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DETERMINATIONS OF THE OVERALL HAEMOSTASIS POTENTIAL AND FIBRIN GEL PERMEABILITY

Method development and application in research and in clinical materials

by

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“Lascia che gli altri parlino sotto la pioggia e le nuvole,
io parlero solo soltanto il sole.”

“Let others speak when it rains and when it's cloudy,
I shall only speak when the Sun shines.”

To my Son

Determinations of the Overall Haemostasis Potential and Fibrin Gel Permeability
- Method development and application in research and in clinical materials -

Aleksandra Antovic

Coagulation Research, Dept of Surgical Sciences and Dept of Woman and Child Health, Karolinska Institutet, Stockholm, Sweden, 2004.

We have previously developed a laboratory method that may screen the ***Overall Haemostasis Potential (OHP)*** in plasma, based on spectophotometric measurement of an area under the fibrin aggregation curve in citrated plasma samples to which tiny amounts of exogenous thrombin and tissue-plasminogen activator (t-PA) are added. When the fibrin aggregation curve is created, the fibrinogen originally present in plasma is gradually converted to fibrin by the generated thrombin. At the same time, plasminogen activation produces plasmin that in turn digests fibrin. Each absorbance value (Abs) represents the fibrin level at the corresponding time point, and the area under the curve, should reflect a balance between the generation and proteolysis of fibrin throughout the measurement period.

To improve the assay sensitivity for the application in clinic or research work, various modifications were introduced. Thrombin in a decreased dose (0.04 IU/mL, compared to 0.2 IU/mL previously used) with or without t-PA was added to plasma. Areas under the two fibrin-aggregation curves i.e OHP and ***Overall Coagulation Potential (OCP)*** were thus created. A difference between the two parameters reflects the ***Overall Fibrinolysis Potential (OFP)***, calculated by (OCP-OHP)/OCP) x 100%.

The modified method has shown its usefulness in detecting hypercoagulation in normal pregnancy, preeclampsia and coronary heart disease. Increased levels of OHP were also found in women with previous thromboembolim especially related to the presence of FV Leiden mutation. Moreover, assay of this parameter can screen immediate changes in the haemostatic system after the injection of low molecular mass heparin (dalteparin) and may be used for monitoring the anticoagulant effects.

To ensure the sensitivity of the assay for determining different severity of hypocoagulation, further modifications were performed by introducing tissue factor and phospholipids to the reaction system. All the factors belonging to the two pathways of coagulation cascade, apart from FXII, affected the OHP outcome. This indicates that the modified assay system is similar to the haemostasis balance in circulating blood, and may thus become a laboratory tool to estimate bleeding tendency in haemophilic patients and distinguish pro-thrombotic cases among patients with FXII deficiency.

OHP assay is thus a quantitative method to determine the fibrin level associated with combined potential of coagulation and fibrinolysis. However studies on fibrin gel porosity may give information about quality of the fibrin network which is important in atherosclerosis. We made modifications in a flow measurement previously established by B Blombäck et al and evaluated the resultant advantages. The essential equipment was simplified and the sample volume minimized which rendered the assay easier to apply in any clinical or research laboratory settings. By using different concentrations of thrombin with, or without phospholipids, it was possible to asses whether the fibrin gel porosity depends on both thrombin generation potential and fibrinogen clotting properties or only on the latter respectively.

The modified flow measurement technique was used to determine the effects of acetylsalicylic acid (ASA, aspirin) effects on haemostasis. Fibrin gel porosity was more markedly increased during treatment with lower doses of ASA, compared to medium- / high-doses. These results are further confirmed by the findings in three-dimensional confocal microscopy where thicker fibrin fibers and larger network pores with irregular structure were observed during the low dose treatment. The greater increase in fibrin gel permeability and alterations in the structure of the fibrin network support the clinical findings of better prevention of arterial thrombosis such as in stroke and cardiovascular disease during treatment with low ASA doses such as 75mg daily, than with the higher doses.

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals (I – VI):

I. **A simple and rapid laboratory method for determination of haemostasis potential in plasma II. Modifications for use in routine laboratories and research work.**

He S, **Antovic A**, Blombäck M.

Thromb Res 2001; 103:355-361

II. **The assay of overall haemostasis potential used to monitor the low molecular mass (weight) heparin, dalteparin, treatment in pregnant women with previous thromboembolism.**

Antovic A, Blombäck M, Bremme K, He S.

Blood Coagul Fibrinolysis 2002; 13:181-186

III. **Persistent increase in overall haemostais potential in women with previous thromboembolism with or without APC resistance.**

Antovic A, Blombäck M, Bremme K, van Rooijen M, He S.

J Thromb Haemost 2003; 1:2531-35

IV. **Possibility of detecting different hypocoagulable states with a global assay of Overall Haemostasis Potential in plasma.**

Antovic A, Blombäck M, Petrini P, Holmström M, He S.

Thomb Haemost 2004; submitted.

V. **Modifications in flow measurement to determine fibrin gel permeability and the preliminary use in research and clinical materials.**

He S, Cao H, **Antovic A**, Blombäck M.

Blood Coagul Fibrinolysis 2004; in press.

VI. **Marked increase of fibrin gel permeability with very low dose ASA treatment. A new argument to use low dose ASA treatment in the prevention of atherotrombotic disease?**

Antovic A, Perneby C, Jacobsson Ekman G, Wallen NH, Hjemdahl P, Blombäck M, He S.

J Thromb Haemost 2004; submitted.

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ABBREVIATIONS

Abs-sum	a summation of the ABS values
APC	activated protein C
aPTT	activated partial thromboplastin time
ASA	acetylsalicylic acid
AT	antithrombin
CHD	coronary heart disease
CLT	clot lysis time
CLT	clot lysis time
Ct	clotting time
CTI	corn trypsin inhibitor
DIC	disseminated intravascular coagulation
DVT	deep venous thrombosis
ELISA	enzyme-linked immunosorbent assay
ETP	endogenous thrombin potential
F1+2	prothrombin fragment 1+2
FII-FXIII	coagulation factors II-XIII
LMMH	low molecular mass heparin
MI	myocardial infarction
NPP	normal pooled plasma
NPP	normal pooled plasma
OCP	Overall Coagulation Potential
OFP	Overall Fibrinolysis Potential
OHP	Overall Haemostatsis Potential
PAI-1,2	plasminogen activator inhibitors 1,2
PC	protein C
PT	prothrombin time
TEG	thromboelastography
TF	tissue factor
t-PA	tissue type plasminogen activator
UHF	unfractionated heparin
VTE	venous thromboembolism
VWF	von Willebrand factor

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INTRODUCTION

The Physiological Haemostatic System

Haemostasis is a physiological mechanism which maintains the normal fluidity of blood in the blood vessels and provides a rapid, potent, but highly localized response to vascular damage.

Normal haemostasis involves a balanced interaction of vasculature (endothelium and subendothelium), blood cells (particularly platelets) and soluble plasma proteins responsible for blood coagulation and fibrinolysis. Under physiological conditions this interaction is an ongoing process which includes a low level of consumption and synthesis of all reactants. After injury, the haemostatic response accelerates markedly in the cascade of enzymatic reactions that convert a group of plasma proenzymes into their active enzyme forms. Thrombin is the terminal enzyme of the process which converts soluble plasma fibrinogen to fibrin, which together with aggregated platelets and other blood cells forms the haemostatic plug – a natural seal to a wound.

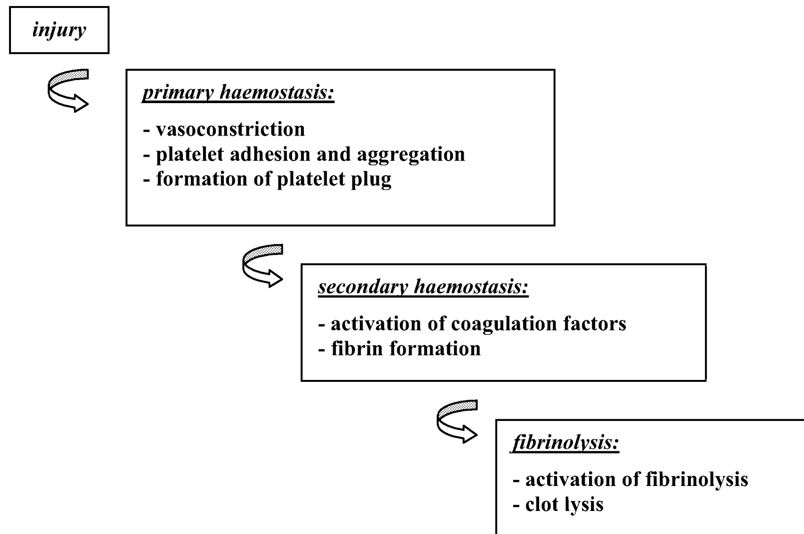


Fig 1. Events in haemostasis

1. Activation of coagulation

The initial models of coagulation introduced by Davie and Ratnoff (1964) and MacFarlane (1964) are based on cascade or waterfall processes that involve two rather independent pathways which converge to a common pathway, with thrombin generation as the end point of reactions.

According to these previously proposed models, components included in coagulation belong either to the tissue factor induced pathway, consisting of tissue factor (TF), factors VII, V, X

and prothrombin (FII), or the contact pathway, comprising prokallikrein (PK), high molecular mass kininogen (HMMK), and factors XII, XI, IX, VIII, X, V and II.

The contact pathway starts *in vitro* with activation of FXII to FXIIa by interaction with negatively charged exogenous surfaces such as glass or caolin; this activates prokallikrein (PK) to kallikrein and in the presence of high-molecular-mass kininogen (HMMK) can convert factor XI to factor XIa. However, deficiencies of FXII, PK or HMMK do not cause a clinical bleeding tendency (Sollo et al, 1985; Lammle et al, 1991) and are therefore unlikely to be required for haemostasis *in vivo*. In addition, if the contact and tissue factor pathways are separate, then the activation of factor X by the tissue factor pathway should compensate for the lack of factor VIII or factor IX. That is not the case, so the absence of factor VIII or factor IX results in bleeding.

Our understanding of the *in vivo* coagulation process has changed a good deal in recent decades. Under physiological conditions, coagulation is now considered to be initiated when a lesion in the vessel wall brings plasma into contact with TF and coagulation activation *in vivo* is believed to be mediated by TF pathway. At present, the so-called cell-based model of coagulation (Hoffman and Monroe, 2001) envisages three overlapping phases of coagulation i.e. initiation, amplification and propagation, as mentioned below:

The cell based model of coagulation

The ***initiation step*** of coagulation's tissue factor pathway occurs on TF-bearing cells such as fibroblasts and monocytes which come into contact with blood at the site of vascular injury (Hoffman and Monroe, 2001). Small amounts of circulating activated factor VII (FVIIa) (Morrissey et al, 1993) bind to exposed TF (Broze, 1982), forming TF-FVIIa complexes which activate inactive factor VII to factor VIIa (FVIIa) as well as factor X (FX) to factor Xa (FXa) (Jesty and Silverberg, 1979) and factor IX (FIX) to factor IXa (FIXa) (Osterud and Rappaport, 1977) on the cell surface. FXa activated by the TF-FVIIa complex is rapidly inhibited by tissue factor pathway inhibitor (TFPI) (Broze, 1987). However, sufficient FXa remains on the cell surface to activate factor V (FV) to factor Va (FVa), to combine with this and to produce small amounts of thrombin, which play an important role in platelet activation (Monroe et al, 1996).

Subsequent reactions, during the ***amplification stage***, are localized on the phospholipid surface of platelets that are activated and localized at the site of vascular injury. Von Willebrand factor / factor VIII complex binds to activated platelets, where factor VIII (FVIII) is released and activated by thrombin to factor VIIIa (FVIIIa) (Hultin, 1985). During activation, platelets also release FV from α granules and FV is then activated by FXa or thrombin (Monkovic et al, 1990).

The propagation stage of coagulation starts with formation of procoagulant complexes on the phospholipid surface. FIXa, together with FVIIIa and Ca^{++}, forms the "tenase" complex, which activates FX to FXa on the platelet surface. Thereafter, the "prothrombinase" complex comprises FXa, FVa and Ca^{++}, leading to the formation of large amounts of thrombin. Thrombin is a potent enzyme with many functions. It converts fibrinogen to fibrin monomers, activates factor XIII to FXIIIa, which is essential for fibrin polymerization, and further promotes coagulation by feedback activation of factor XI (FXI) to factor XIa (FXIa) on the platelet surface (Galiani and Broze, 1991). FXI can then provide additional FIXa on the platelet surface. Subsequently there is a final burst of thrombin. The end event in normal haemostasis is thrombin-catalysed conversion of soluble fibrinogen to insoluble fibrin.

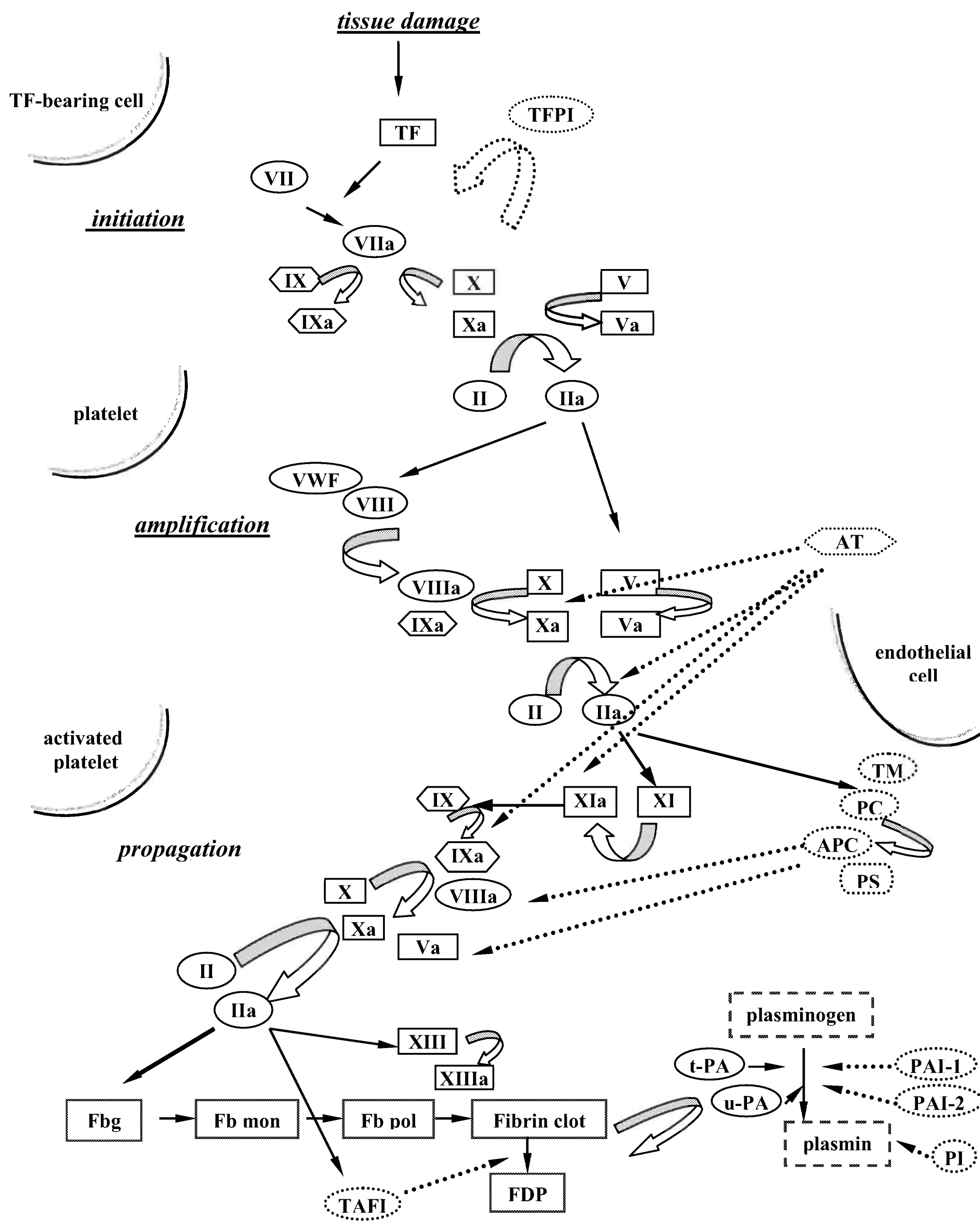


Figure 2. Simplified presentation of cell-based model of coagulation and fibrinolysis.

