a limited ability to prolong clotting time, but are potent enhancers of the inhibition of coagulation factor Xa.

Following subcutaneous administration, the bioavailability of LMWH is high (± 90%) in comparison with UFH (± 15%)[16], which makes it possible to reach therapeutical levels in thrombotic patients without dose adjustments[17]. Analysis of randomized controlled level I trials comparing UFH and LMWH in the prophylaxis of postoperative venous thrombosis demonstrated a clinically important difference in efficacy in favour of LMWH[18]. After intravenous administration LMWH's follow a linear elimination pattern[19-21]. Theoretically, the altered pharmacokinetic properties of LMWH's could result in more predictable levels of heparin activity during aortic reconstructive surgery. The purpose of this study was to compare the pharmacokinetics of intravenously administered LMWH and UFH in patients undergoing elective aortobifemoral bypass surgery.

PATIENTS AND METHODS

patients

The study included consecutive patients undergoing an elective aortobifemoral bypass prosthesis operation. Patients were excluded from the study if they had (previous) bleeding disorders, if they had taken anticoagulant or antiplatelet therapy during 5 days prior to surgery or if they had not given informed consent. All exclusions were documented.

A knitted, gelatin impregnated Dacron graft (Vascutek', Inchinnan, Renfrewshire, Scotland) was used for bypass surgery. Standardized techniques of aortic graft insertion were used in all procedures. The proximal anastomosis to the aortic neck was made either end-to-end when it concerned aortic aneurysmal or end-to-side in case of aortoiliac occlusive disease. The distal parts of the graft were sutured end-to-side to the common femoral arteries just proximal of the bifurcations of the superficial and deep

femoral arteries. Flushing of the graft just before completion of the anastomoses was also part of the procedure.

Study design

The study was performed as a randomized study to compare the pharmacokinetics of LMWH and UFH administered during elective abdominal aortobifemoral prosthesis operations. Included patients were randomly assigned to receive LMWH (dosage: 90 anti-Xa units/kg) or unfractionated heparin (dosage: 80 anti-Xa units/kg). The operating surgeons were kept unaware of the assignment. Heparin was administered as a bolus injection intravenously 3 minutes prior to clamping of the aorta.

Apart of the heparinization, no further attempt was made to alter the surgeon's existing empirical protocol. A solution containing 25 anti-Xa IU UFH per ml normal saline was used for flushing. Protamine was administered if and as desired by the operating surgeon after completion of the anastomosis and removal of the clamps and before abdominal closure. Blood samples were taken after induction of anaesthesia (before heparin administration), 5 minutes after heparin administration and each 15 minutes thereafter until completion of the anastomosis and removal of the aorta clamp. If protamine was administered, a blood sample was taken 5 minutes later.

The study was approved by the medical ethical committees of the University Hospital Rotterdam - Dijkzigt, Rotterdam and the St. Antonius Hospital, Nieuwegein, the Netherlands. Informed consent was obtained from each eligible patient before entering into the study. The study was carried out according to the declaration of Helsinki.

Heparins

- UFH: Thromboliquine^{r} (Organon, Oss, the Netherlands) in prepacked syringes of 5 ml containing 5.000 IU/ml of the highly purified sodium salt of heparin from animal source.
- LMWH: the used LMWH (Kabi 2165, Fragmin^{r}, Kabivitrum AB, Stockholm, Sweden) was obtained by partial depolymerization of heparin from porcine intestinal mucosa with nitrous acid and isolated by ethanol precipitation. Fragmin^{r} has an average

MW of 4000-6000 Daltons and about 90% of the material between MW 2000-9000. The specific anticoagulant activities are about 160 U/mg in an anti-factor Xa assay using the chromogenic substrate S-2222 (Kabivitrum, Stockholm) and about 40 U/mg in the APTT and thrombin inhibitions assays. Aqueous solutions of Fragmin^{r} were supplied in prepacked syringes containing 10.000 IU in a 1 ml solution.

Laboratory assays

- Assays were performed using the FP-910 coagulation system (Labsystems, Helsinki, Finland) as described earlier[4].
- Heparin activity was assessed by measuring anti-factor Xa activity using the chromogenic substrate S-2222, bovine factor Xa and human AT-III concentrate (Coatest heparin, Kabi Vitrum, Amsterdam, the Netherlands)[22]. A standard curve was constructed by testing normal citrated plasma samples (obtained from apparently healthy hospital employees) containing increasing amounts of heparin at concentrations ranging from 0-1 U/ml.
- Activated partial thromboplastin time (APTT) was determined with actin (Merz Dade, Dijdingen, Switzerland)[23].
- Fibrin degradation products (FbDP's) were quantitatively determined in plasma using an enzyme immunoassay (EIA) of the sandwich type (Organon Teknika, Turnhout, Belgium). This EIA is based upon a monoclonal antibody as capture antibody (FDP-14). The tagging antibody was a horse-radish peroxidase (HRP) conjugated monoclonal antibody (DD-13) against the D-dimer[24].
- Platelet count, hemoglobin and hematocrit were determined in EDTA-blood. Thrombotest (TT) was also determined.

Blood samples

Venous blood samples were collected from an arterial line into 5 ml tubes containing 3.2% trisodium citrate (9:1). The first 10 ml of blood was discarded to avoid contamination of the sample with heparin used for flushing. The blood samples were immediately cooled on melting ice and were centrifuged in an Eppendorf centrifuge (3.5 min, 10.000 g) or a Hettich centrifuge (10 min, 3200 g at 4°C). Plasma samples

were stored frozen in small aliquots at -70°C and carefully thawed at 37°C prior to assay.

Blood loss

The intraoperative blood loss was accurately collected and recorded through suction and gauzes.

Statistical analysis

Results are given as mean ± standard deviation unless reported otherwise. Within groups differences between the means were tested using Wilcoxon's signed Rank test for paired samples. Differences between the two groups were tested with the two sample Wilcoxon ranksum test. All tests were two-sided and any probability less than 5% was considered statistically significant. Pearsons correlation test was used for comparison of the APTT and the anti-Xa levels and for comparing the FbDP levels with the anti-Xa levels.

RESULTS

Patients

Fifty three consecutive patients underwent elective abdominal aortobifemoral bypass grafting. Thirteen patients were excluded from the study, one because a history of excessive bleeding during previous surgery, eight because they had been taking anticoagulant or antiplatelet drugs within 5 days prior to surgery and four because they were unwilling to give informed consent.

Forty eligible patients who were willing to give informed consent entered into the study. Twenty patients were assigned to receive UFH and twenty to receive LMWH. The patients characteristics are shown in table 1.

The indications for operation were aortoiliac occlusive or aneurysmal disease of the aorta. Fifteen patients (38%) suffered from aortoiliac occlusive disease (7 of the UFH group and 8 of the LMWH group). Twenty-five patients were electively operated upon because of an aortic aneurysma (13 of the UFH and 12 of the LMWH group). The aorta was cross-clamped above the renal arteries (suprarenal) in 15% of the cases (4 patients in the UFH group and 2 in the LMWH group). The preoperative hematologic

Group	Bodyweight (Kg)	age (yrs)	male/female ratio
UFH	75.7 ± 16.1	61.2 ± 11.1	17/3
LMWH	71.6 ± 8.5	64.9 ± 9.7	18/2
Total	73.6 ± 12.7	63.0 ± 10.3	35/5

Table 1 Characteristics of patients undergoing elective aortobifemoral bypass grafting (mean ± std).

