

**Coagulation Inhibitor Potential:  
a global assay for the detection of thrombophilia**

**by  
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## Abbreviations

APC	Activated protein C
APL	Antiphospholipid antibodies
APS	Antiphospholipid syndrome
APTT	Activated partial thromboplastin time
AT	Antithrombin
AUC	Area under the curve
CAT	Calibrated automated thrombogram
CIP	Coagulation inhibitor potential
CV	Coefficient of variation
DIC	Disseminated intravascular coagulation
DVT	Deep vein thrombosis
ELISA	Enzyme linked immunosorbent assay
ETP	Endogenous thrombin potential
F	Factor
GAG	Glycosaminoglycans
HCII	Heparin cofactor II
kD	kilo Dalton
LA	Lupus anticoagulant
MW	Molecular weight
MTHFR	Methylene tetrahydrofolate reductase
NETA	Norethisterone acetate
OAC	Oral anticoagulants
OC	Oral contraceptives
OCP	Overall coagulation potential
OFP	Overall fibrinolysis potential
OHP	Overall haemostasis potential
PC	Protein C
PCG	ProC global
PCR	Polymerase chain reaction
PE	Pulmonary embolism
PS	Protein S
PT	Prothrombin time
ROC	Receiver operating characteristic
SERM	Selective oestrogen receptor modulator
SLE	Systemic lupus erythematosus
SPSS	Statistical program package of social science
TEG	Thrombelastography
TG	Thrombin generation
TF	Tissue factor
TFPI	Tissue factor pathway inhibitor
t-PA	Tissue type plasminogen activator
TM	Thrombomodulin
U	Units
vWF	Von Willebrand factor
VTE	Venous thromboembolism

# 1. Introduction

## 1.1 Haemostasis

The haemostatic process is activated as a response to vascular injury and aims at restoring vascular patency. It involves interactions between endothelium, platelets and coagulation factors. In “*primary haemostasis*” a vasoconstriction of the damaged blood vessel reduces the blood flow transiently, and platelets adhere to the site of injury to form a haemostatic platelet plug. In “*secondary haemostasis*” a stable platelet plug is formed. A cascade of enzymatic reactions converts proenzymes into their active forms, leading to the formation of thrombin which in turn converts soluble fibrinogen into a fibrin network stabilising the platelet plug. Fibrinolysis is triggered by fibrin which leads to the formation of plasmin. This enzyme may reduce and eventually break down the clot.

## 1.2 Thrombosis

Thrombosis is the formation of insoluble material within the lumen of a blood vessel. This obstructs the blood flow, which may be particularly serious when the thrombosis completely occludes the lumen. Thrombosis involves the same reactions as are involved in haemostasis: activation of platelets and coagulation. Arterial thrombosis most often develops on pathologically changed intima of the vascular wall. Venous thrombosis most often occurs in stagnant blood and without obvious changes in intima. Venous thromboembolism (VTE) comprises deep vein thrombosis (DVT) and pulmonary embolism (PE).

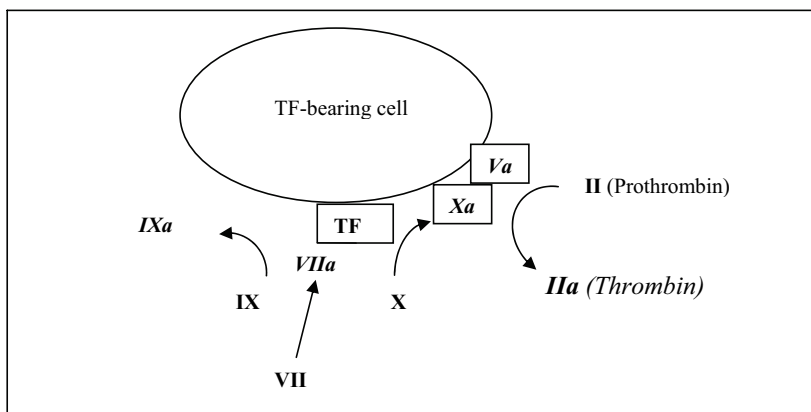
## 1.3 Activation of coagulation

Blood coagulation has traditionally been described as a cascade [1] or waterfall [2] of an ordered series of enzymatic reactions that involved two different activation pathways, the intrinsic (contact activation) and the extrinsic (tissue factor (TF)) induced pathways meeting in a common pathway (for review see [3]). The understanding of coagulation is now changing and it has become clear that these models do not accurately reflect the processes of coagulation *in vivo*.