→ activation or stimulation; ⋯→ inhibition or degradation

TF – tissue factor**; TFPI** – tissue factor pathway inhibitor; **AT** – antithrombin; **VWF** – Von Willebrand factor; **IIa** – thrombin; **Fbg** – fibrinogen; **Fb mon** – fibrin monomer; **Fb pol** – fibrin polymer; **TM** – thrombomodulin; **PC** – protein C; **PS** – protein S; **t-PA** – tissue factor plasminogen activator; **u-PA** – urokinase plasminogen activator; **PAI-1** – plasminogen activator inhibitor - 1; **PAI-2** – plasminogen activator inhibitor – 2; **PI** – plasmin inhibitor; **FDP** – fibrin degradation products, **TAFI** – thrombin activatable fibrinolysis inhibitor.

2. Inhibition of coagulation

Multiple regulatory mechanisms limit the extent of thrombin generation and restrict its formation to areas that require it. The intact endothelial layer constitutes a physiological membrane that prevents initiation of coagulation. After binding to thrombomodulin on endothelial cells, thrombin activates the protein C anticoagulant system, thereby limiting further thrombin generation. Other inhibitors of coagulation are involved in this process, i.e. TFPI, which inhibits the TF-FVIIa-FXa complex, as mentioned above, and antithrombin (AT), which inhibits thrombin, FIXa, FXa and FXIa.

The antithrombin (AT) pathway

AT is a powerful anticoagulant molecule that inactivates thrombin and most of the enzymes generated during activation of the clotting system, i.e. FXa, FIXa, FXIa and TF-FVIIa complex (Rao et al, 1993) and plays a key role in preventing excessive haemostasis and thrombosis. That is why patients with even moderate deficiencies of this anticoagulant display clinical thrombosis. Circulating AT molecules have limited inhibitory activity, which is accelerated a thousandfold by ***heparin***. Such an activity is provided *in vivo* by heparin sulphate proteoglycan molecules (HSPGs), which are present on vascular endothelium and serve to localize and concentrate activated AT on vessel walls (Olson et al, 1992).

The protein C pathway

Together with AT, the protein C pathway is probably the most effective anticoagulant system. Deficiencies in the protein C system are the most frequent congenital disorders associated with thromboembolism. This pathway involves the two vitamin K dependent plasma proteins, protein C (PC) and protein S (PS), as well as two endothelial cell membrane proteins, thrombomodulin (TM) and endothelial cell protein C receptor (EPCR).

The thrombin-TM complex is the major physiological activator of PC (Weiler-Guettler et al, 1998) Therefore, thrombin expresses both procoagulant and anticoagulant effects, depending on the context under which it is generated (Dahlbäck, 2000). At sites of vascular injury, the procoagulant effects of thrombin are fully expressed. However, under physiological conditions, thrombin has an anticoagulation function, since it binds to thrombomodulin and activates PC. Activated protein C (APC) degrades and inactivates FVa and FVIIIa and thereby downregulates prothrombinase complex formation. Efficient inactivation requires the presence of protein S as a cofactor, a phospholipid surface and calcium.

3. Fibrinolysis

Fibrin formation triggers the activation of the fibrinolytic system, which is also based on conversion of zymogen to enzyme. According to the model proposed by Wiman and Collen (1978), fibrinolysis under physiological conditions requires binding of circulating zymogen plasminogen to the fibrin clot, conversion of plasminogen to active enzyme plasmin by tissue type plasminogen activator (t-PA), proteolysis of the clot and finally inactivation of plasmin by circulating plasmin inhibitor. The fibrinolytic system also involves another plasminogen activator, urokinase plasminogen activator (u-PA), as well as the two inhibitors of plasminogen activation, plasminogen activator inhibitor 1 (PAI-1) and placenta produced plasminogen activator inhibitor 2 (PAI-2). Another fibrinolysis inhibitor, described relatively recently (Bajzar et al 1995) and termed thrombin-activatable fibrinolysis inhibitor (TAFI), can be activated by the thrombin-thrombomodulin complex, thereby representing a link between

the coagulation and fibrinolytic systems and giving thrombin another important role in the regulation of fibrinolysis.

The important issues in the present study are reviewed in detail below:

Fibrinogen and fibrin

- *Fibrinogen structure* -

Fibrinogen is a soluble glycoprotein that is synthesized in the liver. It is present in plasma from healthy individuals at a concentration of 2-4 g/L and with a half-life of 3.8 to 4.9 days (Blombäck et al, 1966). Approximately 75% of total fibrinogen is present in plasma (Takeda, 1966) and the rest is distributed in interstitial fluid and in lymph.

The fibrinogen molecule is dimeric (Blombäck and Yamashina, 1958), consisting of three pairs of disulfide bounded polypeptide chains (Aα, Bβ, γ) (Blombäck and Yamashina, 1958; Henschen et al, 1983; Doolittle, 1983), to form a symmetric molecule with a molecular mass of approximately 330.000 Da (Caspary and Kekwick, 1957). The N-terminal region which is highly cross-linked with disulphide bridges (Blombäck et al, 1967) contains two small peptides, fibrinopeptides A and B, present at the N terminal parts of the Aα and Bβ chains, respectively (Blombäck and Yamashina, 1958; Blombäck et al, 1967).

The amino terminal regions of all six chains are tightly folded into the globular dimeric ***central E domain*** that contains fibrinopeptides A and B (Telford et al, 1980). Moving towards each carboxy-terminus of the molecule, each pair of three chains forms a ***coiled-coil region*** (Doolittle et al, 1978), visualized by Rao et al (1991) as a three-stranded rope. The coiled coils continue until globular domains are formed at their carboxy-terminal ends, called the ***D domain*** (Doolittle et al, 1978). Carboxy-termini of Bβ and γ chains fold independently of carboxy-terminal portions of Aα chains, which form a structure termed the ***alpha-C domain*** (Veklich et al 1993).

- *Fibrin formation* -

Three steps in the process of fibrinogen's conversion into fibrin can be considered:

- *release of fibrinopeptides A and B*

is a thrombin-catalyzed release from the respective polypeptide chains, i.e. Aα and Bβ, and results in the formation of ***fibrin monomers*** (Fig 3) (Williams, 1981; Weisel et al, 1981).

- *fibrin assembly*

starts after removal of fibrinopeptides and exposure of new bindings sites on the amino-terminal ends of Aα and Bβ chains, termed "A" and "B", respectively. These binding sites are located in the central E domain of the fibrin monomer and interact with corresponding "a" and "b" binding sites which are present on the γ chains of the outer D domain of another fibrin monomer (Olexa and Budzynski, 1978). Thereby, ***fibrin dimers*** are formed (Fig. 3) and stabilized by noncovalent "A:a" and "B:b" interactions (Weisel, 1986).

The next obligatory intermediates during fibrin assembly are ***protofibrils*** (Fowler et al, 1981) which, beside the "A:a" and "B:b" interactions, contain noncovalent interactions between the distal D domains of fibrin monomers in the same strand of protofibril. After the protofibrils have reached a sufficient length, the lateral association occurs between D domains in laterally aligned protofibrils and thicker ***fibrin fibers*** are formed.

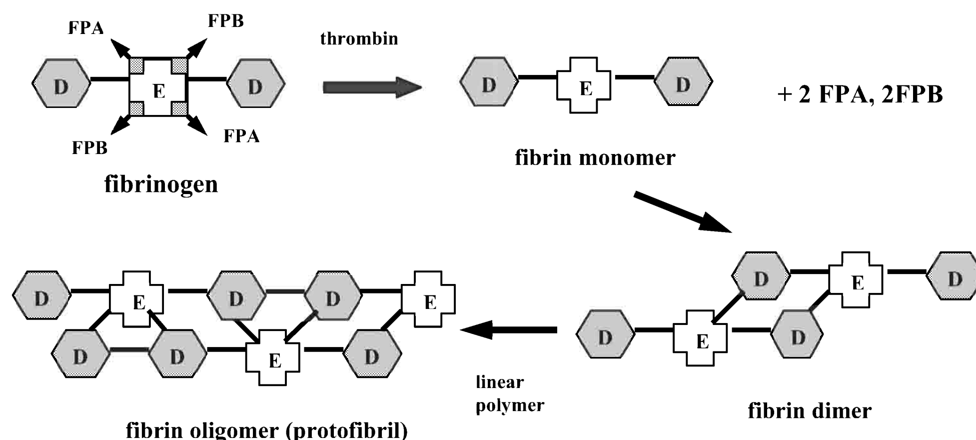


Figure 3. Simplified presentation of processes involved in fibrin polymerization

The final step in the fibrin assembly process is the branching of fibrin fibers, where Hermans et al (1982) have described a novel structure called ***trimolecular branch point*** in which one protofibril forms a junction with two separate protofibrils.

In 1994 Blombäck et al could show in plasma systems that when only a small amount of fibrinogen (3%) was converted to fibrin, a network creating a scaffold into which subsequently activated fibrinogen molecules are deposited. They also could show that the porosity, fiber dimension and architecture of fibrin gels in recalcified plasma on addition of thrombin are within a certain range of thrombin concentration determined by the initial rate of fibrinogen activation.

- *crosslinking of fibrin*

is catalyzed by FXIIIa, which is generated from plasma FXIII by thrombin. FXIIIa links lysine residues from one fibrin monomer with glutamine residues of another (Mosesson at al, 1995). In this way, six crosslinks are formed between one fibrin monomer and its neighbours. The crosslinking occurs more rapidly between lysine and glutamine residues of γ-chains of two adjacent monomers, while α-chain crosslinking is markedly slower. FXIIIa also crosslinks plasmin inhibitor (PI) to the α-chain of fibrin (Sakata and Aoki, 1980). Crosslinking accordingly renders the fibrin clot mechanically stronger and more resistant to enzymatic dissolution.

- *Fibrin degradation* -

Fibrin clot lysis is a process of solubilization of the fibrin network structure. Fibrin formation is accompanied by a dramatic increase in the activation of components belonging to a fibrinolytic system, i.e. in the efficiency of plasminogen activation by tissue plasminogen activator (t-PA) (Hoylaerts et al, 1982). In normal plasma the antigen concentration of t-PA is approximately 5 μg/L and most of t-PA is present in a complex with its primary inhibitor plasminogen activator inhbitor-1 (PAI-1). Fibrin protofibriles have been identified as the smallest molecular species required for enhanced t-PA activation and binding to fibrin. However, efficient generation of fibrinolytic activity requires formation of a ***ternary complex*** composed of t-PA : plasminogen : fibrin (Wiman and Collen, 1978). Plasminogen binds to the

lysine residues in D domains of fibrin (Weisel et al, 1994). As a result, plasmin is generated on the fibrin surface and then cleaves fibrin, exposing new carboxy-terminal lysine residues that enable more t-PA and plasminogen to bind. This positive feedback mechanism is involved in regulating fibrinolysis, ensuring that plasmin activity is targeted to a fully assembled fibrin clot. On the other hand, regulation of fibrinolysis is obtained from the interactions between fibrin, plasminogen and plasmin inhibitor (PI). PI rapidly inactivates plasmin, interferes with plasminogen binding to fibrin and is crosslinked to fibrin by FXIIIa.

Plasmin is a powerful enzyme that cleaves the fibrin network and releases fibrin degradation products (FDP) of different molecular sizes, the smallest being fragments D and E. The smallest product of the series of crosslinked FDPs is ***D-dimer*** (Kopec at al, 1973), which is used in diagnostics. Increased levels of D-dimer indicate increased thrombin generation and fibrin formation as well as subsequent lysis. Plasmin can also cleave fibrinogen and generate fibrinogen degradation products such as fragment X and fragment Y, composed of fragment E and fragment D.

Hypercoagulable conditions

1. Thrombophilia

Any disorder associated with an increased tendency to venous thromboembolism (VTE), either inherited or acquired, is considered to represent thrombophilia. The inherited abnormalities are deficiencies of antithrombin, protein C and protein S, resistance to activated protein C (APC resistance) due to mutation in the gene of factor V and a point mutation in the prothrombin gene (prothrombin 20210A). Mild hyperhomocysteinemia and elevated levels of coagulation factor VIII are also associated with an increased risk of VTE and possible arterial thrombosis, though it is still unclear whether these abnormalities have a genetic background. Acquired thrombophilic disorders include phospholipid antibodies (lupus anticoagulans and cardiolipin antibodies) and cancer.

There is also a group of disorders with reversible or potentially avoidable origins that are seen as acquired thrombophilia. These are surgery, immobilization, pregnancy, or use of oral contraceptives or hormone replacement therapy. In addition, previous venous thrombosis is a risk factor for recurrent VTE.

APC resistance

A decreased response to the anticoagulant function of activated protein C, i.e. APC resistance, is a predominant risk factor for the development of VTE in the Caucasian population. In about 95% of patients, APC resistance is associated with a G to A mutation in the gene of factor V (FV), first reported by Bertina et al (1994), the so-called FV Leiden mutation. The mutated activated FV is degraded more slowly by APC, stimulating thrombin generation that is related to a permanently increased risk of thrombotic events (Ridker et al, 1995; Rosendaal et al, 1995). In addition, APC resistance contributes to thromboembolism by rendering the thrombi more resistant to dissolution by plasmin, due to the increased activation of thrombin activatable fibrinolysis inhibitor (TAFI) (Bajzar et al, 1996).