	UFH	LMWH
Platelet count (x 10^9/l)	222 ± 67	233 ± 57
Haemoglobin (mmol/l)	8.6 ± 1.1	8.9 ± 0.8
haematocrit	0.40 ± 0.05	0.42 ± 0.04
Thrombotest	74% ± 17%	65% ± 23%
APTT (sec)	38.3 ± 12.4	34.8 ± 6.06

Table 2 Preoperative hematologic data of patients receiving UFH or LMWH.

Group	Blood loss mean	s.e.m.	Operation time mean	s.e.m.	Clamping time mean	s.e.m.
UFH	2421	296	235	21	72	10
LMWH	2098	438	207	13	61	6
Total	2255	357	221	12	66	6

Table 3 Intraoperative blood loss (ml), duration of operation (min)and clamping time of the aorta (min) in aortobifemoral bypass grafting. Results are given as mean and standard error of the mean (s.e.m.).

data of both groups were comparable (see table 2). The intraoperative blood loss, the operation time and the clamping time of the aorta in both groups are shown in table 3. The between groups differences of the means were statistically not significant.

Heparin levels

The disappearance curves of the two heparins, as assessed by the levels of anti-factor Xa activity, are shown in figure 1.

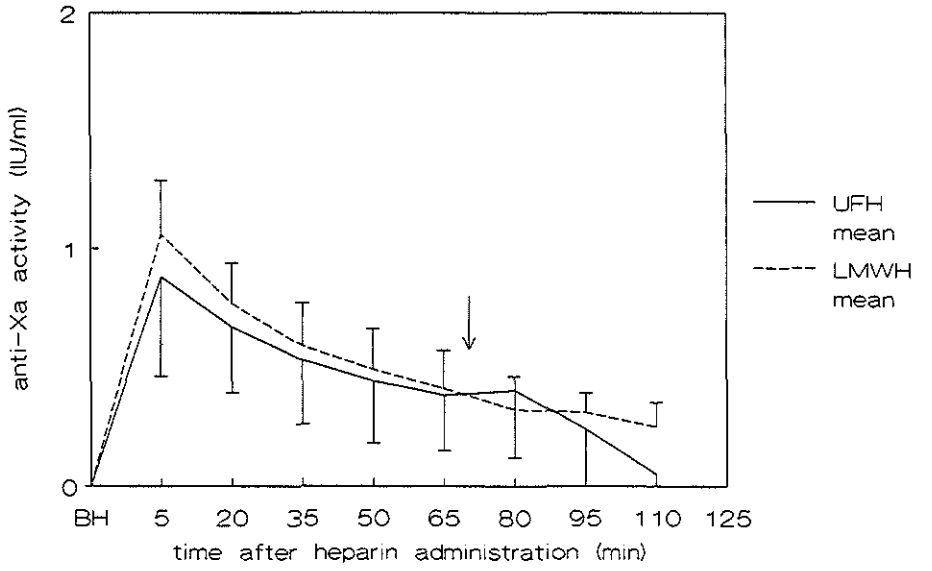

Figure 1 The anti-factor Xa activity (IU/ml) before (BH) and after heparin administration in patients receiving UFH or LMWH (mean ± SD). The arrow indicates an increase of UFH concentrations following flushing of the aorta with UFH preparations.

No anti-Xa activity was measurable in any of the samples before administration of heparin. Five minutes after heparin infusion, the mean heparin level in the UFH group

and the LMWH group were 0.88 ± 0.43 anti-Xa U/ml and 1.06 ± 0.23 anti-Xa U/ml respectively. This difference was not significant.

After removal of the clamps and establishment of flow the mean anti-Xa activity in the LMWH group (0.30 ± 0.14 U/ml) was significantly higher ($p<0.05$) when compared with the UFH group (0.21 ± 0.27 U/ml). The anti-Xa activity (mean ± SD, in U/ml) of the samples drawn at 20, 35, 50, 65, 80 and 95 minutes after heparin administration were: 0.67 ± 0.29 and 0.77 ± 0.18; 0.53 ± 0.28 and 0.59 ± 0.18; 0.44 ± 0.27 and 0.49 ± 0.17; 0.38 ± 0.24 and 0.41 ± 0.17 ;0.40 ± 0.29 and 0.31 ± 0.15; 0.24 ± 0.25 and 0.32 0.09 ± 0.09 for UFH and LMWH respectively.

The anti-Xa levels (mean ± std) at 110 minutes after heparin administration were significantly higher ($p<0.05$) in the LMWH group (0.25 ± 0.11 U/ml) when compared with the corresponding values in the UFH group (0.05 ± 0.09 U/ml).

APTT measurements

UFH group

The intraoperative APTT values in the samples drawn before heparin administration were 51.7 ± 18.2 seconds. Five minutes after heparin administration the APTT was more than 300 seconds (which was the maximal detectable time in our assay) in 14 patients. Six patients had APTT levels ranging from 129 to 160 seconds. After establishment of flow, 9 patients still had APTT levels above 300 seconds. Because the maximum measurable APTT value in our assay was 300 seconds, it was not possible to calculate Pearsons correlation coefficients for comparing the APTT values with the anti-factor Xa activity.

LMWH group

The mean APTT intraoperatively before heparin administration was 51.6 ± 18.4 seconds. The mean APTT value 5 minutes after heparin administration was 156 ± 76 seconds, which is significantly lower ($p<0.001$) when compared with the corresponding values in the UFH group. None of the patients had APTT levels above the maximal detectable level of 300 seconds. After removal of the clamp and establishment of flow the APTT was significantly ($P<0.05$) diminished to 124 ± 46 seconds. No correlation

was observed between the APTT levels and the anti-Xa activity (r=0,22; p=ns).

Fibrin degradation products

The median FbDP levels of the samples drawn before heparin administration were 0.630 ug/ml (range: 0.15-2.3 ug/ml) in the UFH group and 0.798 ug/ml (range: 0.15-13.2 ug/ml) in the LMWH group. As can be seen in figure 2, the mean FbDP levels increased significantly during operation in both groups (UFH group: $p<0.01$; LMWH group: $p<0.05$). After completion of the anastomosis and removal of the clamps, the median and range (in ug/ml) of the FbDP values were 0.956 (0.10 - 3.46) in the UFH group and 1.53 (0.26 - 28.0) in the LMWH group. The between groups differences of the FBDP values were not significant. Pearsons correlation coefficient for the FbDP levels and the anti-Xa activity was 0.33 ($p<0.05$) in the LMWH group. No correlation was found (r=0.014, p=ns) for FbDP levels and anti-Xa activity in the UFH group.

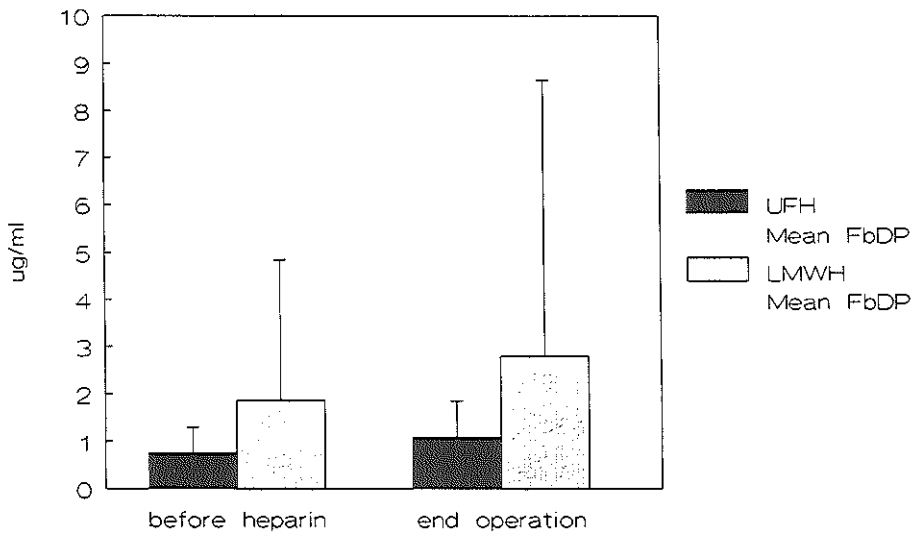

Figure 2 Intraoperative FbDP plasma levels (ug/ml) before heparin administration and at the end of operation.