### *The cell based model of coagulation*

*In vivo* the coagulation reactions occur on cell surfaces in three distinct but overlapping phases: initiation, amplification and propagation [4-6]. The cellular localisation is an important control mechanism to keep coagulation localised to the site of injury.

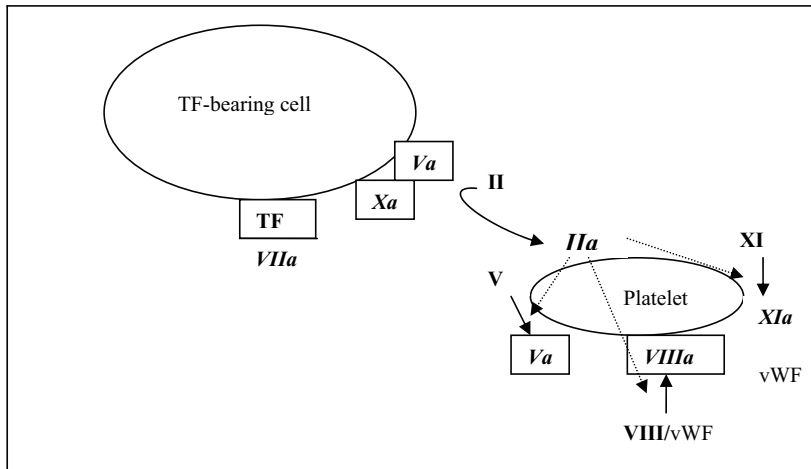
The *initiation* phase of the TF coagulation pathway is triggered when factor VII (FVII) binds to TF-bearing cells that are exposed to circulating blood as a result of vessel wall injury (Figure 1a). TF is an integral membrane protein and is constitutively expressed in different cell types such as fibroblasts and epithelial cells [7] and is normally not in contact with the blood stream. In severe disease states, TF may be expressed on the surface of activated monocytes and may trigger coagulation [8]. TF is also involved in inflammation, angiogenesis and tumour metastasis [9,10]. Small amounts of FVII circulate in blood as an active serine protease (FVIIa) [11]. The formation of TF-FVIIa complex promotes activation of the TF-FVII complex by proteolytic cleavage of the zymogen FVII into the active enzyme FVIIa. The TF-FVIIa complex then converts small amounts of factors IX (FIX) [12] and X (FX) to their active forms (FIXa and FXa) on the cell surface. In plasma, FXa is rapidly inactivated by antithrombin (AT) or by tissue factor pathway inhibitor (TFPI) whereas FIXa is more stable and can dissociate from the cell surface to a nearby platelet. Factor V (FV) can be activated by FXa [13]. FXa that remains on the cell surface can combine with activated FV (FVa) to form the prothrombinase complex to produce small amounts of thrombin. The small amount of thrombin generated on TF-bearing cells also activates nearby platelets [14], exposing receptors and binding sites for activated clotting factors.