Moreover, APC resistance can be found in conditions without the presence of FV Leiden mutation, such as pregnancy (Cumming et al 1995), use of oral contraceptives (Henkens et al, 1995), high factor VIII levels (Laffan et al, 1996), the presence of lupus anticoagulans (Ehrenforth et al, 1995). Such acquired impairment of the protein C anticoagulant pathway is associated with increased thrombin generation and thus connected to thrombotic events (Clark et al, 1999; De Visser et al, 1999).

Since the description of the original assay by Dahlbäck et al in 1993, most screening assays for the APC resistance phenotype have been based on the addition of exogenous APC in various clotting (Kraus et al, 1994) or amidolytic assays (Varadi et al, 1995). Quehenberger et al (1999) described a Russel viper venom clotting time-based assay in which endogenous protein C was activated by the snake venom enzyme. All these assays are sensitive for the factor V Leiden-related APC resistance, but most are not specific for that abnormality. Pre-dilution of test samples in factor V-depleted plasma greatly improves their specificity (Jorquera et al, 1994). Kraus et al (1995) developed an aPTT-based clotting assay, termed ProC® Global assay, which could globally evaluate the functionality of the protein C anticoagulant pathway. After modification using dilution of the patient plasma in factor V-depleted plasma, the ProC® Global assay demonstrated high specificity for FV Leiden mutation and was recommended as a routine screening test for FV Leiden (Quincampoix et al, 2001).

Pregnancy-related thrombophilia

Normal pregnancy is associated with activated coagulation and depressed fibrinolysis, which is pronounced with the course of gestation. These include increases in a number of coagulation factors: fibrinogen, prothrombin, FVII, FVIII, FIX, FXII and von Willebrand factor (Greer, 1994; Hellgren, 2003), as well as decreased protein S levels (Comp et al, 1986). A significant fall in APC activity is found in 45% of pregnant women, demonstrated by an APC sensitivity ratio below the 95th percentile of the normal range for non-pregnant women of similar age (Mathonnet F et al, 1996; Shu H et al, 2000) and this phenomenon has been called “acquired APC resistance”.

Tissue type plasminogen activator (t-PA) activity is decreased during pregnancy (Ishii et al, 1994), not only due to increased levels of plasminogen activator inhibitor-1 (PAI-1), but also, and probably mainly, to the rising levels of plasminogen activator inhibitor-2 (PAI-2) (Wright et al, 1988; He at al, 1995). While this physiological shift in the overall balance towards hypercoagulability may be important for minimizing intrapartum blood loss, it contributes to an increased risk of thromboembolism (VTE) during pregnancy and the post-partum period (Bremme, 2003).

VTE is a leading cause of illness and death during pregnancy and puerperium and remains a diagnostic and therapeutic challenge (Pabinger and Grafenhofer, 2002). Pregnancy increases the risk of VTE in the general population approximately 5-fold. In individuals with well-defined hereditary thrombosis risk factors, such as the FV Leiden mutation, the prothrombin: G20210A variation, antithrombin-deficiency or protein C-deficiency, the risk of pregnancy-associated VTE is increased even more (Gerhardt A et al, 2000).

2. Preeclampsia

Preeclampsia is an important pathological syndrome of pregnancy, affecting 3-5% of normal pregnancies (Roberts et al, 2001). Preeclampsia is induced by an impaired utero-placental circulation and is characterized by hypertension and proteinuria. Another common finding in preeclampsia is activation of coagulation (Perry et al, 1992), accompanied by depressed fibrinolysis due to increased levels of PAI-1 in both plasma and placenta (He et al, 1995).

3. Coronary heart disease (CHD)

CHD is a leading cause of morbidity and mortality in Western society. A central roll in the pathogenesis of CHD is played by the development of coronary artery thrombosis. Occlusion of these arteries is a consequence of atherosclerotic process, which remains asymptomatic until significant plaques have formed. Disruption of unstable plaques triggers acute thrombotic events, causing myocardial infarction and death.

Haemostatic factors participate in the pathogenesis of atherosclerosis and thrombosis (Loscalzo, 1992). Fibrinogen (Wilhelmsen et al, 1984), FVII (Heinrich et al, 1994), PAI-1 (Hamsten et al, 1985) and von Willebrand factor (Silveira et al, 1992) have been shown in epidemiological studies to be independent risk factors for atherosclerotic cardiovascular disease. One study (Wu et al, 1992) has shown increased FVIII activity in cases with CHD, but FVIII was not found to be an independent risk factor.

The precise mechanisms by which prothrombotic factors promote atherosclerosis are not known; but these prothrombotic determinants do facilitate the extracellular deposition of fibrinogen and its degradation products, which in turn induce endothelial disorganisation (Kadish et al, 1979), stimulate the proliferation and migration of smooth muscle cells (Ishida and Tanaka, 1982), provide absorptive surfaces for low density lipoproteins and its oxidized derivates (Tompson and Smith, 1989) and modulate endothelial cell production of prostacyclin (Watanabe, 1984). Moreover, impaired fibrinolysis is present due to direct inhibition of t-PA by lipoprotein (a), which is known to be a powerful risk factor for atherosclerosis (Simon et al, 1991). Wiman at al (2000) demonstrated that the increased levels of t-PA / PAI-1 complex in plasma seem to be a good biochemical marker predicting recurrent myocardial infarction.

Hypocoagulable Conditions

(Inherited Bleeding Disorders)

A wide range of inherited bleeding disorders has been described, originating from inadequate platelet function as seen in Glanzmann`s disease and the Bernard-Soulier syndrome (Shapiro, 2000) or caused by deficiency of procoagulant proteins such as von Willebrand factor, fibrinogen, FII, FV, FVII, FVIII, FIX, FX, FXI and FXIII (Peyvandi et al, 2002). Coagulation factor deficiencies (other than FVIII and FIX) that cause a bleeding disorder are inherited as autosomal recessive traits. Deficiencies of coagulation factors, except those of von Willebrand factor, FVIII and FIX are generally rare, with prevalences in the general population of between 1:500.000 and 1:2.000.000.

The relatively frequent deficiencies are FVIII deficiency - haemophilia A (1:10.000) or FIX - haemophilia B (1:25.000 to 1:30.000), both X-linked recessive diseases characteristically recognized almost exclusively in males. Females with one abnormal gene copy are carriers because the other X chromosome has a normal copy of the gene. However, in approximately one-third of haemophilia A cases, *de novo* mutations in FVIII gene are found which seem not to be present in the X chromosome of their mothers. Classification of the severity of haemophilia has been based on either clinical bleeding symptoms or plasma coagulation levels (White et al, 2001). Approximately 50-60% of patients have severe haemophilia with FVIII / IX activity < 0.01 IU/mL (< 1% of normal); these patients develop frequent spontaneous bleeding into joints, muscles and internal organs. Moderately severe haemophilia occurs in approximately 25% to 30% of patients; the FVIII / IX activity is 0.01 – 0.05 IU/mL (1% - 5% of normal) and the similar bleeding tendency exsist, though in milder extent. Mild

haemophilia, which occurs in 15% to 20% of patients, is associated with FVIII / IX activity between 0.05 and 0.40 IU/mL, and there is bleeding only after major trauma or surgery.

The successful treatment consists of replacement of FVIII or IX to haemostatically adequate plasma levels. Early treatment, at the first onset of symptoms, limits both the amount of bleeding and the extent of the consequent tissue damage. Replacement products are derived from human normal pooled plasma or nowdays mostly from cell lines genetically engineered to synthesize recombinant human proteins. During the past three decades, prophylaxis with clotting factor concentrate has been accepted as the treatment of choice for young boys with severe haemophilia (Petrini, 2002). Primary prophylaxis is initiated at the age of 1 to 2 years, before the occurrence of repeated joint bleeding, the recommended dose being 20-40 U/kg bodyweight three times weekly in patients with haemophilia A, and 30-40 U/kg twice weekly in those with haemophilia B.

In general, 30% to 50% of normal factor levels are required for most less severe bleeding episodes. Treatment or prevention of life-threatening haemorrhage, or surgical bleeding, requires between 50% and 100%, or more, of activity levels.

Coagulation Screening Tests and Determination of the Fibrin Gel Porosity

Screening assays are performed in order to get an overview of the entire coagulation system, including enzymes, cofactors and inhibitors, and also of the influence of anticoagulant drugs on blood coagulation. The most commonly used tests are:

1. Activated partial thromboplastin time (aPTT)

Langdell and colleagues (1953) developed a plasma clot-based assay, in which the observation of fibrin strands formation (i.e. clotting time) is the endpoint. Later, the aPTT assay was incorporated in the first-line investigation of subjects in whom a bleeding disorder has been clinically diagnosed (Erban et al, 1989). It is considered to be sensitive for the deficiencies of components included in the intrinsic system (HMMK, PK, FXII, FXI, FIX, FVIII, FX, FV, FII and fibrinogen) as well as for deficiency of von Willebrand Factor (VWF). aPTT is often employed as a screening test for lupus anticoagulant (Triplett, 1989) and for monitoring direct anticoagulants such as heparin (Banez et al, 1980; Van den Besselaar et al, 1990; Kitchen and Preston, 1996) or hirudin – a direct thrombin inhibitor (Nowak G, 2001).

The reagents nowadays used to assess aPTT contain a contact activator (a suspension of negatively charged particles such as kaolin or silica) and phospholipids (synthetic or isolated from animal tissue such as rabbit brain) apart from calcium-chloride.

The major limitation of the aPTT assay is its strong dependence on reagents and instruments (D`Angelo et al, 1990). Even lot-to-lot variations in the same reagent have been confirmed (Brill-Edwards et al, 1993). Therefore, each laboratory should test plasma from healthy subjects (40 is the recommended number) in order to determine its own normal range, which is considered to be 4 to 6 seconds around a mean aPTT of about 30 seconds. The common mistakes made in the preanalytical phase (inappropriate blood sampling or errors in the storage of samples) may lead to activation of the contact pathway *in vitro* and artificial shortening of aPTT (Contant et al, 1983; Ray, 1991). Because elevated FVIII levels (acute phase protein) can also shorten the aPTT, the results must be interpreted with particular care in inflammatory disease.

Different aPTT reagents show variable factor sensitivity. Most reagents will show prolonged aPTT when FVIII or IX are $< 30\%$ (Triplett, 2000), while the sensitivity for the other factors

is much more inconsistent. However, the clinically significant deficiencies (levels of examined factor < 10%) are usually identified.

Another a drawback of the aPTT assay is its insensitivity to some clinically important bleeding disorders such as FXIII deficiency and plasmin inhibitor deficiency. These diseases are admittedly rare in the general population but they may cause life-threatening bleeding; even so, an aPTT assay would show normal values. At the same time, prolonged aPTT is found in some factor deficiencies that are clinically irrelevant and do not cause bleeding (FXII, PK, HMMK) (Chee at al, 2003).

For monitoring heparin treatment, Basu et al (1972) suggested a therapeutic range of 1.5 – 2.5 for the aPTT ratio (control-patient) and this was confirmed by Hull et al (1992). However, aPTT reagents differ with respect to heparin responsiveness, mainly due to their phospholipid components (Kitchen et al, 1999). Moreover, a poor correlation has been found between heparin concentration and aPTT (Kitchen et al, 1996), i.e. samples with the same aPTT ratio can have markedly different heparin concentrations.

2. Prothrombin time (PT)

The PT assay, developed by Quick (1935), is widely used as a screening test to identify deficiencies of factors II, V, VII, X and fibrinogen. It is also used to monitor oral anticoagulant therapy, since these drugs lower the levels of factors II, VII and X.

The test is initiated by recalcifying citrated plasma in the presence of “thromboplastin” and clotting time is observed as the endpoint. Currently, three types of “thromboplastins” (PT reagents) are in use: 1) tissue factor (TF) containing extracts from TF - rich tissues (either rabbit brain or human placenta); these extracts are often also rich in phospholipids which are necessary to make TF functional. 2) recombinant human TF supplemented with phospholipids (van den Besselaar and Bertina, 1993). 3) TF from rabbit brain containing phospholipids and supplemented with adsorbed bovine plasma, deficient of factors II, VII and X. The latter type of reagent is generally called a combined thromboplastin reagent. The interassay variability is high especially for the “plain” reagents (1 and 2, sometimes called Quick PT reagents), not containing adsorbed bovine plasma. This variability is mainly dependent on differences between the various thormboplastin preparations but also to some extent on the clot detection techniques used. Manual techniques have largely been replaced with automated instruments that detect the endpoint (clotting) as an optical or electromechanical change. Although it is recommended that each laboratory determines its own reference range, for most combinations of reagent and method the reference interval is a few seconds around a mean PT between 11 and 15 seconds.

The combined thromboplastin reagents were introduced by Owren in the 1950-ties. The addition of adsorbed plasma makes the PT insensitive to deficiencies in factor V and fibrinogen. Most likely the added bovine plasma reduces the matrix effects i. e. differences in concentrations of various enzyme inhibitors and differences in plasma colour, when the test is performed on plasma. The fact that the modern Owren PTs works with a final plasma dilution of 1+20 makes the test less influenced by differences in matrix effects introduced by differences between samples.The combined reagents allow dilution of samples. Consequently they can be calibrated by dilutions of normal plasma, similar to the way one-stage clotting assays are standardized. The PT activity could thus be expressed as ***% of normal PT activity***.

Quick PT assays cannot be calibrated in the same way. In order to introduce a comparable expression of results the World Health Organization (WHO) in 1983 suggested that the results should be given as INR (International Normalized Ratio). In order to be able to express results as INR each instrument-reagent combination had to be calibrated according to a WHO

protocol leading to establishment of an ISI (International Sensitivity Index) for each instrument-reagent combination. By use of this ISI the local clotting-time ratio could be transformed to INR.

However, the introduction of INR was delayed for many years in the Nordic countries, where the Owren PT test was used, mainly due to the latter's much simpler standardization. However after a careful investigation a fixed relation between the Owren PT, expressed as percent activity, and INR using the manual WHO reference procedure could be established allowing a joint introduction of local INR calibration throughout Sweden. Thus the Owren type assay could be calibrated with normal plasma samples to give results in INR and this has improved assay precision, both between and within laboratory CVs. (Hillarp et al, 2004; Lindahl et al 2004). The established therapeutic INR range, which is widely used for monitoring oral anticoagulation, is 2-3 (Hirsh et al, 1992). It represents a safe dividing line between the risk of recurrent thrombosis and increased bleeding.

3. Thrombin time – thrombin clotting time (TCT)

TCT is a simple screening test for the conversion of fibrinogen to fibrin, performed by adding thrombin, usually of bovine origin, to citrated plasma. When relatively low or moderate thrombin concentrations are used, the observed clotting time is a function of fibrinogen concentration and quality.