Reversal of heparin activity

Protamine chloride was administered in 19 cases (8 receiving UFH and 11 receiving LMWH). The dosages varied between 20 and 40 mg. Two patients received protamine when heparin activity had already disappeared. No heparin was detectable after protamine administration in UFH-patients. Six of the 12 UFH-patients who did not receive protamine still had measurable heparin levels (range: 0.1-0.66 IU/ml) at the end of operation. Heparin activity was measurable in 9 of the 11 LMWH-patients who had received protamine (range: 0.05-0.42 IU/ml). At the end of operation heparin was detectable in all LMWH-patients who had not received protamine (range: 0.05-0.46 U/ml).

Intraoperatively, no thrombotic or bleeding complication occurred in any of the patients. Postoperatively, two patients of the LMWH group died because of a poor lungfunction and asystole respectively. One patient in the UFH group underwent relaparotomy because of postoperative blood loss. The cause of blood loss was not found. The anti-Xa activity at the end of operation in this patient was 0.65 U/ml and the corresponding APTT value was more than 300 seconds. The preoperative APTT value in this patient was within the normal range.

The APTT value intraoperatively before heparin administration was 47.3 seconds. Five minutes after heparinization the APTT value reached the maximum detectable value of 300 seconds and remained on this level until the end of operation.

DISCUSSION

Bypass grafting has become the most widely applied and effective method of treating aortoiliac disease[25]. In aortobifemoral bypass grafting systemic heparinization is common practice that attempts to prevent distal thrombotic complications during and after temporary occlusion of the aorta. The use of heparin in these procedures is mainly based on empirical grounds and its value has never been evaluated in a controlled prospective trial.

Intraoperative heparin monitoring showed that the current methods for administering heparin may not provide an adequate degree of anticoagulation in all patients[2-4,7].

Information gained from heparin monitoring provides insight into the actual level of anticoagulation and may be used to predict the amount of additional dosages of heparin if required. Whether this is effective in reducing the risks of complications due to large individual differences in heparin response and elimination rate needs to be determined.

Another approach to overcome some of the heparin related complications may be the use of heparins with different pharmacological properties. In the current study we compared the pharmacokinetics of intravenously administered LMWH and UFH during aortobifemoral bypass prosthesis operations. At five minutes after heparin administration, no difference was observed in the anti-Xa activities induced by UFH and LMWH. The interindividual variation of anti-Xa activity in the LMWH group was smaller (SD:0.23) than in the UFH group (SD:0.43) and the anticoagulant effect, expressed as anti-factor Xa activity, was more sustained in the LMWH group: at 110 minutes after administration, the levels were still in the therapeutical range of 0.2-0.5 IU/ml as defined by others[26,27].

Surprisingly, the disappearance curves of the two heparins used in this study showed quite a similar shape as measured with the anti-Xa assay. This has not been reported before.

We are aware of only one study that compared the effect of a LMWH and UFH during aortic graft surgery[28]. In that study heparin was not administered intravenously before clamping, but injected into the aorta just distal to the clamp, which resulted in delayed appearance of peak levels of heparin activity.

Which conditions existing in this study could have affected the pharmacokinetic behaviour of the heparin fragments? Firstly, shortly before completion of the anastomosis, the graft was flushed with variable amounts of a fluid containing 25 IU of UFH per ml normal saline solution. Since the two heparin assays employ different calibration curves, it is possible that the flushing of the graft is only detected in the UFH calibration curve (see arrow in figure 1) and hence may influence the pharmacokinetic profile of UFH rather than that of LMWH.

Secondly, the pharmacokinetics of the two heparins probably have a different metabolic basis. It has been suggested that elimination of heparin from the circulation is the result

of a combination of a saturable and a linear clearance curve[14]. The saturable mechanism involves binding of heparin to endothelial cells and uptake into the reticulo-endothelial system[29-31]. The non-saturable mechanism ("linear clearance") could be related to renal clearance[32]. High molecular weight forms of heparin are preferably eliminated by the saturable mechanism[33], while LMWH is predominantly cleared from the circulation by the non-saturable renal route[34]. This model is supported by the observation in patients that severe chronical renal insufficiency did not alter the half-life of UFH, but strongly prolonged that of LMWH[35]. Also, the prolongation of the half-life of LMWH in nephrectomized rabbits was much more pronounced than that of UFH[36]. Therefore it is conceivable that the large amounts of fluids infused during aortic surgery could have affected the renal clearance route of heparin, which would especially alter the pharmacokinetic behaviour of LMWH and not that of UFH. This is in agreement with the observation by others that the decay curves of UFH in patients undergoing vascular surgery are similar pre-and intraoperatively[10].

The finding that LMWH prolongs the APTT to a lesser extent than UFH and that the APTT values were not correlated with the anti-factor Xa levels in the LMWH group is in accordance with other studies[21,37,38].

FbDP levels in plasma, i.e. cross-linked and non cross-linked derivatives of fibrin, increased significantly in both groups. This has also been reported by others using a different assay specific for cross-linked fibrin degradation products[39-42]. The peroperative rise of FbDP levels could be explained by the surgical trauma which is known to trigger blood coagulation mechanism[43] but may also be caused by lysis of e.g. intravascular thrombi. The large standard deviation of the FbDP levels in the LMWH group (6.01 ug/ml) can be explained by the observation of very high FbDP values in one patient (13.2 ug/ml before heparin administration and 28.0 ug/ml after removal of the clamp). No clinical abnormalities were found in this patient.

This study confirms earlier reports that administration of protamine on empirical basis is inadequate[4,11] and that the anti-factor Xa activity of LMWH is, in contrast to that of UFH, not completely reversible by protamine (detectable levels ranged from 0.05 to 0.42 IU/ml). This has clinical relevance because observations from clinical studies

showed that plasma concentrations exceeding 0.4 anti-factor Xa IU/ml produce a critically enhanced risk of bleeding[44]. In our study no bleeding accident occurred in any of the patients receiving LMWH and the blood loss was comparable in both groups. In conclusion, following an intravenous bolus injection, the pharmacokinetic profiles of LMWH and UFH show a similarity in patients undergoing aortobifemoral bypass grafting.

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Chapter 6

DIAGNOSTIC VALUE OF QUANTITATIVE TESTS FOR FIBRIN DEGRADATION PRODUCTS IN DEEP VENOUS THROMBOSIS

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SUMMARY

Laboratory diagnosis of deep venous thrombosis (DVT) has regained interest through development of enzyme immuno assays (EIA's) based on monoclonal antibodies for detection of fibrin degradation products (FbDP's) in plasma. This study was undertaken to evaluate the diagnostic value of these new assays in the diagnosis of DVT on the basis of reported data in literature using receiver operating characteristics (ROC) methodology and Bayesian analysis. Comparing test characteristics of the D-dimer assay for DVT at cutoff values as reported in literature showed a significant difference between test performance in hospitalized and outpatients. By applying Bayes' rule on pooled data of hospitalized and outpatients separately, the relationship was calculated between D-dimer values, pre-test and post-test probabilities of DVT. It was concluded that test performance of the D-dimer assay for DVT is different in outpatients and hospitalized patients. Current diagnostic concepts concerning FbDP assessment in patients suspected for deep venous thrombosis should be adapted.