**Figure 1a. Initiation phase. Cell based model of coagulation.**

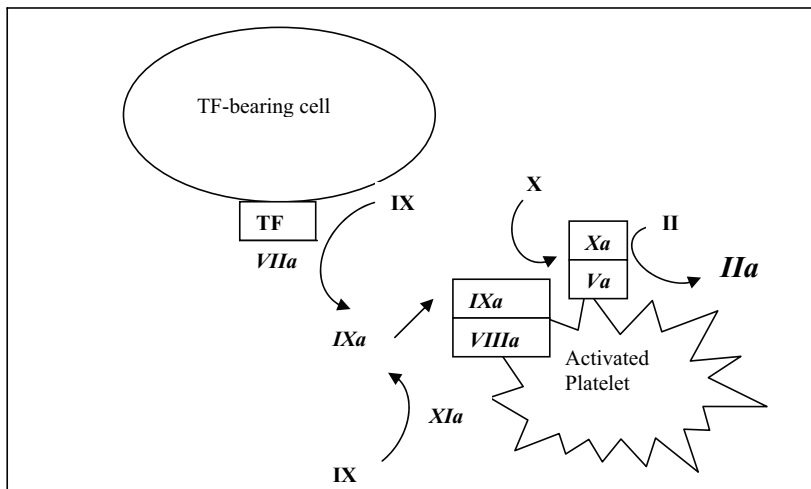


The *amplification* phase takes place on the phospholipid surface of the platelets at the site of vascular injury (Figure 1b). FV and factor VIII (FVIII) are procofactors which are converted into their active forms by thrombin or FXa [13,15]. Prior to activation FVIII circulates bound to von Willebrand factor (vWF), but activation promotes dissociation of the FVIII/vWF complex. Activated platelets release FV from  $\alpha$  granules onto their surfaces. Factor XI (FXI) is also activated on the platelet surface by thrombin to its active form FXIa [16].



**Figure 1b. Amplification phase. Cell based model of coagulation.**

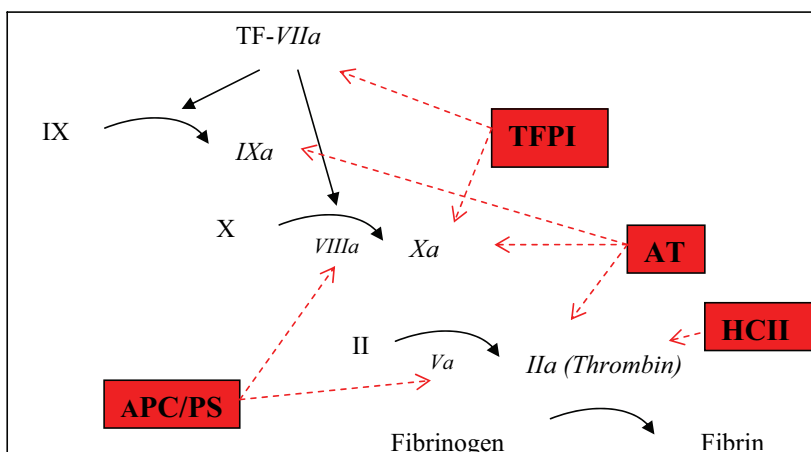
In the *propagation* phase procoagulant complexes assemble on the surface of activated platelets to accelerate thrombin generation (Figure 1c). FIXa binds to FVIIIa to form the tenase complex which activates FX. The FXIa can provide additional FIXa for the formation of FXa. FXa associates with platelet surface FVa to form the prothrombinase complex. Prothrombin is then converted to prothrombin fragments 1+2 (F1+2) and a burst of thrombin which converts fibrinogen into a fibrin clot.