Shortening of TCT is rare and when found, usually due to by mistakes during blood sampling and sample storage. Prolongation of TCT can either indicate hypo- or afibrinogenemia (Galanakis, 1992) or be due to the presence of circulating inhibitors against either thrombin or fibrin polymerization. TCT is therefore useful for monitoring treatment with direct thrombin inhibitors (Bithel, 1993) and fibirinolytic agents (Cadroy at al, 1991).

If plasma contains heparin, the complex of heparin and AT rapidly neutralizes a certain amount of added thrombin and prolongs the TCT time (Triplett, 2000). A prolongation is also found in the presence of relatively high concentrations of fibrin(ogen) degradation products. To minimize interference from heparin and fibrin(ogen) degradation products, a high thrombin concentration is used to initiate clotting. This modification of the TCT assay, developed by von Clauss (1957), is commonly used to measure the plasma fibrinogen concentration. The TCT test is not standardized and is strongly method dependent.

4. Recently developed global haemostasis assays

The above desctibed plasma clot-based assays, in which the observation of fibrin strands is the endpoint, have been most commonly used. Although these assays are serviceable for identifying congenital deficiencies of coagulation factors, their utility for evaluating prothrombotic states and anticoagulant therapy is rather limited. Studies on whole blood by Brummel et al. (2002) have demonstrated that cleavage of fibrinopeptide A and subsequent clot formation occur just prior to the propagation phase of coagulation. In other words, the formation of detectable fibrin clots occurs at around 3-5% of the total amount of thrombin produced. This means that the subsequent haemostatic reactions or possible abnormalities in the haemostatic process cannot be observed by a simple clotting endpoint (Mann et al, 2003).

A new generation of global haemostatic assays has recently been developed and modified in an attempt to overcome the drawbacks of classical screening tests. Some of the most widely used are described below:

Modified aPTT assay - transmittance waveform (TW) analyses

It was recently realized that clotting assays such as aPTT or PT can provide more information than just the clotting time. The use of advanced technology and coagulation analysers with photometric signalling of clot formation have led to the graphic representation of an optical profile of "clot waveform" as a function of time (Downey et al, 1997).

The assay was described by the same authors as simple, rapid and robust, sensitive method for diagnosing disseminated intravascular coagulation (DIC). It is only in patients with this disorder that the normal sigmoidal appearance of aPTT-TW is replaced by a biphasic profile. Patients with congenital factor deficiencies, acquired coagulation antibodies, or those on anticoagulant treatment did not manifest the biphasic pattern (Toh, 1999). An important observation was that the changes in wave form were independent of the aPTT reagent or influences from preanalytical variables. However, Toh et al (2000) subsequently showed that the PT assay of TW is reagent-dependent. Nevertheless, the analyses of PT are promising for the detection of lupus anticoagulants (Su et al, 2002).

Finally, complete waveform analysis of aPTT appears to provide additional information for defining the clinical severity of haemophilias compared to the standard aPTT clotting assay or one-stage factor assays (Shima et al, 2002). Shima (2004) also reported the usefulness of the assay in monitoring haemostatic effects of recombinant FVIIa in haemophilic patients with inhibitors.

Endogenous thrombin potential (ETP)

The ETP assay, developed by Hemker et al (1986), is based on the assumption that thrombin generation in examined plasma or whole blood reflects the sum of the activities and concentrations of procoagulant and anticoagulant substances. In the simplest version, thrombin generation was measured after addition of a suitable trigger (tissue factor, phospholipids and calcium) by sampling tested plasma at fixed time intervals in a solution containing a thrombin specific chromogenic amydolytic peptide substrate (S2238). The amount of generated thrombin was plotted against time to construct a thrombin generation curve (thrombogram), from which additional parameters were calculated, i.e. lag time, peak height, time to peak and the area under the thrombin generation curve – ETP.

In 1993, the ETP assay was modified (Hemker at al) by employing thrombin specific substrate with slow reactivity (methylmalonyl-methylanalyl-arginyl-pNA – SQ 68), which allows photometric measurement of the para-nitroaniline split by thrombin without any subsampling procedure. With this modification, the test was fully automated. However, defibrination of test plasma, which is required before testing, remained a major drawback of the assay. In a later version (Hemker et al, 2000), the chromogenic substrate was replaced by a fluoregenic substrate, whereby the fluorescent signal is not disturbed by the optical turbidity from fibrin formation. This modification meant that tested plasma could be used without defibrination; together with the introduction of a microtiter-plate fluorometer, it rendered the ETP assay applicable in less specialized clinical laboratories. The result of the ETP assay is still dependent on many variables, of which the concentration of TF and phospholipids used for the initiation of the reaction system are particularly important (Chantarangkul et al, 2003).

The latest modification of ETP assay may find wide applications for monitoring oral anticoagulation, direct thrombin inhibitors (Hemker et al, 2000; Bostrom et al, 2003) and heparin or heparin derivates (Beguin et al, 1988; Bendetowicz et al, 1994) but also for demonstrating the altered thrombin generation in hyper- as well as hypocoagulable disorders. ETP appeared to be increased in prothrombotic states, in patients with antithrombin

deficiency, prothrombin mutation A20210G (Kyrle et al, 1998) and acquired APC resistance due to pregnancy (Eichinger et al, 1999) or use of oral contraceptives (Rosing et al 1997; Rosing et al, 1999). High ETP was significantly associated with the incidence of stroke at a young age (Faber et al, 2003). At the same time, decreased ETP values (<10% of normal) were found in severe deficiencies of factors V, VII, VIII, IX or X (Al Dieri et al, 2002).

However, it seems that the assay system is still technically complicated, definitely not suitable for bed-side monitoring and does not measure the final step of coagulation reaction, i.e fibrin formation.

Tissue factor dependent blood clotting in minimally altered whole blood

In 1994, Lawson at al established a whole blood model system, containing physiological concentration of phospholipids and procoagulants, to study the kinetics of coagulation reactions when initiated by low concentration of TF. The progress of the reactions is monitored by variety of assays, which permits the reconstruction of intermediate / product concentration in different points during the reaction. The results are viewed in terms of thrombin generation. By employing such model, different phases in the TF-induced pathway can be observed, such is the generation of tiny amounts of FXa and FIXa at the end of initiation phase, or the rapid increase in thrombin concentrations formed in propagation phase of coagulation process. A mathematical simulation of the TF pathway to the generation of thrombin has been developed (Jones and Mann, 1994). The authors suggested the use of this combined empirical and mathematical model in order to explore the influence of alterations in the concentrations of any coagulation factor or inhibitor on coagulation reaction.

This approach was even more utilized by Holmes et al (2000), who designed a point-of-care clotting assay to assess the relevant plasma and cellular events involved in physiological blood coagulation and its management by pharmacological agents. Clotting is initiated by a low concentration of TF, while the contact pathway activation is inhibited with corn trypsin inhibitor (CTI, a specific inhibitor of FXII). Although the assay is based on the observation of activated clotting time (ACT), it has some advantages compared to classic assays of this kind. It is performed in the presence of the cellular components of blood; the influence of contact activation is eliminated and it is highly sensitive for direct thrombin inhibitors, factor Xa inhibitors and potent antiplatelet agents such as glycoprotein IIb/IIIa inhibitors.

Thromboelastography (TEG)

The thromboelastographic principle for recording viscoelastic changes during coagulation was introduced by Hartert (1948). However, in the early years, the thromboelastographic apparatus was very sensitive to the changes in the surroundings. As a test for general clotting function, TEG has been used for more than half a century. Unlike clotting assays, TEG monitors haemostasis as a whole dynamic process, rather than revealing information about isolated parts of different pathways (Chandler, 1995). By following the change in elasticity during clot formation with a mechanical detection system, the assay provides data about clot formation and its physical strength and stability, in addition to any dissolution. Recently, a new portable TEG instrument (roTEG Coagulation Analyser) was established by Calatzis at al (1996). This provides continuous data that are readily used for further calculations.

The preferred sample is citrated whole blood (Bowbrick et al, 2000) but plasma can also be used for experimental work (Schroeder et al, 2001). The whole-blood based assay makes it possible to observe the interaction of plasma factors, platelets and other cells, as well as the influences of many drugs on haemostasis. Hence, this method may more closely reflect the *in vivo* clotting situation. Traditionally, thromboelastography is performed without a specific test reagent except for $CaCl_2$ and can therefore easily be used for bedside monitoring. TEG is

sensitive for detecting diminished platelet count (Oshita et al, 1999), fibrinogen polymerization disorders and deficiency (Chandler, 1995), lack of coagulation factors, heparin effects and hyperfibrinolysis (Vorweg et al, 2001). TEG's good ability to predict bleeding makes it applicable for monitoring relevant treatments and can lower costs, particularly by reducing the infusion of platelet concentrates, cryoprecipitate or fresh frozen plasma (Shore-Lesserson et al, 1999; Spiess et al, 1995).

In 2002, Sorensen et al modified the assay system by adding minimal concentrations of tissue factor as an initiator of coagulation. A new system for data calculation and display of whole-blood clotting profiles was also introduced. They also shortened the total time of analyses so that this was considered acceptable for routine clinical applications. The same group of authors (2004) suggested that thromboelastography based on low TF activation might be a suitable method for monitoring a haemostatic intervention with recombinant FVII in patients with haemophila A and rare coagulation disorders.

However, several disadvantages appear to be associated with TEG. They are connected with sample preparation, i.e. storage time, especially during the first 30 minutes after venipuncture (Vig et al, 2001). In addition, TEG measurements should be performed within 8 hours after blood sampling (Camenzind et al, 2000) and frozen-thawed samples cannot be used.

Clot onset time (COT) measurement using free oscillating rheometry (FOR)

The assay based on free oscillating rheometry to measure clotting properties of blood and plasma after recalcification of citrated blood was presented by Ungerstedt et al. (2002). It is based on measurement of the frequency and dumping of oscillations of investigated samples using the ReoRox instrument invented by Bohlin (1994). These variables are related to the changes in the sample's viscosity and elasticity. During the coagulation process, the increase in viscosity will cause a decrease in the frequency of oscillations.

The endpoint – clot onset time – is derived from the deviation from the initial viscoelastic properties of an oscillating sample, allowing identification of the onset of coagulation rather than the latter steps of coagulation, e.g. thrombin formation. COT in recalcified blood and plasma covariated positively with Owren PT (INR), indicating measurement of components involved in the tissue factor dependent pathway. It appears to be quick, easy to handle and suitable for point-of-care testing of hypo- and hypercoagulable states in environments where quick analyses are needed. However, like other classic screening assays, the endpoint of COT represents a major disadvantage of this assay.

A simple and rapid laboratory method for determining haemostasis potential in plasma

A broad spectrum of laboratory tests is available for identifying imbalances in the multifaceted haemostatic system and locating the underlying causes. However, a definite conclusion can hardly be drawn from just a routine analysis of one or even several variables. Determining hypercoagulation in a simple way has proved particularly difficult (Francis, 1998). Much effort has been invested in recent decades in providing a global method for the diagnosis and differentiation of haemostatic disorders. Each of the described methods clearly has its advantages and drawbacks and there is still no ideal system that can provide a global parameter that shows an increase for every kind of hypercoagulation and a decrease for hypocoagulability.

In an attempt to contribute to this search, we developed (He et al, 1999) a laboratory method for screening the ***Overall Haemostasis Potential in plasma (OHP)***. The assay is based on the spectrophotometric registration of a fibrin-aggregation curve made with citrated plasma, into which small amounts of exogenous thrombin, tissue plasminogen activator (t-PA) and

calcium chloride have been added. The area under the fibrin aggregation curve, calculated as sum of absorbance (Abs-sum), is regarded as a laboratory parameter for OHP determination. The preliminary findings have demonstrated the logical variations in Abs-sum values after addition of purified pro- and anticoagulants to examined plasma *in vitro*. Moreover, the levels of the Abs-sums were higher in samples obtained from normal pregnant than in in nonpregnant women and further increased in samples from preeclamptic patients. However, the levels of this parameter in coagulation-deficient plasma were around 40% of the normal, which was unexpectedly high. We consider that exogenous thrombin in a dose of 0.2 IU/mL, not only initiate the generation of thrombin from pro-thrombin by a feedback effect in vitro, but also may catalyse the transformation of fibrinogen to fibrin, leading to inadequate sensitivity for determining hypocoagulable states.

To ensure the sensitivity and suitability of the assay for clinical or research work, various modifications were made in the latter study reported in ***paper I***. Briefly, thrombin in a decreased dose (0.04 IU/mL) with or without t-PA was added to plasma for initiation of fibrinogen clotting. Areas under two fibrin-aggregation curves i.e., above-mentioned OHP and ***Overall Coagulation Potential (OCP)*** were thus created (Fig 4-I and 4-II).

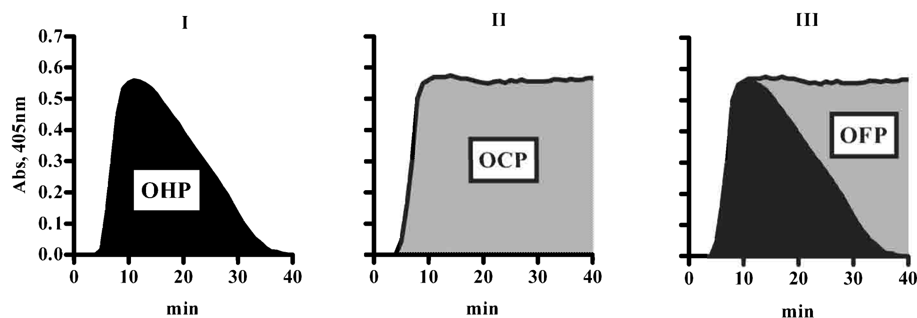


Fig 4. OHP / OCP / OFP assayed in normal pooled plasma sample

A difference between the two parameters reflects the ***Overall Fibrinolysis Potential (OFP)***, calculated by ((OCP-OHP)/OCP) x 100%) (Fig 4-III). By employing a 96-well microplate and an ELISA reader commonly used in any laboratory, 27 samples can be analysed in around 1 hour, starting from buffer preparation and including the calculation of results. The calculations are performed with a simple form in a Microsoft Excel program. Thus, the OHP assay seems to be simple, time-saving and suitable for most of coagulation and routine laboratories.

The sensitivity of the assay for detecting hypercoagulation in normal pregnancy, in preeclampsia and in coronary heart disease was further evaluated. Extremely low or undetectable levels of OHP and OCP were found in samples of Factors VIII-, IX-, VII-, V-, X- or II-deficient plasma, showing an improved power of the method in identification of hypocoagulability.

After introducing the last modification (***paper IV***) which involved a platelet reagent containing tissue factor and phospholipids, this assay system has become more similar to the haemostasis balance in vivo, and thus hopefully serve as a laboratory tool to find different severity of hypocoagulation, especially in patients with haemophilia A or B.