INTRODUCTION

Laboratory assessment of fibrin and fibrinogen degradation products (FDP's) in deep venous thrombosis (DVT) has been practised for several years using different techniques (1-7). Most of these assays could only be performed on serum samples using polyclonal antibodies which cross-react with fibrinogen. Because serum is a source of artefacts (8), results obtained by these serum-based assays for FDP's should be interpreted with caution (9). As a consequence, the initial suggestion that determination of FDP's in serum could be of value in the diagnosis of DVT was abandoned.

The issue of measuring FDP's in patients with DVT has regained interest through the development of quantitative enzyme immuno assays (EIA's) for their assessment (10-15). These EIA's employ monoclonal antibodies against specific fibrin and fibrinogen epitopes which do not crossreact with fibrinogen. Furthermore, these assays permit discrimination between degradation products of fibrin (FbDP's) and fibrinogen (FgDP's) and can be performed on plasma samples, thereby avoiding the problems inherent in the use of serum. Clinical experience with the new assays is steadily growing and it is becoming clear that diagnostic concepts concerning FbDP assessment in patients suspected for DVT may have to be adapted. Therefore an evaluation of the diagnostic characteristics of these assays in patients suspected for DVT seems appropriate.

Diagnostic tests are often characterized using the indices of sensitivity, which is the probability that the test result is positive in patients who have the disease (also called true positive rate) and specificity, which is equivalent to the probability that the test is negative in patients who do not have the disease (also called true negative rate). Most of the published studies concerning DVT and the new assays for FbDP assessment have reported sensitivity and specificity based on single cutoff points to distinguish between diseased and non-diseased. The use of different cutoff points makes direct comparisons between studies difficult. Furthermore, the appropriate cutoff value in clinical practise depends on the patient population in which the test is used.

By dichotomization of test results, the test is either positive or negative, valuable information may be lost, eg. suppose a hypothetical cutoff point of 20; in this case a

test result of 10.000 will be interpreted in exactly same way as a value of 21, although the probability of disease may be considerably higher. A useful parameter to characterize clinical information without dichotomization is the likelihood ratio (LR), which is the ratio of the probability of a test result in diseased persons and the probability of the same test result in nondiseased persons (21). Another way, although also based on dichotomization, to obtain more information upon a diagnostic test is to determine sensitivity and specificity for different cutoff points. This is possible with EIA's for FbDP's since they are quantitative tests yielding results distributed on a continuous scale, which may be reduced to an ordinal scale by categorizing the test variable. In this way, a variety of pairs is generated which can be plotted as a set of points in a unit square. This constitutes a receiver operating characteristic (ROC) curve. The area under a ROC curve is a measure of test performance and may be used for comparing tests (16-20).

The post-test probability of disease can dramatically be affected by the prevalence or pre-test probability of disease: increase or decrease of disease prevalence will be accompanied respectively by a rise and fall of post-test probability. Hence, when interpreting test results one should be aware of disease prevalence in the studied population. The relation between test result, pre-test and post-test probability of disease can be determined with Bayes' Theorem (21,22).

The aim of this study was to evaluate the diagnostic value of the new assays for DVT by use of ROC methodology and Bayesian analysis on published data obtained in patients presenting with symptoms suggesting DVT.

MATERIALS AND METHODS

Materials

The English language medical literature was searched for articles that have assessed diagnostic test characteristics of EIA's for fibrin degradation products in DVT. Because the first report that described an EIA for fibrin degradation products based on monoclonal antibodies was published in 1983 (11), we conducted a MEDLINE search

from january 1983 to january 1991 for articles discussing quantitative assessment of fibrin degradation products in plasma and deep venous thrombosis. In addition we reviewed the bibliographies of the articles found by the above method. Each article resulting from this search was screened and assessed for study design. Our criteria for inclusion of the articles in this study were: a blind comparison of the results of the index test with the current gold standard (ascending venography); a prospective evaluation of consecutive patients presenting with signs or symptoms suggesting DVT; a blind interpretation of test results and clear definition of criteria for test positivity of both index test and gold standard test.

Methods

ROC curves were constructed on the basis of data presented in each study fulfilling the inclusion criteria. True positive rates (or sensitivity) were plotted on the abcis, false positive rates (or 1 minus specificity) on the ordinate. Cutoff points were chosen in such a way that the pairs of true-positive rates and false negative rates were evenly spaced along the ROC curves. The area under the ROC-curve was calculated by connecting the calculated points and adding the areas of the trapezoids underneath each part of the curve. The standard errors associated with the areas were determined according to the Hanley-McNeil algorithm (17). In comparing the area under two ROC curves, the two sample t-test was used. Within studies that determined test characteristics for hospitalized and outpatients separately, a comparison was made between the proportions of nonDVT inpatients and nonDVT outpatients with D-dimer levels exceeding the reported cutoff value. In testing differences between proportions, the Chi-square test for independent samples was used. All reported significance levels were two-sided and any probability less than 0.05 was considered to represent a significant difference.

Post-test probabilities of DVT were calculated for different prevalences of DVT by use of the Odds form of Bayes' rule (21,22) which can be written as:

$$\frac{P(DVT|T)}{P(noDVT|T)} = \frac{P(DVT)}{P(noDVT)} \times \frac{P(T|DVT)}{P(T|noDVT)}$$

where P(DVT|T) is the post-test probability of DVT given a test result T and P(DVT) is the prevalence of DVT in the population under investigation; P(T|DVT) is the probability of a test result given the presence of DVT; P(noDVT|T) is the probability of DVT absence given a test result and P(T|noDVT) is the probability of finding a test result given the absence of DVT. The quotient of P(T|DVT) and P(T|noDVT) is called the likelihood ratio (LR).

RESULTS

From a total of fifteen retrieved papers (13,14,23-35), four studies met our criteria for inclusion (32-35). Nine papers were not included in our study because test performance was evaluated in a population consisting of patients with confirmed DVT (13,14,23-29), one because impedance plethysmography was used to confirm DVT (30) and one because only results of the semiquantitative latex agglutination assay were reported (31). The results of the published reports that were included in our study are summarized in Table 1. All studies used the D-dimer assay for assessment of FbDP plasma levels. Results were calculated for hospitalized and outpatients separately in case this had not been done by the authors and could be extracted from the available data. Figure 1 shows the relative frequency distributions of the categoric D-dimer values in outpatients and hospitalized patients separately. As can be seen, most outpatients without DVT has lower D-dimer levels compared with nonDVT inpatients, whereas D-dimer levels of inpatients with confirmed DVT were in general higher than those of outpatients. The areas of the ROC curves of individual studies obtained in outpatients were statistically not different ($0.2 < p < 0.35$). The same was true for the ROC areas of inpatients ($p=0.23$). Data of individual studies were pooled for hospitalized patients and outpatients separately. Comparison of the areas of the two pooled ROC curves showed that the ROC curve for inpatients (area: 0.80 ± 0.05) was

Table 1 Test characteristics of the D-dimer assay in patients suspected for DVT as reported in literature