**Figure 1c. Propagation phase. Cell based model of coagulation.**

#### 1.4 Regulation of coagulation

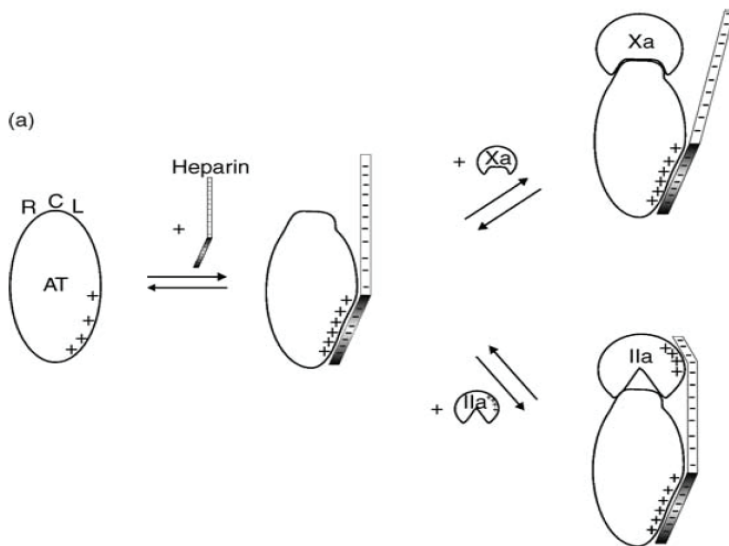
In normal haemostasis there is a natural balance between procoagulant and anticoagulant forces. Various inhibitory mechanisms prevent the coagulation process beyond the site of vascular injury that may otherwise result in thrombosis. The coagulation process is controlled by three inhibitory pathways: the AT pathway, the protein C (PC) pathway and the inhibition of TF by TFPI (Figure 2).



**Figure 2. Inhibitors of coagulation.** Continuous lines: activation, dotted lines: inhibition.

### *The antithrombin (AT) pathway*

AT is the main inhibitor of thrombin [17] circulating at a high concentration (2.5  $\mu$ M) in blood plasma. It is a powerful anticoagulant molecule. FXa, FIXa [18] and FXIa [19] generated during activation of the clotting process are also inactivated by AT. AT is a glycoprotein of approximately 58 kD which is synthesised in the liver [20]. Circulating AT molecules have slow inhibitory activity, but are accelerated considerably by specific interactions with glycosaminoglycans (GAG) such as heparin and heparan sulphate [21]. *In vivo* AT might localise to heparan sulphate chains on the endothelium or bind to the localised release of heparin. Heparin may be released from the granules of mast cells which are found lining the vasculature. Binding of a specific pentasaccharide sequence in heparin induces a conformational change in the AT molecule [22] (Figure 3).



**Figure 3. Inactivation of factor Xa and thrombin by AT and heparin.** AT undergoes a conformational change upon interaction with a specific pentasaccharide fragment in heparin (left). This accelerates interaction with FXa via the reactive centre loop (RCL). Interaction with thrombin requires heparin chains long enough to bridge AT and thrombin. Adopted with permission from Huntington et al [23].

This sequence is present in about one percent of the heparan sulphate in the vessel wall and about one third of mast cell heparin [24]. The change in structure of AT substantially

accelerates the interaction with serine proteases such as FXa and FIXa [25], but not thrombin. Rapid inhibition of thrombin requires heparin chain lengths of at least 18 monosaccharides which contain the pentasaccharide sequence. A ternary complex must be formed between heparin, AT and thrombin [26]. This may accelerate the interaction of AT with thrombin up to a thousand fold [27].

#### *Heparin cofactor II*

Heparin cofactor II (HCII) contributes to the inhibition of thrombin, but its physiological function remains unclear [28]. Thrombin inhibition by HCII is accelerated and enhanced by GAGs. The GAG specificity however, is much less discriminating than that of AT. HCII is activated by dermatan sulphate, heparin, and heparan sulphate [29]. In comparison with AT, much higher concentrations of heparin or heparan sulphate are necessary to activate HCII [30]. The heparin binding mechanism of HCII is similar to that of AT [31]. HCII is synthesised in the liver and the mature protein is approximately 66 kD. Like AT, HCII circulates at a high concentration in plasma (1.2  $\mu$ M) [32].