Andersen at al (2003) developed a modification for the approach of OHP in attempt to increase the assay sensitivity for hereditary thrombophilia. Besides the addition of exogenous thrombin and t-PA, a protein C activator i.e. Protac (Exner and Vaasjoki, 1988) and antithrombin activator i.e. heparin pentasaccharide (Beguin S et al, 1989) were used, due to which the difference of OHPs between the patients and controls were increased.

5. Qualitative determination for the constitutive properties of fibrin clot by a flow measurement

The OHP assay is a quantitative method for determining the level of fibrin and thereby demonstrating the combined effect of coagulation and fibrinolysis. Information about the quality of fibrin in examined samples can be obtained by studying fibrin gel porosity. Both assays contribute to a better understanding of the formation and constitutive properties of fibrin, which is both the final product of coagulation and the main substrate for fibrinolysis.

The fibrin gel (or clot) is an infinite network structure (Blombäck et al, 1994). Ferry and Morisson (1947), who were the first to perform systematic studies on fibrin gel structures, distinguished between two types of gel: fine types formed of more or less individual fibrin polymers that cross-link to form the gel, and coarse gels where the polymers are laterally aggregated and cross-linking occurs between the bundles of polymers. Most information on fibrin gel structure has been obtained by studying optical and mechanical properties of the gels.

Carr et al (1977) examined the thickness of fibrin strands in fibrin gels using liquid permeation technique. Using this approach, Okada and Blombäck (1983) studied factors that influence fibrin gel structures. The permeation and turbidometric analyses provided information on the porosity of the gels, the fiber mass/length ratio and the average strand width, thereby supplementing the qualitative information obtained by microscopy. The permeability coefficient (Ks) became an established parameter that provides information on the overall gel structure and reflects the size and shape of the pores in the gel.

Physiologically, the most important determinants of fibrin gel structure are the concentrations of thrombin and fibrinogen, i.e. the clotting potential (Blombäck et al, 1989; Blombäck et al, 1994). Other factors of importance are pH, ionic strength and plasma proteins like albumin, fibronectin and thrombospondin. On this basis, modifications have been performed to render the method suitable for different study aims: a tiny dose of thrombin (0.05 IU/ml) with phospholipids, or a higher dose of thrombin alone (0.4 IU/ml), is used to make the clot for assessing whether the fibrin porosity is changed by alterations of thrombin generation potential and fibrin clotting property or only by the latter, respectively.

Relevant Treatments

1. Anticoagulant treatment

At present, the two most widely used classes of anticoagulant drugs for the prophylaxis and treatment of thromboembolic disease are heparin and coumarins. Heparin is a heterogeneous mixture of glycosaminoglycans extracted from mast cells of pig intestinal mucosa or bovine lung, and has been used in anticoagulant therapy since the 1940s (Best, 1959).

Standard unfractionated heparin (UFH) is a heterogeneous mixture of polysaccharide molecules (average MM 15.000 to 18.000 daltons). It is widely used in prevention and treatment of VTE, for the management of arterial disease, in extracorporeal circulation, for the flushing of indwelling catheters and in many other hospital procedures (Hirsh et al, 2001).

Major drawbacks associated with the use of UFH include an increased risk of haemorrhage and heparin-induced thrombocytopenia (HIT) and thereby a need for anticoagulant monitoring and dose adjustment.

Low molecular mass heparins (LMMHs) were suggested as potentially preferable to standard heparin. Several LMMHs have been evaluated and are used in clinical settings. They are obtained from standard heparin by enzymatic or chemical cleavage to fragments with an average MM of approximately 5.000 daltons. LMMHs preferentially inhibit FXa and have antifactor Xa to antifactor IIa ratios of 2 to 4:1 as against 1:1 for standard heparin. Due to improved bioavailability and more predictable dose responses, LMMHs can be administered once or twice daily without laboratory monitoring (Hirsh and Levine, 1992).

Sanson et al (1999) postulated that LMMHs are a safe and attractive alternative to UFH anticoagulant treatment during pregnancy because the former carry a lower risk of bleeding, osteoporosis and HIT. Patients with a hereditary antithrombin deficiency, phospholipid antibodies, a combined abnormality or a history of a severe thrombotic event (pulmonary embolism, extended deep vein thrombosis) should be advised to use prophylactic heparin during pregnancy, starting during the first trimester (Blombäck et al, 1998 a) and b)). Post-partum prophylaxis should be, in principle, given in all women with an increased risk for VTE.

2. Acetylsalicylic acid (ASA) therapy

Acetylsalicylic acid (ASA) is an effective antithrombotic agent for preventing a variety of cardiovascular diseases. This effect is commonly explained by acetylation of a serine residue of platelet cyclooxygenase (COX-1), with consequent inhibition of the biosynthesis of the platelet activating and vasoconstricting compound, thromboxane A_2 (TXA_2) (Roth and Majerus, 1975).

However, it has been reported that ASA also acetylates lysine residues of several proteins in the coagulation system, such as procoagulants like fibrinogen (Bjornsson at al, 1998) and prothrombin (Szczeklik et al, 1992) on the one hand, and anticoagulants like antithrombin on the other (Villanueva and Allen, 1986). In the fibrinogen molecule, the lysine residues are involved in the cross-linking reaction of fibrin fibers promoted by Factor XIII, leading to formation of a mechanically stable clot which is more resistant to fibrinolysis. Thus, it can be assumed that the acetylation of fibrinogen renders the structure of the derived fibrin network more porous.

The available evidence from clinical trials favours the use of antiplatelet therapy with 75 - 150 mg ASA daily for patients at risk of occlusive vascular events (Antithrombotic Trialists` Collaboration, 2002). Previous laboratory investigations by Williams at al (1998) showed more favourable effects on the permeability of the fibrin network during treatment with a lower dose of ASA (75 mg vs 320 mg daily). This might contribute to the better clinical efficacy of low compared to higher doses of ASA.

AIM OF THE STUDY

The ***general aims*** of this study were:

1. To offer a simple, time-saving, but still global quantitative method for use in research work and routine laboratories, which can demonstrate combined effects on coagulation and fibrinolysis in examined plasma, verify the imbalance in haemostasis and contribute to monitoring the effect/safety of relevant therapies.

2. To make the the flow measurement method for determining fibrin network porosity suitable for use by more groups with various laboratory conditions, sample materials or study purposes.

The ***specific aims*** of the study were:

- Utilization of the OHP assay in the assessment of hypercoagulable states due to increased thrombin generation and/or depressed fibirnolysis during normal pregnancy; in patients with pregnancy-related deep venous thrombosis (DVT); in patients with preeclampsia and in patients with coronary heart disease (CHD).
- Utilization of the OHP assay for a better understanding of the mechanism behind the risk of recurrence of DVT in asymptomatic women with a history of pregnancy-related DVT due to presence of APC resistance and/or FV Leiden mutation.
- To study whether the OHP assay could be used as an alternative method for monitoring anticoagulant treatment with low molecular mass heparin (dalteparin).
- To assess whether the OHP method after modification is powerful enough for the detection of different hypocoagulable states caused by coagulation factor deficiencies.
- To study the influence of different doses of ASA on fibrin gel porosity and on qualitative properties of fibrin network, using the modified flow measurement and confocal laser three measure-dimensional (3D) imaging.

PATIENTS, MATERIALS AND METHODS

Patients

All studies were approved by Ethical Committee of Karolinska Hospital.

A group of healthy subjects, 70 men (24-75 years, mean age 45.0 years) and 72 women (24-73 years, mean age 46.9 years), was selected to form the control group in ***paper I***.

In 27 healthy pregnant women, included in ***paper I***, samplings were carried out in gestation week 12 in the first trimester, week 20 in the second and weeks 28, 32 and 37 in the last trimester, and 5-7 weeks post-partum. Samples taken in week 32 were also used in ***paper II***.

Seventeen female patients, mean age 61 years (range 48-70), with the diagnosis of chronic coronary heart disease (CHD) were included in ***papers I and V***. They had been hospitalised several years earlier due to acute myocardial infarction and/or angina pectoris. None of them had an unstable condition of the disease when the blood samples were collected.

Ten previously healthy women with preeclampsia in gestation weeks 37 (n=1), 32 (n=6) and 20 (n=3) were also included in ***paper I***.

Fifteen youths (20-35 years, mean age 25 years; 8 females and 5 males) were recruited from the hospital staff and medical students and included in ***paper I***. They received acetylsalicylic acid (ASA; Trombyl®, Pharmacia & Upjohn, Uppsala, Sweden) 75 mg once daily over a 21-day period. Blood samples were drawn before (day 0) and at the end of treatment (day 21), and in weeks 1 and 2 after withdrawal.

Five pregnant women, aged 27-35 years (mean 31 years), with previous deep venous thrombosis were included in ***paper II***. They were on prophylactic treatment with LMMH, dalteparin (Fragmin®, Pharmacia, Sweden). Kinetic investigations were performed in gestation weeks 32-35, twice, and sampling was performed before dalteparin injection and after 1, 2, 4, 6, 8, 10, 12, 14, 16 and 24 hours.

The control group in ***paper III*** comprised 25 healthy women aged 18–33 years (mean 25 years).

A case group in ***paper III*** was formed of 88 women, aged 23–47 years (mean 35 years), who had previously experienced DVT during pregnancy or soon after delivery. With reference to findings for the nAPC ratio and factor V Leiden mutation, three subgroups of patients were formed. Group 1 comprised 56 cases without either FV Leiden mutation or APC resistance. Group 2 was made up of 7 cases with APC resistance but no detectable FV Leiden mutation. The 25 cases in Group 3 had both the FV Leiden mutation and APC resistance; they were all heterozygous for the gene defect. The sampling was performed 8 months to 13 years after the last thromboembolic episode.

In ***paper IV*** 30 patients with haemophilia A (23 with severe, 5 with moderate and 2 with mild disease) were included. Samples from 6 patients with severe and 3 with moderate haemophilia B were also included. All severe cases of haemophilia A or haemophilia B were on regular prophylactic treatment with Factor VIII concentrate (Recombinate, 1000 IU, 2-3 times / week), or Factor IX concentrate (Immunine, 2000 IU, once / 2 weeks), respectively, while one patient with moderate haemophilia A took Factor VIII concentrate on demand. For the others, relevant treatment was required in the event of surgery or in risk situations. Samples from all severe haemophiliacs were taken immediately before the replacement treatment. No bleeding complication was found in any of the patients who participated in this study when blood sampling was carried out.

Five patients with deficiency of Factor XII (FXII) were included in ***paper IV***. Low FXII concentration was detected at the routine preoperative coagulation investigation after finding prolonged activated partial thromboplastin time (aPTT): four of them had F XII conc. < 0.01 IU/mL and one had 0.32 IU/mL.

Blood samples from 29 healthy volunteers (19-63 years), recruited from the laboratory staff, were used as a reference group in ***paper IV***.

Fifteen healthy male volunteers, aged 22-39 years, participated in the study of ***paper VI***, which was designed as an open cross-over study to compare baseline and treatment with three different regimens of ASA: 37.5 mg/day for 10 days (half a tablet Thrombyl®, Pharmacia Sverige AB 75mg); 320 mg/day for 7 days (one tablet Alka Seltzer®, Bayer, Germany) followed by a single dose of 640 mg (two tablets of Alka Seltzer®). The sampling procedure is presented in detail in Fig 1, ***paper VI***.

Blood collection

Blood samples included in papers I – VI were drawn into 0.129 mol/L trisodium citrate (1 part trisodium citrate + 9 parts blood, pH 7.4).

The citrated blood samples were centrifuged in different ways:

- at 2000 x g for 20 min at room temperature (***papers I, IV and V***)
- at 10,000 g for 20 min at +4°C (***paper II***)
- the control samples were centrifuged at 1430 g for 20 minutes at 10ºC and the patient samples at 2500 g for 20 min at room temperature. Platelet count has been adjusted to be $<10x10^6$/mL in both (***paper III***)
- at 1400 x g for 10 min at 4°C (***paper VI***)

The plasma was stored deep-frozen in aliquots of 0.5 mL at - 70ºC until test performance in papers I - VI.

Materials

Normal pooled plasma (NPP) from 13 blood donors was calibrated against the international standards and used to control the experimental quality in ***papers I, II, III and V***.

Coagulation factors IX, VIII, VII, V, X or II deficient plasmas (concentration of the respective factor < 0.001 U/mL) obtained from patients with the respective deficiency and purchased from Helena Bioscience, Sunderland, UK, were used in ***paper I***.

Standard human plasma used in ***paper IV*** was from Dade Behring, Marburg, Germany. It was obtained from pooled citrated plasma collected from selected healthy blood donors and calibrated against the WHO standard.

In ***paper IV*** we also used coagulation factors XII, XI, X, IX, VIII, VII, V or II deficient plasmas (Dade Behring, Marburg, Germany), which were manufactured by immunoadsorption from normal plasma and therefore free from antigen of the respective coagulation factor.

The platelet reagent (Unicorn Diagnostics Ltd, London UK) used in ***paper IV*** is a lyophilised platelet membrane preparation, derived from a suspension containing washed normal human

platelets. The reagent was reconstituted with 3 ml of distilled water, giving a final platelet count of $70x10^6$/mL (based on counting by the manufacturer before lyophilising). The reconstituted reagent was stored in small aliquots at -70ºC.

The platelet reagent used in ***paper V*** was prepared in our laboratory from fresh platelet concentrates in plasma obtained from the Blood Centre of Karolinska Hospital, Stockholm. The concentrates, containing around $1500x10^9$/L platelets, were washed and the platelet number was adjusted to around $6000x10^9$/L. The washed platelets were kept in small aliquots at -70ºC until used.

Methods

1. Determination of Overall Haemostasis Potential (OHP), Overall Coagulation Potential (OCP) and Overall Fibrinolysis Potential (OFP) in plasma

In ***papers I, II and III*** a modified method was used:

Thrombin in a decreased dose (0.04 IU/mL, previously 0.2 IU/mL) with or without tissue-type plasminogen activator was added to plasma for initiation of fibrinogen clotting.

Sixty µl of plasma in each well of the microplate (Imm.-IB; Dynex Technologies, Chantilly, USA) was mixed with 50 µl of the respective buffer in the assay of OCP or OHP (final conc. of CaCl2 17 mmol/mL and thrombin 0.04 IU/mL in both; t-PA 300ng/ml in the latter). Absorbance (Abs) was recorded each minute for 40 minutes to construct two fibrin-aggregation curves (***Figures 3-I and 3-II, paper I***).

The areas under the curves were expressed as a summation of the Abs values (Abs-sum) and regarded as laboratory parameters for determination of OHP and OCP, respectively. The difference between the two areas shown in Figure 3-III, ***paper I***, represents the Overall Fibrinolysis Potential (OFP), calculated as OFP=(OCP-OHP)/OCP x 100%.