Size	Population (ref)	Capture antibody	Cutoff value (ng/ml)	Prevalence of DVT	Sensitivity	Specificity
50	inpatients (32)	DD-3B6/22	250	52%	100%	17%
54	outpatients (32)	DD-3B6/22	250	35%	100%	66%
108	outpatients (33)	DD-3B6/22	200	36%	97%	65%
108	outpatients (33)	anti-FDP-D.D.	500	36%	97%	62%
25	outpatients (34)	anti-FDP-D.D.	200	20%	100%	50%
25	outpatients (34)	anti-FDP-D.D.	500	20%	86%	72%
28	inpatients (34)	anti-FDP-D.D.	200	50%	100%	7%
28	inpatients (34)	anti-FDP-D.D.	500	50%	100%	14%
58	in+outpatients (35)	DD-3B6/22	200	41%	100%	47%

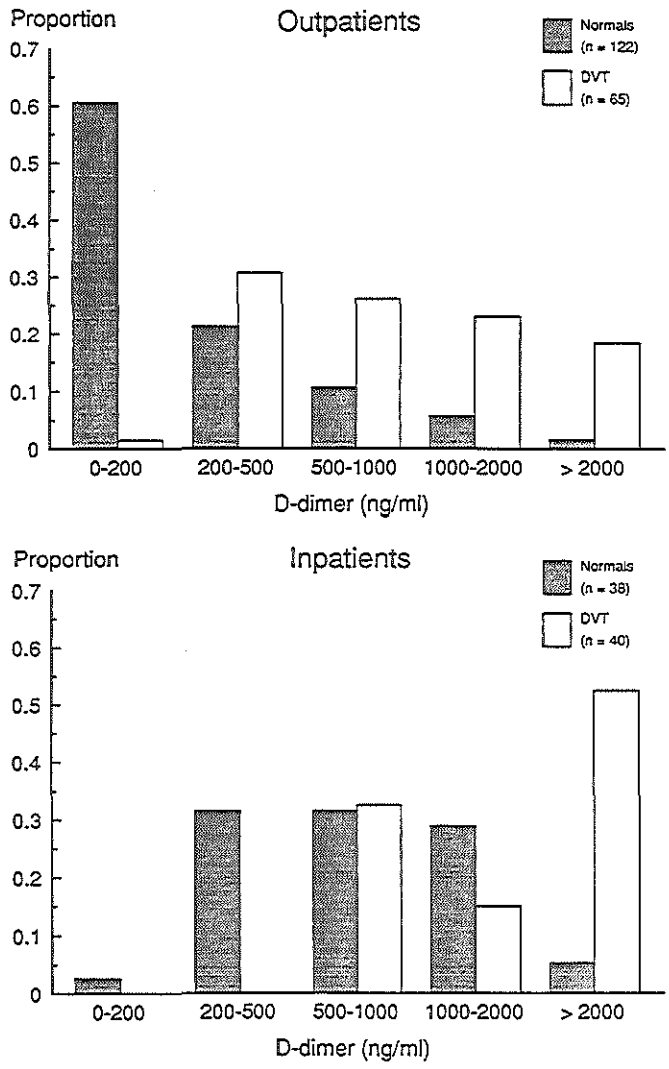

Figure 1 Relative frequencies of categoric D-dimer values in outpatients and inpatients with symptoms suggesting DVT.

not as good as for outpatients (area: 0.86 ± 0.03), although the difference was statistically not significant (p=0.14). The area of the ROC curve based on reported data obtained in a mixed population of hospitalized and outpatients (35) was not

significantly different as compared with that of pooled ROCs of inpatients (p=0.48) and outpatients (p=0.16). The pooled ROC curves are shown in Fig 2.

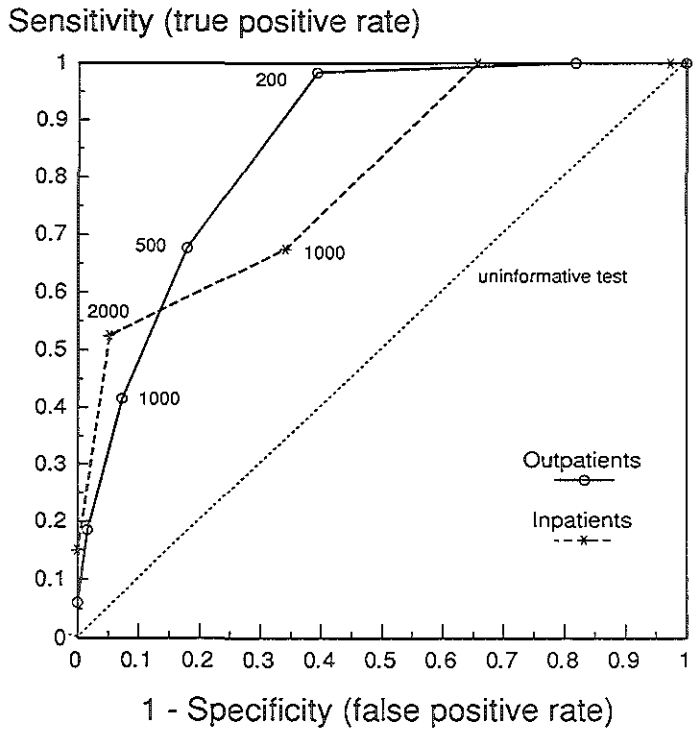

Figure 2 Roc curves of D-dimer in outpatients and inpatients for detection of DVT.

Two studies determined test characteristics of the D-dimer assay for hospitalized and outpatients separately (32,34). One of these studies showed that the proportion of nonDVT patients with D-dimer levels above the reported cutoff value of 250 ng/ml was significantly higher (X^2=11.48, p<0.001) in the subgroup of inpatients when compared with outpatients (32). This finding was confirmed in the other study (34) using cutoff values of 200 ng/ml (X^2=4.89, p<0.05) and 500 ng/ml (X^2=6.64, p<0.01). The relationship between D-dimer value, pre-test and post-test probabilities of DVT is shown in Table 2. Because of the relatively small sample size of inpatients, LR's in this

A. Outpatients

Pre-test probability of DVT	Post-test probability of DVT D-dimer levels				
	<200	200-500	500-1000	1000-2000	>2000
10%	0.3%	14%	21%	31%	56%
15%	0.4%	20%	30%	42%	67%
20%	0.6%	27%	38%	50%	74%
25%	0.8%	33%	45%	57%	79%
30%	1%	38%	51%	63%	83%
40%	2%	49%	62%	73%	89%
50%	3%	59%	71%	80%	92%
60%	4%	69%	79%	86%	95%
70%	6%	77%	85%	90%	96%

B. Inpatients

Pre-test probability of DVT	Post-test probability of DVT D-dimer levels				
	<200	200-500	500-1000	1000-2000	>2000
10%	1%	2%	5%	11%	86%
15%	2%	4%	7%	16%	91%
20%	3%	5%	10%	22%	93%
25%	3%	7%	13%	27%	95%
30%	4%	9%	16%	32%	96%
40%	7%	13%	23%	43%	97%
50%	10%	18%	31%	53%	98%
60%	14%	25%	40%	62%	99%
70%	20%	34%	51%	72%	99%

Table 2 Relationship between D-dimer levels (ng/ml), pre-test and post-test probability of DVT in outpatients (A) and inpatients (B) presenting with symptoms suggesting DVT.

subgroup were obtained after applying logistic regression analysis on the square roots of the values. As can be seen from table 2, a rise of disease prevalence is accompanied by an increase of disease probability in each categoric D-dimer value. Furthermore, the presence of disease becomes more probable with higher D-dimer values for each of the

pre-test probabilities.