For OCP, the intra-assay CV was 3.08% (n=30), 2.84% (n=27), 3.04% (n=29), 2.04% (n=17) or 4.81% (n=10); the inter-assay CV was 4.17% (n=5). For OHP, the intra-assay CV was 8.65% (n=30), 8.72% (n=27), 7.77% (n=29), 7.92% (n=17) or 8.27% (n=9); the inter-assay CV was 5.13% (n=5) (***paper I***)

In order to increase the assay's sensitivity, further modifications were introduced in ***paper IV***: besides the exogenous thrombin, a platelet reagent containing phospholipids as well as TF was added to the reaction system to initiate coagulation. 70 µl of plasma sample and 10µl of the reconstituted platelet reagent in each well of the microplate were mixed with 50 µl of working buffer, prepared as described, for the determination of OCP and OHP. A correction to the mean value of NPP used as the control in the assay was performed to obtain normalised results i.e. *n-OHP, n-OCP* or *n-OFP = result from unknown sample / result from NPP.*

For n-OCP, the intra-assay CV was 1.85% (n=5), 5.48% (n=4), 2.86% (n=4), 4.30% (n=4); the inter-assay CV was 4.30% (n=4). For n-OHP, the intra-assay CV was 5.10% (n=5), 3.62% (n=4), 1.26% (n=4), 3.61% (n=4); the inter-assay CV was 5.13% (n=4) (***paper IV***).

2. Determination of the fibrin gel porosity

Flow measurement for determining fibrin gel permeability (papers V and VI)

The plasma samples (at least 250 µL) were dialyzed at 4ºC against dialyzing buffer (TNE-buffer, pH 7.4, 0.05 mol/L Tris, 0.1 mol/L NaCl, 1mmol/L EDTA, aprotinin 5 KIU/mL) for 3

hours, with change of outer fluid at 1-hour intervals. Thereafter, 200 µL of dialyzed plasma were transferred into a plastic test tube. Depending on the concentration of thrombin used for fibrinogen clotting, two different procedures were used:

1) 10 µL of diluting buffer (pH 7.4, 0.05 mol Tris, 130 mmol/L NaCl) containing 0.42 mol/L $CaCl_2$ and 8.3 IU/mL thrombin were added to the dialyzed plasma, giving final concentrations of 20 mmol/L for $CaCl_2$ and ***0.4 IU/mL for thrombin***.
2) 10 µL of diluting buffer (pH 7.4, 0.05 mol Tris, 130 mmol/L NaCl) containing 0.44 mol/L $CaCl_2$ and 1.04 IU/mL thrombin were added to the dialyzed plasma, containing platelet reagent of $4400x10^6$/mL. Thus, the final concentrations were ***$200x10^6$/mL*** for platelets, 20 mmol/L for $CaCl_2$ and ***0.05 IU/mL for thrombin***.

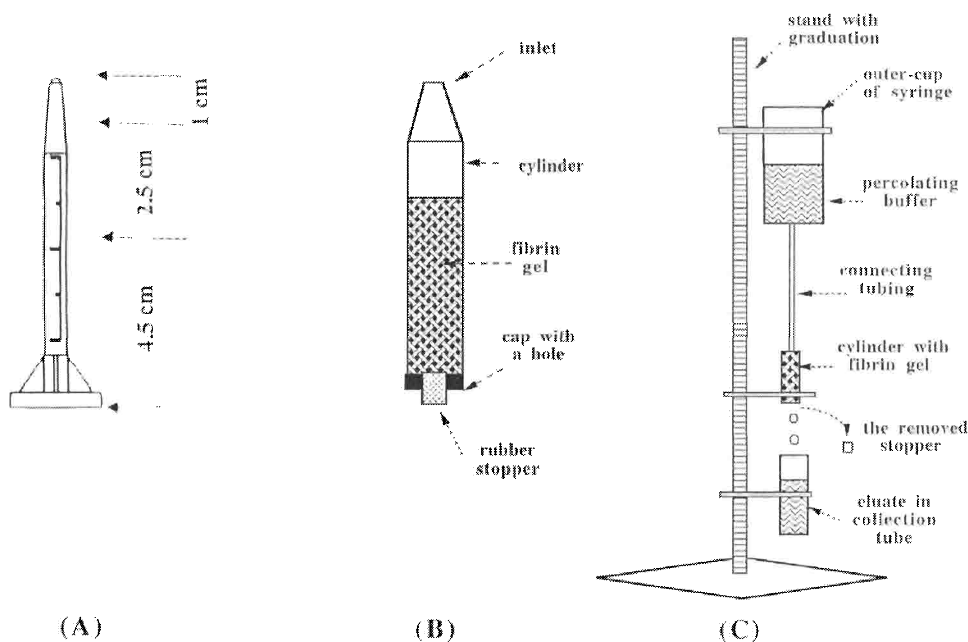


Fig 5. Abridged diagram of equipment used for the flow measurement
A: a multipipette (Eppendorf Combitip® Plus), used to make a plastic cylinder
B: the plastic cylinder (2.5cm) made of "A", in which the fibrin gel had been formed
C: the plastic cylinder with the fibrin gel placed vertically on a stand with a ruler. The upper opening was connected to the outer cup of a syringe containing percolating buffer that passed through the gel and eluted from the lower opening.

The previously described equipment for permeability measurements (Blombäck at al, 1989) was modified. The plastic cuvette was replaced by a plastic cylinder (Fig 5), made according to the design described in paper V. After the cylinder had been pre-treated with purified

fibrinogen, 180 µL of the above mixture were transferred and the cylinder was kept in a standing position in a moist atmosphere overnight.

Flow measurements were preformed at different pressures on the gel; pressure was calculated from the distance between the upper buffer level in the reservoir and the bottom of the gel. Percolating buffer (pH 7.4, 0.02 mol/L Tris, 0.02 mol/L imidazol, 0.1 mol/L NaCl) passed through the gel and the eluates were collected under each of 5 alternative hydrostatic pressures after indicated time. The permeability coefficient or Darcy constant (Ks) was calculated from the equation given by Carr et al (1977). It provides information on the network structure (shape and size).

Determination of fibrin fiber/mass ratio (µ) (paper VI)

The value of µ was calculated from Ks values with certain assumptions regarding hydration of the fibrin fibers, and with the function relating Ks to the fractional volume of fibers, i.e. to the fibrinogen concentration (Carr et al, 1977). Thus, high Ks and µ values indicate thick fibrin fibers and a porous gel.

Study of the fibrin network with 3-dimensional microscopy (paper VI)

The analysis by 3D microscopy was in accordance with an earlier report (Blombäck et al, 1989). Briefly, fibrin gel was formed in a microchamber, made in our laboratory, by adding 120 µL dialyzed plasma mixed with thrombin and $CaCl_2$, giving final concentrations of 0.4 IU/mL and 20 mmol/L, respectively. The gel in the microchamber was left to mature at room temperature overnight. The fibrin gels were percolated with fluorescein isothiocyanate (FITC) in Tris- NaCl buffer (0.01 mol/L Tris, 0.1 mol/L NaCl and 1mol/L EDTA, pH 8.0) for 2 hr. After extensive washing, the 3-dimensional structure of the labelled fibrin gel was observed using a confocal laser scanning microscope, Leica TCS SP2, equipped with one argon and two HeNe lasers (Leica Microsystems, Mannheim, Germany). Excitation of FITC was obtained with the 488 nm laser line, and emitted light was detected over the wavelength region 500 – 650 nm. The 3-dimensional structure was reconstructed by scanning the fibrin gel at a volume equal to the maximal scanning depth of the system (166 µm) and with 1 µm between optical sections.

3. Other laboratory methods

Fibrinogen: a modified assay by Von Clauss (Von Clauss, 1957), employing Fibri Prest Automate reagent (Diagnostica Stago, Asnieres, France) and the Sysmex CA 1500 apparatus from Dade Behring, Marburg, Germany (***papers I, III, V and VI***).

Anti-Xa activity: an amidolytic assay with the chromogenic peptide substrate S-2222 (Chromogenix Mölndal, Sweden), according to the method described for unfractionated heparin (Teien et al, 1976) (***paper II***).

Soluble fibrin: an amidolytic assay (Wiman at al, 1986) using Coatest Fibrin Monomer (Chromogenix Mölndal, Sweden) (***paper II***).

F1+2: a sandwich ELISA technique with Enzygnost F1+2 kit (Behringwerke, Marburg, Germany), (Pelzer at al, 1991) (***paper II***).

APC resistance: APTT-based assay using a commercial kit from Chromogenix, Mölndal, Sweden (De Ronde at al, 1994) (***paper III***).

Factor V Leiden mutation: analysis of the FV gene was performed with a polymerase chain reaction (Bertina et al, 1994) (***paper III***).

Clotting time: measured with the same fibrin aggregation curve as for the determination of OCP. The Abs was recorded each minute up to the time point when the curve reaches a plateau. A tangent to the steepest part of the sigmoidal turbidity curve was drawn. Its intersection with the time axis is defined as clotting time (Blombäck et al, 1982) (***paper III***).

Clot lysis time: measured with the same fibrin aggregation curve as for the determination of OHP and defined as the time from the maximum absorbance to the midpoint of the maximum turbid-to-clear transition, as described by Von dem Borne et al (1995) (***paper III***).

FVIII concentration: an amidolytic chromogenic method with Coamatic FVIII kit, Chromogenix, Mölndal, Sweden (***paper IV***).

FIX concentration: a one-stage clotting method with Actin FS (Activated PTT reagent) from Dade Behring, Liederbach, Germany (***paper IV***).

Tissue factor antigen: a human tissue factor ELISA kit from Chemo-Sero-Therapeutic Research Institute, Japan (***paper IV***).

APTT assay: Sysmex 1500-CA apparatus (Dade Behring, Marburg, Germany), and reagents, including cephalin phospholipid from animal brain and FXII activator, i.e. PTT Automate, from Diagnostica Stago, Asnieres France (***paper IV***).

4. Statistical analysis

Using software StatView for the Macintosh computer or GraphPad Prism 4 software for PC computer, statistical differences were tested:

- between two groups of non-parametric values (***papers I and V***) using Mann-Whitney test
- between two groups of parametric values (***papers IV and VI***) using t-test
- between two groups with continuous variables (***papers I, II and V***) using Wilcoxon signed-rank test
- between three and more groups with continuous variables (***paper III***) using Kruskal-Wallis test (Dunn`s multiple comparison test) or repeated-measures ANOVA (Dunn`s multiple comparison test) in ***paper VI***.

Associations were estimated by calculating Spearman correlation coefficients (***papers I, III and VI***) and linear regression coefficients (***paper IV***).

$P < 0.05$; < 0.01 and < 0.0001 were considered to be significant.

RESULTS

The reference levels of OHP, OCP and OFP (various percentiles) for a general population were based on investigations regardless of age or sex and presented in Table 1, ***paper I***. New reference levels of the three parameters are also shown as different percentiles (Table 1, ***paper IV***).

1. Validity of the OHP assay in estimation of hypercoagulable conditions

In paper I

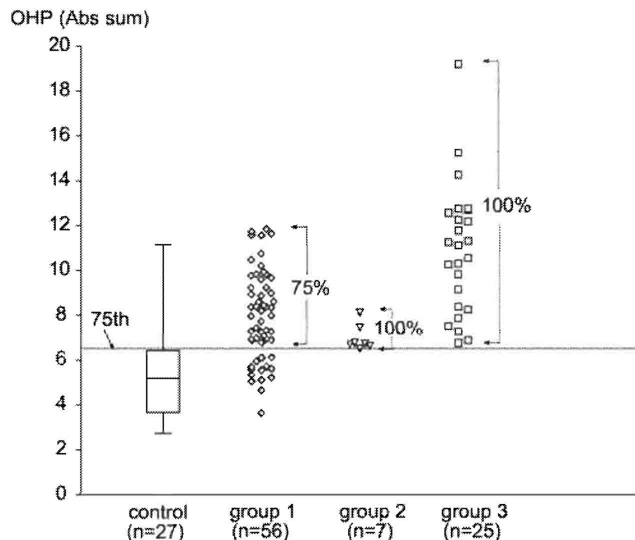
Compared to reference levels, in a ***normal pregnancy group*** OHP and OCP rose (Figs 2-A and B) while OFP fell (Fig 2-C) during the course of gestation. By 5-7 weeks after delivery, all the parameters showed levels within the reference ranges for the non-pregnant individuals.

In ***women with preeclampsia,*** levels of OHP were greatly increased (> 97.5th) in 3, moderately increased (85-90th) in 1 and close to the median (50-65th) in 6 compared to the percentiles (th) in matching stages of healthy gestation. Increased OCPs (>97.5th in 5; 90-95th in 4; 55-60th in 1) and decreased OFPs (<2.5th in 6; =2.5th in 2; 5-10th in 1; 15-20th in 1) were found (Table 2).

In ***female patients with CHD,*** increased levels of OHP and OCP, as well as decreased levels of OFP, were found compared to levels in the healthy senior women (Figure 4-I). The fibrinogen concentrations in patients were significantly increased (P < 0.01) compared to the controls. They varied in significant correlation to those of OHP (r_s=0.66) and of OCP (r_s=0.67) in samples from both groups.

In paper III

In ***all women with previous thromboembolism***, OHP levels (obtained from modified OHP method, reported in paper I) were significantly higher than in the controls (Table 1).



Overall haemostatic potential (OHP) levels in women with previous thromboembolism (patients) and healthy young women (controls)

If the 75th percentile of OHPs in the control group (6.43) was used as the cut-off, higher levels were found in 75% of the women in Group 1 (without both APC resistance and FV Leiden mutation), while all the women in Groups 2 (acquired APC resistance) and 3 (APC resistance due to FV Leiden mutation) had OHP levels above this cut-off .

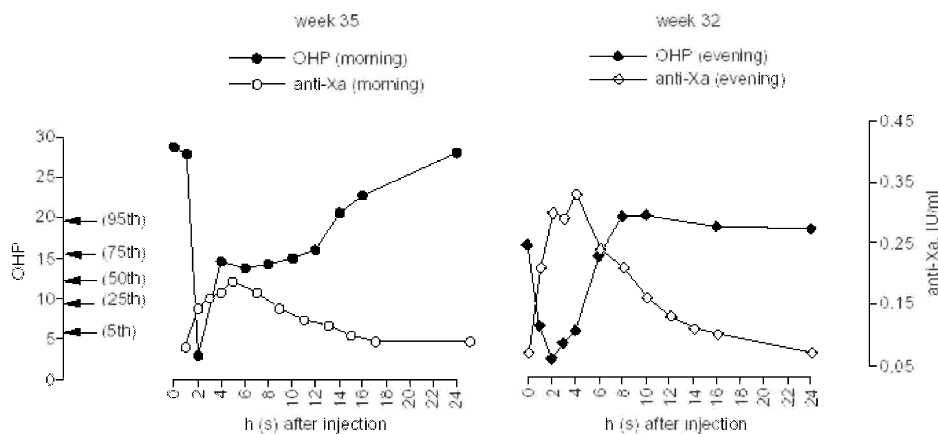
Clotting times were significantly shorter in Group 3 compared to the controls ($p<0.05$). Clot lysis times were longer in the patients compared to the controls, particularly in Group 3 (Table 1).

The fibrinogen concentration did not differ either between controls and cases or between the different groups of patients. Moreover, the OHP levels were unrelated to the fibrinogen concentrations (Fig 2).

2. Validity of the modified OHP assay (reported in paper I) in monitoring anticoagulant and antithrombotic treatment

In paper II: OHP used to monitor dalteparin treatment

Levels of OHP, as well as those of OCP and OFP, in samples drawn before dalteparin injection from treated women, were within the ranges obtained for the control group in the respective stage of gestation, except for one patient (patient 5) (Table 2).



Levels of overall haemostasis potential (OHP) and antiX-a in one pregnant women with previous thromboembolism (patient 5)

OHP in all patients varied in converse relation to anti-Xa activity (Figs 1 and 2); the lowest value for the former and the highest for the latter appeared at almost the same time point (mean±SD: 3.4±1.78 and 3.7±0.95 hours, respectively). By 24 hrs after injection, both parameters had returned to levels around those before the administration of the drug.

Before dalteparin injections, the concentrations of F1+2 (1.54-3.50 µg/L) in all patients were within reference levels for F1+2 in pregnancy weeks 32-35 (1.30-6.10 µg/L). The levels of soluble fibrin were also normal in patients 1-4 (9-23 nmol/L) but slightly enhanced in patient 5 (28 nmol/L), compared to reference levels (< 25nmol/L). There were no significant variations in concentrations of F1+2 and soluble fibrin during the observation period.

In paper I*: *OHP used to monitor acetylsalicylic acid (ASA) therapy

During treatment with ASA, OHP levels decreased ($p<0.05$) compared to the levels before. By week 1 after withdrawal, this parameter had returned to levels similar to those before ASA administration (Fig 5). Further increases in OHP levels were noted 2 weeks after withdrawal of the drug but did not differ significantly from the original level on day 0.

3. Validity of the further modified OHP assay (reported in paper IV) in estimation of hypocoagulable conditions

In Paper IV, n-OHP levels (mean ± SD, n=3) were assayed in the reconstructed plasma samples with different severities of coagulation factor deficiencies (0%, 1%, 5%, 10%, 15%, 30%, 50%, 70% or 100%) (Figs 2-5). Progressive increases in n-OHP along with rising concentrations of the coagulation factor were seen in all kinds of the reconstructed samples, showing a high correlation: the correlative coefficients ranged between 0.87 - 0.99 (Figs 2-5). However, in FXII deficient plasma, OHP levels remained between the 5th and 50th percentile of the normal range regardless of the concentration of F XII (Fig 6).

After incubation of NPP with recombinant Annexin V, an inhibitor of phospholipids, the n-OHP levels were undetectable during the observation period of 40 min (no occurrence of clotting).

In the reconstructed FXI deficient plasma samples or in NPP, n-OHP levels were unaffected by the incubation with CTI (Fig 2).

Levels of OHP, as well as n-OCP and n-OFP, were assayed in reconstructed plasma with different concentrations of FVIII or FIX. n-OCP levels showed the same tendency as n-OHPs (Figs 4 and 5) but n-OFPs varied inversely.

The normal range in our laboratory of n-OHP, n-OCP and n-OFP, obtained from the 29 healthy volunteers, are shown as different percentiles (Table 1). The 2.5^{th} and 97.5^{th} percentiles were used as cut-off values for determining whether levels of the parameters were normal, or abnormally low or high. The 30 patients with haemophilia A (Table 2) were divided into two groups according to n-OHP levels < or ≥ the 2.5^{th} percentile of the normal range. In the group with n-OHP below the cut-off, n-OFP was significantly higher ($P < 0.05$) than in that with normal levels of n-OHP.

Reference levels for aPTT in our laboratory are 28-40 sec. aPTTs were measured in the reconstructed plasmas with FVIII or FIX deficiency (0%, 1%, 5%, 10% or 15%) and in samples from 20 patients with haemophilia A and 7 with haemophilia B (Fig 7). All the aPTTs were abnormally prolonged (> 40 sec) and varied in a logarithmic correlation with the levels of FVIII or FIX. All patients with FXII deficiency had prolonged aPTT (> 40 sec.) compared to the reference interval (Table 3). n-OHP levels were evidently increased (>97.5^{th} percentile) in three patients, while 2 had levels within the normal range. OFP levels were decreased in all of them (< 2.5^{th} in 4 patients and 2.5^{th} -5th in one).

4. Modifications of flow measurement assay for determining fibrin gel permeability

In Paper V, the porosity of the fibrin network is evaluated by analyses of gel permeability using flow measurement. To make the method suitable for use by different research groups, we simplified the essential equipment and also minimized the sample volume to 250 μL, compared with 3000 μL in the previous assay procedure.

In three experiments with NPP to which ***0.4 IU/mL thrombin*** was added, the mean Ks levels were 12.8 (n=3), 13.2 (n=3) and 12.1 (n=3); variation coefficients were 3.3%, 6.0% and 4.5%, respectively, for intra-assay and 4.3% for inter-assay. In the three experiments with ***platelet reagent and thrombin 0.05 IU/mL***, the mean Ks levels were 17.1 (n=3), 19.5 (n=3) and 17.2 (n=3), respectively; variation coefficients were 9.9%, 2.4% and 4.5% for intra-assay and 7.6% for inter-assay.

In the normal pooled plasma samples, Ks levels showed an inverse correlation ($r = -0.98$, $P < 0.01$) to the final concentrations of thrombin used to initiate fibrinogen clotting (Fig 2).

In the experiments with thrombin ***0.05 IU/mL and the platelet reagent*** (Fig 3), Ks levels (median and range) were significantly lower ($P < 0.01$) in plasma samples from 13 patients with previous MI (9.7; 3.7-21.0) than in those from 12 controls (19.3; 9.8-27.1).

When ***0.4 IU/mL*** of thrombin was used in samples from 10 healthy individuals, Ks levels were significantly increased during treatment with ASA ($p < 0.01$), compared to before therapy (Fig 4). Fibrinogen concentrations did not differ before and after ASA administration.

In fibrin gels derived from NPP with thrombin 0.2, 0.3, 0.4 or 0.5 IU/mL, Ks values were 12.4, 9.5, 8.4 or 7.5, respectively (Fig 5). When percolating buffer containing t-PA had seeped into the four gels, total amounts of D-dimers in the pooled eluates collected under 5 hydrostatic pressures were 0.27, 0.18, 0.07 or 0.05 mg/mL, respectively. The correlation between the levels of Ks and D-dimers was significant ($r=0.98$, $P < 0.05$).

5. A very low dose of acetylsalicylic acid leads to a large increase in fibrin gel permeability as determined by a modified flow measurement

In ***paper VI,*** the fibrinogen concentration in plasma did not change over time, and was not affected by ASA treatment at any dose. The fibrinogen concentrations were within the reference range of our laboratory (2-4g/L).

The permeability of fibrin gels (Ks) was similar in all three samples obtained during baseline conditions (Fig 2a), and Ks was inversely correlated to fibrinogen concentrations ($r = 0.66$, $P < 0.01$) (Fig 3a). The thickness of the fibrin fibres (expressed as the fibre mass/length ratio [μ]) was similar in all three baseline samples (Fig 2b) as was the structure of the fibrin network as examined by 3D confocal microscopy (not shown).

Fibrin gel permeability (Ks) was increased by ASA (all doses) compared to baseline ($P < 0.0001$). Ks values and μ values were similar in the samples obtained *during* the 24 hour dosing interval of ASA (Figs 2a and 2b).

Treatment with low dose ASA, 37.5 mg daily, increased Ks values by +44%, ($P < 0.0001$) and μ values by +30% ($P < 0.01$), compared to baseline values (Figs 4a and 4b). However, the increase in permeability was more pronounced during the administration of 37.5 mg compared to the higher doses ($P < 0.01$), since Ks levels increased by only 21% during medium and 31% during high dose ASA treatment, respectively ($P < 0.01$ for both compared to no ASA; Fig 4a). There was no significant difference in fibrin fibre thickness (μ) during medium or high dose ASA treatment compared to baseline (Fig 4b).

The 3-D confocal microscopy images (a typical experiment of 3 performed for each dose is presented in Fig 5 a-d), showed that thicker fibrin fibers, in more irregular arrangements, form fibrin networks with larger pores during low dose ASA treatment (Fig 5b), compared to treatment with the higher doses of ASA (Figs 5c and 5d).

The inverse relationship between Ks and fibrinogen concentrations found at baseline was abolished by ASA treatment at any dose (Fig 3b and 3c).

DISCUSSION

Modification of Laboratory Assay for Determination of Overall Haemostasis Potential in Plasma

Paper I

In view of the finding of undetectable OHP related to X- or II-deficiency, we consider that the smaller amount of added thrombin (0.04 IU/mL) is sufficient to trigger the coagulation cascade via a feedback reaction, and that the clotting of fibrinogen is exclusively caused by the generated thrombin in vitro. Plasminogen activation, on the other hand, depended almost exclusively on the exogenous t-PA, because t-PA function is extremely low in the citrated plasma. After addition of t-PA, plasminogen is converted into plasmin, which digests fibrin into its soluble fragments. Fibrin and fibrin breakdown products are able to raise the catalytic property of t-PA by 50-fold (Doolittle, 1994), so fibrinolytic function should progressively rise with increasing fibrin generation and accumulation of the breakdown products. Fibrin generation may therefore be superior to the rate of fibrin lysis in the early period of observation, as indicated by the increasing absorbance values (Abs). Under the stronger effect of fibrin and fibrin breakdown products, the t-PA became more active in plasminogen activation and fibrin lysis was accelerated, leading to a decline in the Abs values.

This description implies that each ABS value on the curve represents a level of fibrin at the corresponding time point, and that the area under the curve, expressed by Abs-sum, gives general information about fibrin generation and lysis throughout the measurement period, i.e. 40 minutes. The area under the curve is regarded as a laboratory parameter for the Overall Haemostasis Potential (OHP) in plasma, which is determined by the thrombin generation potential, the fibrinogen concentration and clotting properties as well as by the fibrinolytic potential of plasmin and may therefore may be considered a global parameter.

In the healthy pregnant women, our results support earlier findings (Greer, 1992), i.e. pregnancy is associated with activated coagulation and depressed fibrinolysis, which is pronounced in the course of gestation. The placenta provides an additional source of plasminogen activator inhibitor type 1 (PAI-1). When an insufficient blood supply in the placental circulation leads to preeclampsia, the haemostatic disturbance becomes more severe, with a characteristic further increase in PAI-1 activity (He et al, 1998). In the preeclamptic women, the clear decline in OFP (Fig 6) – the main reason for the elevation of OHP – may point to this complication being more related to the impairment of fibrinolysis than to that of coagulation.

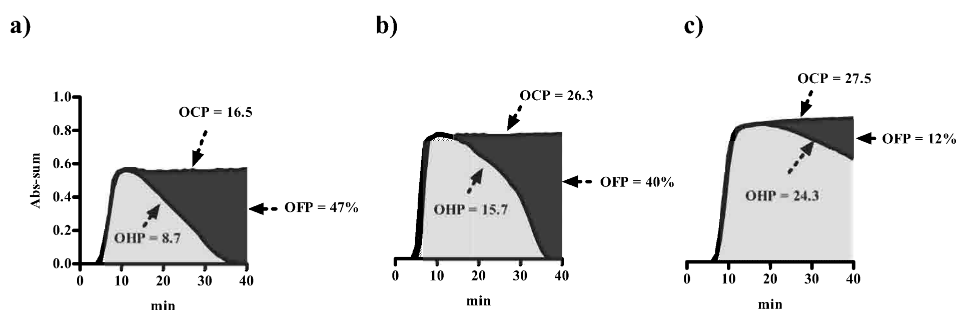


Fig 6. OCP / OHP / OFP assayed in normal pooled plasma sample (a), in sample from healthy pregnant women (b) and in sample from women with preeclampsia (c).

Despite no recent acute event of myocardial infarction and/or angina pectoris in the women with chronic CHD, using the 90th percentile in the controls as the cut-off value showed that 81% of them had increased OHPs. This finding indicates that the diagnostic power of the OHP assay is sufficiently strong, although the reference level shows a wide range. An explanation for the large variations in the reference levels could be that the test results are affected by many plasma proteins with different properties of pro-/anti-coagulation.

During treatment with ASA, the falling levels of OHP may be evidence of the depressed haemostasis, supporting clinical experience with regard to the benefit of ASA in the prevention of arterial thromboembolism (Antiplatelet trialists´ collaboration, 1994; Patrono and Roth, 1996). After withdrawal, the OHPs returned to levels similar to those before he initiation of the therapy, which explains why acute myocardial infarctions sometimes occur after cessation of ASA (Fatah et al, 1996).

The OHP Assay Used to Monitor Dalteparin Treatment

Paper II

After the long-term treatment with dalteparin by gestation weeks 32-35, the samples drawn before drug injection showed normal OHPs in 4 patients. After injection, the OHPs declined in response to the increases in anti-Xa**,** after which both parameters returned to levels around those before the drug administration. These findings suggest that during prophylaxis or treatment with LMMH, the simple and quick assay of OHP can indicate the druge influence on the general potential of haemostasis.

From 0 to 24 hrs after injection, the absence of significant variations in the thrombin generation markers (F1+2 and soluble fibrin) may be viewed in relation to the "half-life times", i.e. 60-90 min for F1+2; 15-30 hrs for soluble fibrin (Blomback et al, 1998).

No bleeding complications occurred in patients 1-4, who for 1-13 hrs had levels of OHP < the 5th percentile. DVT recurrence was successfully prevented during pregnancy and postpartum despite the increased levels of OHP in several samples from patient 5. The repeated administration of dalteparin, once daily for more than ten weeks, should lead to an accumulation of dalteparin in the endothelium and thus to protection against thrombosis (Bremme et al, 2001).

Levels of OCP and OFP were determined in patient 5 to search for the underlying causes of the increase in OHP. As demonstrated in Table 1, both increases in OCP and decreases in OFP were connected with the OHP levels above the 95th percentile of normal pregnancy. The depressed OFP probably indicates that this woman is more prone to thrombotic complications, despite treatment with dalteparin and absence of thromboembolic events.

Increased OHP in Women with previous DVT

Paper III

Compared with the healthy individuals, the most significant increase in the levels of OHP was found in patients with APC resistance due to factor V Leiden mutation, while a pro-coagulant state was also found in women who do not have a thrombophilic genotype.