DISCUSSION

This study was undertaken to determine the role of measuring FbDP's plasma levels in the diagnosis of DVT. We used ROC methodology and Bayesian analysis on published data to assess diagnostic test performance of EIA's for FbDP's in patients clinically suspected of having DVT. ROC analysis is a convenient method for comparing diagnostic test performance and may provide insight into the practical use of diagnostic tests, because it shows various pairs of sensitivity and specificity which can be obtained by applying different cutoff values. Although test characteristics are claimed to be independent of pre-test probability, reported sensitivities and specificities differ greatly between inpatients and outpatients (Table 1). After transforming data into ROC curves, it was demonstrated that the areas of individual studies obtained in outpatients and inpatients were comparable, which suggests that test results are comparable within these subgroups.

As mentioned, the area of the pooled inpatients ROC curve was smaller as compared with that of outpatients, although this difference was not significant. Comparing sensitivities and specificities in hospitalized and outpatients at reported single cutoff points showed, however, that test performance of the D-dimer assay was not comparable between these subgroups. This is illustrated in Table 2: In case the prevalence of DVT is 20%, the finding of a D-dimer value between 200 and 500 ng/ml predicts the presence of DVT in outpatients with a probability of 27%, whereas the probability for DVT in hospitalized patients is only 5%. The D-dimer distributions of both DVT and nonDVT inpatients are shifted to the right when compared to outpatients (Fig.1). A possible explanation for this difference may be a higher incidence of co-morbodity interfering with the coagulation system in hospitalized patients suspected for DVT.

In the diagnostic process the clinician, after having been supplied with a numerical value of the D-dimer test result, is interested in the probability of DVT. This post-test probability, however, depends on two conditions: 1. given a certain test value, the post-

test probability of disease will change in the same direction with the prevalence. 2. given a certain pre-test probability, the probability of disease varies with the test value. The relation between test result, pre- and post-test probability can be calculated with Bayes' rule and is illustrated in Table 2. Suppose an outpatient with a pre-test probability for DVT of 20% (prevalence in outpatients); in this case the finding of a low D-dimer value, eg <200 ng/ml, implies that the probability of DVT reduces to 0.6% (i.e. the probability for DVT absence is ± 99%), whereas a D-dimer value of 1500 ng/ml indicates the absence of DVT with a probability of 50%. Even so, in case of a fixed D-dimer result of 400 ng/ml, the probabilities for absence of DVT may range from 23% to 86%, depending on the pre-test probability (10%-70%) in the studied outpatient population (Table 2). After the test result is known, the physician must choose between doing nothing (ruling out the diagnosis), obtain additional diagnostic information or treat. The decision to withhold therapy depends on the no-treatment threshold, which is a measure of the balance between the benefits of treatment to diseased patients (B) and the risks of treatment to healthy patients (R). The optimal cutoff point of a diagnostic test can be determined by applying Bayes' rule and the specification of a risk/benefit ratio. The latter is difficult to estimate because life expectancy and quality of life should also be taken into account in assessment of a treatment risk/benefit ratio. However, it is obvious that the treatment threshold probability for DVT is low, i.e. it has high benefit to patients with DVT (prevention of fatal pulmonary embolism) and relatively low risk (bleeding) to nonDVT patients. The relationship between post-test probability for DVT and the risk/benefit ratio is described in the following formula:

$$\frac{P(DVT|T)}{P(noDVT|T)} = \frac{R}{B}$$

Suppose the benefit of treatment is 5 times higher than the risk, then the threshold post-

test probability for DVT is ± 17%. The corresponding cutoff value can now be derived from Table 2 for each pre-test probability of DVT, e.g. in hospitalized patients with a pre-test probability of 40%, the appropriate cutoff value would be 500 ng/ml; the treatment threshold is not reached in case the pre-test probability for DVT is 70%. The relationship between post-test probability and D-dimer values for the above mentioned pre-test probabilities of DVT in hospitalized patients is shown in Figure 3.

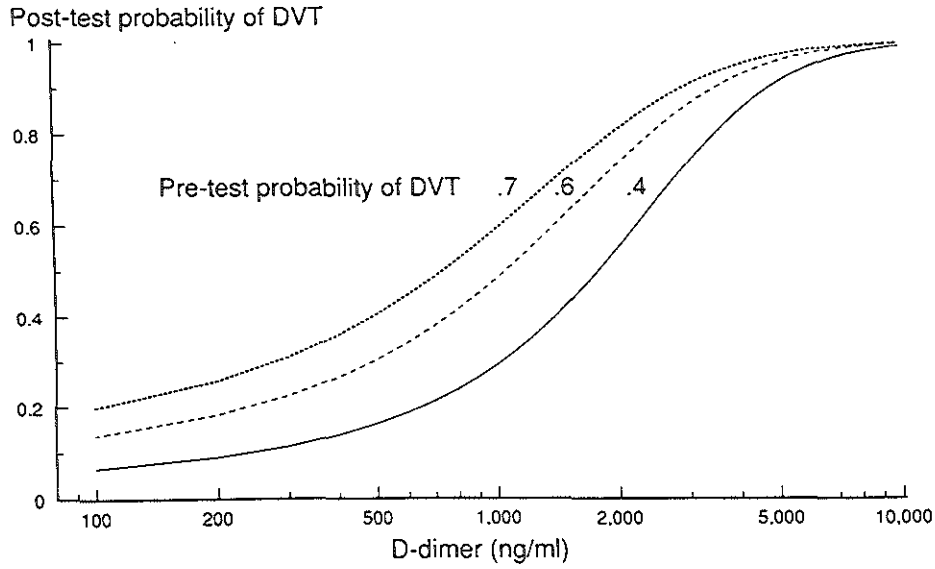

Figure 3 Relationship between post-test probability and D-dimer levels (ng/ml) at fixed pre-test probabilities of DVT in hospitalized patients.

This is just an example of how a cutoff point could be assessed and does not necessarily imply that this should be the optimal cutoff point for clinical use. The appropriate cutoff point depends on the patient population in which the test is used: eg. the pre-test probability of DVT in hospitalized patients after total hip replacement is considerably higher as compared with that of outpatients, hence the cutoff point

necessary to obtain an equal post-test probability for absence of DVT must be higher in the hospitalized group.

In conclusion, evaluating D-dimer test results with ROC methodology on reported data demonstrated that D-dimer test characteristics were comparable for individual studies in outpatients and hospitalized patients separately. The D-dimer assay performs significantly different in outpatients when compared with hospitalized patients in the diagnosis of DVT at reported cutoff points.

By applying Bayes theorem on pooled data of in- and outpatients, posterior probabilities for DVT could 3be calculated in relation to categorized D-dimer values and different prior probabilities. Based on this analysis, the optimal cutoff point can be calculated by combining Bayes rule and estimating a treatment risk/benefit ratio. A reconsideration of the clinical utility of FbDP assessment in the diagnosis of DVT seems warranted on the basis of available evidence on test characteristics.

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Chapter 7

Discussion

Summary

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Acknowledgements

Curriculum vitae

DISCUSSION

During the last decades insight into the mechanism underlying the formation of fibrin and its degradation has increased markedly. This progress has provided a basis for the development of techniques for assessment of the two opposing processes of coagulation and fibrinolysis.