OHP is a parameter that may be affected by changes in the potential of thrombin generation, fibrinogen concentration as well as plasminogen activation. Our earlier findings have shown a significant correlation between levels of fibrinogen and OHP in patients with coronary heart disease. In the present study, however, no such significant correlation was found and the fibrinogen concentrations were similar in the subjects with or without previous DVT. It is thus unlikely that the increase in OHP levels in the patient groups was determined by the amount of fibrinogen.

The clotting time signals the beginning of fibrin gel formation in connection with the generation of thrombin in vivo. An increase in the thrombin generation potential would lead to a shortening of the clotting time (Blombäck et al, 1994). The findings in the patients with previous thromboembolism may therefore serve as evidence that thrombin stimulation plays a vital role in creating an imbalance in the overall haemostasis potential in patients with previous DVT, especially in those with APC resistance related to factor V Leiden mutation.

Increased thrombin activity can lead to a tighter fibrin network. When a sluggish blood flow leads to a reduced transportation of fibrinolysis-promoting components through the small pores of fibrin gel, fibrin degradation is probably down-regulated. Our findings of prolonged clot lysis times, implying decreased clot dissolution by plasmin, in plasma samples from patients with previous thromboembolism may support this hypothesis. In addition, as reported in the literature (Bajzar et al, 1996; Antovic and Blombäck, 2002), APC resistance may initiate activation of thrombin activatable fibrinolysis inhibitor (TAFI), thereby impairing the binding of plasminogen to fibrin. We thus consider that suppression of the fibrinolysis potential served as another promoter of the persistent increase in OHP in DVT associated with APC resistance.

OHP Used to Estimate Hypocoagulable Conditions

Paper IV

In the present study, we designed a simple model to assess the influence of each single coagulation factor on the haemostatic process, shown as changes in OHP. This model includes a series of reconstructed plasma samples, i.e. commercial plasmas with different coagulant deficiencies individually mixed with NPP in different proportions. In these reconstructed plasma samples, OHP levels varied in highly significant correlations to the concentrations of coagulants (FVII, FXI, FII, FV, FX, FIX or FVIII), demonstrating that all the factors in the two coagulation cascade pathways can affect the OHP outcome in the assay system.

To initiate clotting in the examined plasma, 0.04 IU/ml of exogenous thrombin was added. In that the OHP level was undetectable in plasma samples with 0% of FX or FII (Fig 3), we consider that such a tiny amount of added thrombin is only enough to trigger a feedback reaction, while the fibrinogen clotting is solely caused by the thrombin generated in vitro. In FVII deficient plasma (0%), the prolonged clotting time (Fig 1) and the low levels of OHP (Fig 2) demonstrate the impaired thrombin generation in the absence of this coagulant.

Moreover, as TF was found to be present in the platelet reagent, the increase in OHP levels with increasing concentrations of FVII in the reconstructed plasmas should be a sign that activation of FVII in a complex with TF released from platelets had taken place.

The prolonged clotting time (Fig 1) and the low levels of OHP (Fig 2) were also detected in FXI deficient plasma. This suggests that when FXI is absent, thrombin generation can be initiated via the tissue factor pathway but a full thrombin burst is precluded due to lack of XI activation by the feedback reaction (Keularts IM, et al 2001). Since no specific inhibitor of XII was used in our assay system (Hojima Y, et al 1980), a surface reaction may contribute to the activation of FXI. However, this presumption was disproved by the experiment (Fig 2) in which reconstructed plasmas containing different concentrations of FXI (0-100%) were incubated with the specific inhibitor of FXII (CTI) before the OHP levels were assayed: OHP levels in the samples were similar with and without the treatment. We therefore consider that no FXII activation is present or at least no significant influence on the assay system from a surface response.

Even for comparatively common hereditary bleeding disorders, such as haemophilia A or B, no method can precisely reflect the clinical picture (Ingerslev et al, 2003). Among the patients with severe haemophilia (FVIII / IX <1%) (White et al, 2001), the clinicians found that patients with the same phenotype may differ in their predisposition to bleed. In the present study, haemophilic patients had abnormally prolonged aPTTs, yet none of them suffered from bleeding complications at the time of sampling. In the FVIII/FIX deficient plasma samples mixed with NPP, FVIII/FIX should be the exclusive variable, while all other coagulation factors are kept constant (Figs 4 and 5). The aPTT levels in the reconstructed plasmas were rather similar to those from the patients (Figs 7-A and 7-C), implying that aPTTs in the patients predominantly depend on the function of the relevant coagulant. However, OHP levels, like the haemostatic balance in the circulating blood, are determined not only by FVIII or FIX but also by numerous other factors in the coagulation cascade as well as by fibrinogen clotting properties and plasminogen activation. For this reason, the similar results of aPTT (Figs 7-A and 7-C) and a less close relationship of OHPs between the haemophilic patients and reconstructed plasma (Figs 7-B and 7-D) may mean that OHP reflects the haemostatic potential in vivo more accurately than aPTT, which is merely associated with the function of contact pathway. We are therefore interested in introducing the OHP assay to clinical practice for estimating the bleeding risk in such patients. For example, if the 2.5^{th} percentile of the normal OHP range is used as a cut-off to determine whether the haemostatic conditions are normal or abnormal (Fig 7), the patients with higher levels of OHP should be relatively safer than those with lower levels. Further studies are required to prove this hypothesis.

It has been previously observed that F XII deficiency is more commonly associated with thromboembolic complications than with a bleeding tendency (Mannhalter et al, 1987). In spite of this, a regular finding in patients with FXII deficiency is prolonged aPTT, similar to what is found in hypocoagulable disorders with deficiency of FVIII, FIX or FXI. This disagreement in patients was also observed in our investigation: none of the patients expressed a bleeding tendency even though aPTT was prolonged in them all due to decreased FXII levels and one of them was on oral anticoagulant treatment due to previous thrombotic events. Thus, as there was a noticeable enhancement of OHP in plasma from the patient group, we assume that the OHP assay may assist in determining the real haemostatic balance in patients with this disorder. Moreover, OFP levels were notably low in all the patients with XII deficiency, supporting the hypothesis that impaired fibrinolysis may contribute to the development of a prothrombotic condition in such patients (Levi et al, 1991).

Determination of Fibrin Gel Permeability by Modified Flow Measurement Technique

Paper V

Our results confirmed previous findings by Blombäck et al (1989) of an inverse relation between Ks levels and the amount of thrombin used for fibrin gel formation. No or just a tiny addition of exogenous thrombin (0.05 IU/mL) led to increases in Ks levels as high as 32.7 or 22.2, respectively. In the presence of phospholipid surface from exogenous platelet reagent, Ks levels decreased to 15.1 or 13.2, respectively. As phospholipid surface is essential for the activation of prothrombin, it is most likely that the above decline in fibrin gel porosity resulted from thrombin generation in vitro even in the absence of exogenous thrombin.

However, when higher doses of thrombin (0.2, 0.3, 0.4, 0.5 IU/mL) were added, fibrin network permeability was almost unaffected between the samples with or without platelet reagent. This implies that if the concentration of thrombin rises to a certain limit, thrombin generation in vitro may not occur at all. Hence, fibrinogen clotting is catalysed in principle by the exogenous thrombin; fibrin gel porosity relies on the quantity and/or quality of fibrinogen, the major material for construction of the fibrin network (Blombäck et al 1994).

Thus, we consider that the modified reaction system with added phospholipids and thrombin in a concentration as low as 0.05 IU/mL may be regarded as a global assay, revealing the influence of the endogenous generation potential and fibrinogen clotting ability on fibrin gel porosity. In studies aiming to assess whether fibrin gel permeability results from modifications of fibrinogen clotting properties, the higher dose of thrombin, 0.4IU/ml, is recommend.

Marked Increase in Fibrin Gel Permeability with Very Low Dose ASA Treatment

Paper VI

The mechanism underlying effects of ASA on fibrin gel structure was proposed to be acetylation of lysine residues in the fibrinogen molecule that disturb the fibrin fiber assembly, leading to enhanced fibrin gel porosity. A looser fibrin network should be less stable and therefore more prone to fibrinolysis (Bjornsson et al, 1998).

The results from this study demonstrated that 37.5 mg of ASA increased the permeability of the fibrin network more markedly than did 320 mg or 960 mg (320 mg + 640 mg). Our methodological study (paper V) suggested that fibrinogen clotting is exclusively induced by the exogenous thrombin if the final concentration of the enzyme is higher than 0.2 IU/ml; under such conditions, fibrinogen function should be the chief contributor to variations in fibrin gel structure. The favourable changes in fibrin gel permeability during low-dose ASA therapy should thus be caused by modifications of the fibrinogen clotting properties by acetylation. As regards the interesting phenomenon that Ks increases more markedly with lower doses of ASA (Williams et al, 1998 and present study), an explanation may be that fibrin porosity is not solely determined by how many lysine residues – the functional sites for cross-linking of fibrin fibers – have been blocked; but also by changes in charge distribution or protein conformation due to acetylation.

The 3D images showed that thicker fibrin fibers in more irregular arrangements formed fibrin networks with larger pores during low compared to high dose ASA treatment. Thus, strong support was obtained for the assay results, indicating an increased fiber mass/length ratio and increased network permeability. This morphological demonstration is indeed the first report showing visible changes in the fibrin network structure under the influence of ASA treatment.

Regardless of the ingested dose of ASA, the levels of permeability showed no daily variability. Thus, although the plasma concentration of ASA decays with a short half-life, i.e. 15-20 min (Patrono et al, 2001), acetylation-induced changes in fibrin gel structure persist for at least 24 hours, and probably last throughout the duration of fibrinogen persistence, with a half-life in plasma of 3.8-4.9 days (Blombäck et al, 1966).

CONCLUSIONS

General conclusions**:**

1. The OHP assay is a simple global method, which opens up the possibility of determining the imbalance in haemostasis and monitoring the effect/safety of relevant therapies. The OCP and OFP are supplementary parameters of OHP, providing details of underlying changes in the coagulation and/or fibrinolysis system. We thus suggest a combination of the three approaches to assist in pathological/pharmacological studies of haemostasis and thrombosis.
2. Any interested laboratory ought to be able to repeat the simplified assay of fibrin gel permeability to determine fibrin clot stability in pathological / pharmacological investigations with different study aims.

Specific conclusions:

- The sensitivity of the modified assay for detecting hypercoagulation in normal pregnancy, in preeclampsia and in coronary heart disease was further evaluated.
- The increase in OHP persists for a long time after a thromboembolic event. It might be clinically important to screen patients with previous thromboembolism for the need of thromboprophylaxis during their next pregnancy, regardless of the presence of APC resistance and / or factor V Leiden mutation.
- OHP can screen immediate changes in the haemostatic balance after dalteparin injection and is therefore sensitive for monitoring the anticoagulant effects, rather than the levels of F1+2 and soluble fibrin. Moreover, the OHP assay can demonstrate the combined effect of coagulation and fibrinolysis in the examined plasma, while anti-Xa levels only reflect the amount of heparin present in plasma.
- After the latest modification of OHP method, the assay system became more similar to the haemostasis balance in vivo, and thus hopefully serves as a laboratory tool to find different severity of hypocoagulation, especially in patients with haemophilia A or B.
- The modified flow measurement technique may be used to determine ASA effects on coagulation and provide further explanations for the finding that medium/high doses of ASA are less effective than low doses in preventing arterial thrombotic complications.

SAMMANFATTNING PÅ SVENSKA

Vi har tidigare utvecklat en metod, som kan mäta den totala hemostas balansen i plasma- Overall Hemostasis Potential (OHP), baserad på spektrofotometrisk mätning av området (arean) under den fibrin aggregations kurva som erhålles i citratplasma till vilken satts spårmängder av trombin och vävnads plasminogen aktivator (t-PA) Fibrinogenet i plasma omvandlas då gradvis till fibrin av det trombin som genereras. Samtidigt producerar t-PA plasmin som i sin tur degraderar bildat fibrin. Varje absorbans värde som erhålles representerar fibrin nivån vid just den tidpunkten och arean reflekterar genereringen och nedbrytningen av fibrin under mätningstiden.

För att förbättra metodens känslighet för användbarhet i klinik och i forsknings arbete har vi gjort ett flertal modifikationer. Trombin i en låg dos (0.04 IE/mL) med eller utan t-PA sattes till plasma. Arean under de två fibrin aggregations kurvorna: OHP och Overall Coagulation Potential (OCP) kunde så erhållas. Differensen mellan dessa speglar fibrinolyspotentialen (OFP = Overall Fibrinolytic Potential) .

Den modifierade metoden har visat sig användbar för att studera normal graviditet, havandeskapsförgiftning och patienter med hjärt-kärl sjukdom. Ökade OHP-nivåer hittade vi också hos kvinnor med tidigare trombos speciellt hos dem med den s.k. FV Leiden mutationen. Vidare kan medoden mäta förändringar i hemostassystemet under behandling med lågmolekylärt heparin (dalteparin) och kan således användas för att följa behandlingseffekt.

För att förbättra känsligheten för olika svårighetsgrader vid hypokoagulation modifierade vi metoden ytterligare genom att tillsätta vävnadsfaktor och fosfolipid till systemet. Alla faktorer, som hör till det ytaktiverade och det vävnadsfaktor aktiverade systemet förutom FXII påverkade OHP, vilket visade att denna modifikation kunde användas för att uppskatta blödningstendensen hos blödarsjuka patienter och upptäcka vilka FXII brist patienter som var protrombotiska. Vi anser att OHP metoden är en enkel global metod som öppnar möjligheter för att bestämma balans och obalans i hemostasen och för att följa effekten av olika behandlingar.

OHP är således en metod att bestämma hemostasen och också i princip fibrinogen nivån, men studier av fibrin gel porositet med flödesmetodik kan ge information om fibrinogenets kvalitet vilket är av betydelse t.ex. för ateroskleros. Vi har modifierat metoden som tidigare beskrivits av Birger Blombäck och medarbetare; vi har förenklat apparaturen och minskat volymen så att den bättre går att använda i praktiken. Genom att använda olika koncentrationer av trombin med eller utan fosfolipid kunde vi bestämma huruvida fibrin gelens porositet beror på både trombin genererings potentialen och fibrinogenets klotting potential eller bara på den senare.

Denna modifierade flödesmetodik har använts för att studera acetylsalicylsyrans (ASA) = aspirinets effekt på hemostasen. Vi har visat att fibrin gelens porositet var kraftigt ökad under behandling med låga doser ASA jämfört med medium/höga doser. Dessa resultat har konfirmerats med hjälp av 3 dimensionell konfokal mikroskopi där vi fann tjockare fibrin fibrer och större porer i fibrin gel nätverket med en oregelbunden struktur. Den större ökningen i fibrin gel porositeten och förändringarna i strukturen noterade i mikroskopet vid olika doser av ASA stöder de kliniska fynden att lägre doser av ASA, såsom 75 mg dagligen, ger en bättre skyddande effekt vad avser arteriell tromboembolism, såsom vid hjärt-kärl sjukdom och stroke, än högre doser.

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