There are many components in blood which influence the overall fibrinolytic activity which can be measured in a clinical setting. However, three markers of fibrinolysis have emerged as being of paramount importance. These are tissue plasminogen activator (t-PA), plasminogen activator inhibitor (PAI) and fibrin/fibrinogen degradation products (FDP). While speculation may still exist about the role of measuring activators and inhibitors of plasminogen, there is agreement that fibrinolysis is reflected by generation of fibrin and fibrinogen degradation products. Therefore, the most tangible evidence for ongoing fibrinolysis in blood is the formation of fibrin an fibrinogen degradation products.

The main difference between the formerly used serum based assays for FDP assessment and the newly developed assays is, that the latter assays can be carried out in plasma using monoclonal antibodies, which makes it possible to avoid serum artifacts. Furthermore, these new plasma assays permit discrimination between degradation products of fibrin and of fibrinogen (chapter 1).

We found that fibrinogen degradation products (FgDP) are generally well correlated with degradation products of fibrin (chapter 2). One may speculate about the origin of the plasma FgDP fraction: it is unknown whether this FgDP fraction originates from circulating fibrinogen or the fibrin-fibrinogen complex in the circulation. It is possible that plasmin, which lysis fibrin, also digests a part of the fibrinogen molecule before its action is terminated by α_2-antiplasmin. The observation of good correlations between the FgDP fractions and degradation products of fibrin suggests that primary fibrinogen digestion in the circulation is a frequent event, which is associated with secondary fibrinolysis.

We examined the role of the D-dimer assay for the diagnosis of deep venous

thrombosis (DVT). Analysis of published studies showed that test performance of the D-dimer assay for DVT at reported cutoff values was different in hospitalized patients when compared with outpatients (chapter 6). This can be explained by the higher incidence of co-morbidity interfering with the coagulation system in the first group. It was demonstrated that very low levels of D-dimer could predict the absence of DVT, whereas very high levels of D-dimer confirmed the presence of DVT. This finding suggests that the D-dimer assay may be used for the diagnosis of DVT, but is in contrast to the results of our study with the D-dimer assay in outpatients, which was performed at the Thrombosis Service Centre of Rotterdam (chapter 3). The test characteristics of the D-dimer assay in this study (sensitivity 92%, specificity 21%, negative predictive value 88% ,positive predictive value of 28%) were disappointing. This discrepancy may be explained that the fact that IPG was used to confirm DVT instead of ascending venography, which is less sensitive detecting calf vein thrombosis. The effectiveness and feasibility of the D-dimer assay for the diagnosis of deep venous thrombosis can be demonstrated by a management study. Other clinical applications of the assays for fibrin and fibrinogen degradation products may concern the monitoring of treatment (chapter 5) and the course of disease (chapter 4). In general, detection of fibrin(ogen) derivatives by monoclonal antibody based assays provides valuable information on haemostasis in diseases associated with an activated state of coagulation.

SUMMARY

This thesis comprises clinical and methodological studies employing newly developed plasma assays for assessment of fibrin and fibrinogen degradation products (FDP's) on patients with diseases or conditions characterized by an activated state of coagulation. In the introduction the mechanism of the haemostatic balance is briefly summarized based on current insights and it is described that disturbance of this balance may have two different clinical outcomes: bleeding or thromboembolism. The importance to obtain information regarding the state of the haemostatic balance in patients at risk for developing thromboembolism and/or bleeding is emphasized. The aims of the studies are defined.

In chapter 1 the disadvantages of existing serum FDP assays, based on polyclonal antibodies, are described. The newly developed FDP assays employ monoclonal antibodies which do not cross-react with fibrinogen and can be performed on plasma samples, thereby avoiding the disadvantages inherent in the use of serum samples. A summary is given of the currently available FDP assays in plasma and their clinical relevance has been reviewed as reported in literature. The aims of the studies were defined on the basis of this review.

In chapter 2 we have evaluated four different plasma FDP assays in healthy volunteers, patients with cirrhosis of the liver and patients suspected for deep venous thrombosis (DVT). Plasma FDP levels, as assessed by different assays, showed a good correlation in each (sub)group of patients. In general, the correlations between plasma FDP assays were lower in normals than in patients. It was first seen that secondary fibrinolysis appeared to be associated with fibrinogenolysis in cirrhosis of the liver and DVT.

In chapter 3 the diagnostic value of the D-dimer assay for deep venous thrombosis (DVT) is studied in 232 outpatients clinically suspected of having DVT. The Sensitivity and specificity of the D-dimer assay for DVT were 92% and 21% respectively. The DVT prevalence was 25% and the predictive value of a negative and positive test result were 88% and 28% respectively. It was concluded that the D-dimer assays has limited value, either to confirm or to exclude DVT.

In chapter 4 the fibrinolytic response in patients with gram-negative septic shock is studied. In the past, disturbance of haemostasis in septic shock was considered to depend largely upon extreme activation of the coagulation pathway. It has recently been suggested that fibrinolysis may be impaired during septic shock. Measurement of plasma FgDP and TDP plasma levels in patients with septic shock showed that the fibrinolytic activity was comparable in survivors and nonsurvivors. The process of ongoing fibrinolysis during septic shock is evidenced by elevated TDP and FgDP plasma levels. It was concluded from this study that the fibrinolytic system remains activated during septic shock.

In chapter 5 we focused on heparinization during vascular surgery. During aortic graft surgery, heparin is administered on empirical basis and is associated with a wide variation in patient response and elimination rate which makes it difficult maintain predictable or adequate levels of anticoagulation for all patients. The recently developed low molecular weight heparins and the FDP plasma assays may be used to place heparin administration during vascular surgery on a rational scientific basis. We studied the pharmacokinetics of low molecular weight heparin (LMWH) and unfractionated heparin (UFH) during aortobifemoral bypass grafting. It was demonstrated that plasma FbDP levels showed a significant rise intraoperatively in both patients groups, which could be explained by lysis of intravascular thrombi. FbDP levels correlated moderately with anti-factor Xa in the LMWH group, but not in the UFH group. Whether this may be used to define dose regimen for heparin administration needs to be determined by further studies. It was concluded that the pharmacokinetic profiles of UFH and LMWH showed a similarity after intravenous bolus injection in patients undergoing aortobifemoral bypass grafting and that the anti-Xa activity in the LMWH group was more sustained as compared with the UFH group.

In chapter 6 the value of plasma FDP assays for the diagnosis of disease is evaluated in a broader sense. This chapter comprises an analysis of published studies on the diagnostic value of the plasma FDP assays for deep venous thrombosis (DVT). Test performance of the D-dimer assay for DVT at reported cutoff values was different in hospitalized patients when compared with outpatients. The relationship between D-

dimer levels, pre-test and post-test probabilities of DVT were calculated. Very low levels of D-dimer could predict the absence of DVT, very high levels of D-dimer confirmed the presence of DVT. It was concluded that the D-dimer assay may be used in the diagnosis of DVT and current diagnostic concepts concerning FDP assessment in patients suspected for DVT should be adapted.

In the discussion has been outlined that assessment of fibrin and fibrinogen degradation products is the most tangible evidence for ongoing fibrinolysis, and secondary fibrinolysis appears to be associated with fibrinogenolysis. Whether assays for fibrin degradation products can be used in daily clinical practise for diagnosis of deep venous thrombosis can be determined by management studies. Application of the assays on patients with diseases associated with an activated state of coagulation allows detailed information on the haemostatic balance.

SAMENVATTING

In dit proefschrift worden klinische en methodologische studies beschreven naar fibrine en fibrinogeen afbraakprodukten in ziekten of condities met een gestoorde haemostase. De studies zijn uitgevoerd met recent ontwikkelde, op monoclonale antilichamen gebaseerde enzyme immuno assays.

De inleiding gaat in op het mechanisme van het haemostase evenwicht. Een verstoring van deze balans kan twee verschillende klinische gevolgen hebben: bloedingen of trombo-embolieën. Om therapeutische redenen is het van belang te weten waar het evenwicht ligt bij patiënten met een verhoogd risico op bloedingen of trombo-embolieën. De doelstellingen van de studie worden uiteengezet.

Hoofdstuk 1 geeft een literatuuroverzicht van de beschikbare laboratoriumtesten voor fibrine en fibrinogeen afbraakprodukten (FDP). De nadelen van reeds langer bestaande serum FDP assays worden besproken. De recent ontwikkelde FDP testen zijn gebaseerd op monoclonale antilichamen, die niet kruisreageren met fibrinogeen, en kunnen worden uitgevoerd met plasma samples. Hierdoor worden de nadelen, welke inherent zijn aan het gebruik van serum samples, vermeden.

In hoofdstuk 2 worden vier verschillende plasma FDP assays geëvalueerd in patiënten met levercirrhose, patiënten die worden verdacht van diep veneuze trombose (DVT) op basis van het klinisch beeld, en in niet zieke vrijwilligers. De plasma FDP concentraties, bepaald door verschillende assays, correleren goed met elkaar in de verschillende patiënten groepen, doch minder goed in de groep van niet zieke vrijwilligers. Op theoretische gronden mag worden verwacht, dat de berekende som van de FbDP (fibrine afbraakprodukten) en FgDP (fibrinogeen afbraakprodukten) concentraties gelijk is aan de bepaalde TDP (totaal aan fibrin and fibrinogeen afbraakprodukten) concentratie. Wij hebben echter geen verklaring kunnen vinden voor de discrepantie tussen de TDP waarden en de berekende som van de FbDP en FgDP concentraties in 4 patiënten met DVT en 1 patiënt met klinische symptomen van DVT, waarbij de diagnose niet kon worden gesteld. In deze studie wordt voor het eerst aangetoond, dat secundaire fibrinolyse gepaard gaat met fibrinogenolyse in levercirrhose

en DVT.

In hoofdstuk 3 wordt de diagnostische waarde van de D-dimer assay voor DVT bestudeerd. De studie werd geïnitieerd omdat er behoefte bestaat aan een simpele niet-invasieve laboratoriumtest voor het vaststellen van DVT. 232 poliklinische patiënten met verdenking op een trombose been zijn onderzocht. De sensitiviteit en specificiteit van de D-dimer assay zijn respectievelijk 92% en 21%, de voorspellende waarde van een positief en negatief test resultaat bedragen respectievelijk 88% en 28% bij een DVT prevalentie van 25%. Uit het onderzoek wordt geconcludeerd, dat de D-dimer een geringe diagnostische waarde heeft voor DVT.

In hoofdstuk 4 wordt de fibrinolytische respons beschreven in patiënten met een gram-negatieve sepsis. In het verleden werd de verstoring van de haemostase bij septische shock voornamelijk toegeschreven aan sterke activatie van de stollingscascade. Recent onderzoek suggereert, dat de fibrinolyse geremd kan zijn tijdens septische shock. Deze studie werd uitgevoerd om de mate van fibrinolytische activiteit te bepalen door het meten van de eindprodukten van de fibrinolyse. Plasma FgDP en TDP concentraties zijn verhoogd, maar niet significant verschillend in survivors en nonsurvivors van een septische shock. De studie toont aan, dat de fibrinolyse geactiveerd blijft tijdens gram-negatieve septische shock en dat de mate fibrinolytische activiteit in survivors en nonsurvivors van een septische shock vergelijkbaar is.

In hoofdstuk 5 wordt ingegaan op het probleem van heparine toediening bij aortobifemorale bypass chirurgie. Tijdens deze ingrepen wordt op empirische basis ongefractioneerd heparine (UFH) toegediend. Dit gaat gepaard met een grote variatie in patiënt respons en eliminatiesnelheid, waardoor het moeilijk is voorspelbare en adequate anticoagulantie te verkrijgen in alle patiënten. In de studie wordt de farmacokinetiek van UFH en laag moleculair heparine (LMWH) vergeleken tijdens aortobifemorale bypass operaties. Intra-operatief vindt een significante stijging plaats van de FbDP concentraties in plasma, hetgeen verklaard kan worden door lysis van intravasculaire trombi. FbDP concentraties correleren matig met de anti-Xa activiteit in de LMWH groep, maar niet met de anti-Xa activiteit in de UFH groep. Nader onderzoek is nodig om te bepalen, of de FbDP assay een bijdrage kan leveren bij het

vaststellen van een adequate LMWH dosis. Het farmacokinetisch profiel van LMWH en UFH blijkt vergelijkbaar na een intraveneuze bolus injectie. De anti-Xa activiteit in de LMWH groep houdt langer aan dan die in de UFH groep.

In hoofdstuk 6 wordt de waarde van de plasma FDP assays voor de diagnose van DVT geanalyseerd op basis van gepubliceerde studies. De testeigenschappen van de D-dimer assay voor DVT bij vermelde cutoff points zijn verschillend bij klinische patiënten in vergelijking met poliklinische patiënten. Dit kan worden verklaard door een hogere incidentie van co-morbiditeit, die interfereert met het stollingssysteem in klinische patiënten. Zeer lage D-dimer waarden zijn vrijwel bewijzend voor de afwezigheid van DVT, terwijl zeer hoge D-dimer waarden de diagnose DVT bevestigen. Plasma FDP assays kunnen een belangrijke rol gaan spelen in de laboratoriumdiagnose van DVT. In de discussie wordt onderstreept, dat de nieuwe testen voor fibrine en fibrinogeen afbraakprodukten waardevolle informatie opleveren over de heamostase in verschillende ziektebeelden. Als directe klinische toepassing wordt de diagnostiek van DVT genoemd. Andere mogelijke toepassingen betreffen het monitoren van het beloop van ziekten die gepaard gaan met een gestoorde haemostase en het monitoren van therapie.

NAWOORD

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Curriculum vitae

De schrijver van dit proefschrift werd geboren op 27 januari 1955. De studie Geneeskunde werd in 1975 aangevangen aan de Erasmus Universiteit te Rotterdam, waar hij in 1976 het propaedeutisch examen behaalde. Van 1976 tot 1983 onderbrak hij de studie voor werkzaamheden in het bedrijfsleven. Na hervatting van de studie in 1983 was hij als student-assistent werkzaam op de afdeling Anatomie van de Erasmus Universiteit te Rotterdam en op de afdelingen Interne Geneeskunde I en II van het Academisch Ziekenhuis Rotterdam-Dijkzigt. In oktober 1988 behaalde hij het artsexamen.

Van januari 1989 tot mei 1991 was hij als wetenschappelijk onderzoeker werkzaam op de afdeling Interne Geneeskunde II (hoofd: Prof. J.H.P. Wilson) van het Academisch Ziekenhuis Rotterdam-Dijkzigt en op het Centrum voor Klinische Besliskunde (hoofd: destijds Prof.dr.J.Lubsen, later Prof.dr.ir. J.D.F. Habbema) van de Erasmus Universiteit te Rotterdam. Aansluitend volgde hij de opleiding Interne Geneeskunde (opleider: Prof. J.H.P. Wilson) in het Academisch Ziekenhuis Rotterdam-Dijkzigt tot november 1991. Sedertdien is hij werkzaam als verzekeringsgeneeskundige bij de Gemeenschappelijke Medische Dienst te Rijswijk.