#### *The protein C (PC) anticoagulant pathway*

The PC system is an effective and important way of regulating the activities of the cofactor FVIIIa in the tenase complex, and the cofactor FVa in the prothrombinase complex. In addition to anticoagulant properties PC also has anti-inflammatory and antiapoptotic properties [33]. PC is synthesized in the liver with a MW of approximately 62 kD [34]. It circulates in the blood as a zymogen. Binding of thrombin to its cofactor thrombomodulin (TM) on the endothelial cell surface changes the enzymatic specificity of thrombin and activates PC by enzymatic cleavage [35]. The endothelial PC receptor helps to orient PC to the thrombin-TM complex and provides further stimulation of the activation of PC [36]. Activated PC (APC) inhibits coagulation by inactivating surface bound FVa and FVIIIa [37]. The anticoagulant activity of APC is supported by free protein S (PS) which functions as a cofactor for APC in the degradation of FVa [38]. Degradation of FVIIIa by APC requires cofactor activities of both PS and FV [39]. APC is a specialised enzyme and cleaves FVa in three sites at positions Arg306, Arg506 and Arg679 [40]. The Arg506 cleavage is kinetically favoured over the Arg306 cleavage.

### Inhibition by *tissue factor pathway inhibitor (TFPI)*

TFPI is a plasma serine protease inhibitor. Inactivation of the TF-FVIIa complex by TFPI is dependent on FXa and involves a formation of a quaternary complex containing FXa-TFPI-TF-FVIIa [41]. TFPI consists of three Kunitz-type domains followed by a long carboxy-terminus [42]. The first Kunitz domain interacts with FVIIa and the second domain binds FXa [41]. TFPI recognises FXa most effectively when the enzyme is in the prothrombinase complex [43]. Once this occurs, FXa can only be produced by the tenase complex. TF-FVIIa may also be inactivated by AT-heparin complex [44], but inactivation by TFPI is the main physiological inactivation mechanism.

In plasma TFPI exists both in full-length and variably carboxy-terminated truncated forms. TFPI is mainly produced by microvascular endothelial cells and the total normal plasma concentration is 1.0-2.5 nM [45,46]. The major fraction of circulating TFPI is bound to lipoproteins, in particular low-density lipoproteins [47]. The most predominant forms of plasma TFPI have a molecular weight of 34-41 kD [48].

### **1.5 Disseminated intravascular coagulation (DIC)**

DIC is an acquired disorder characterised by systemic activation of blood coagulation and subsequent exhaustion of anticoagulation mechanisms and the fibrinolytic system. In its fulminant form, this may lead to simultaneous widespread microvascular thrombosis and profuse bleeding from various sites [49]. Ongoing activation of coagulation leading to vascular fibrin deposition, thereby compromising an adequate blood supply may contribute to multiple organ failure.

The mechanisms involved are TF dependent initiation of coagulation, amplification of thrombin generation caused by consumption of platelets, coagulation factors and inhibitors and propagation of fibrin deposition in the microvasculature caused by insufficient fibrin degradation as a result of an inhibited fibrinolytic system.

DIC is not a disease in itself, but is always secondary to an underlying disorder. Clinical conditions that may be associated with DIC are severe infections, major trauma and tissue damage, obstetrical complications, cancer, immunological and haematological disorders [50].

## 1.6 Thrombophilia

Thrombophilia can be defined as an increased persistent tendency to thrombosis and can be acquired or inherited. Inherited thrombophilia may be caused by several different genetic defects that mostly result in reduced levels of inhibitors of coagulation in the blood. This leads to a hypercoagulable state and an increased risk of VTE. The most common forms of venous thrombosis are DVT of the leg and PE [51]. Arterial thrombosis is only rarely triggered by thrombophilia.

### 1.6.1 Inherited thrombophilia

Deficiencies of the natural coagulation inhibitors AT, PC and PS are rare and can be found in less than 1% of the general population. Deficiencies which are associated with increased procoagulant activity, FV Leiden and prothrombin gene mutation are more common causes of inherited thrombophilia.

#### *Antithrombin (AT) deficiency*

AT accounts for about 80% of the thrombin inhibitory activity of plasma. Inherited AT deficiency as a cause of familial thrombophilia was first reported in 1965 by Egeberg [52]. AT is a single chain glycoprotein, belonging to the serine protease inhibitor (serpin) superfamily. Its concentration in plasma is 2.5  $\mu\text{M}$  [53]. AT deficiency is a heterogeneous disorder and can be classified as type I and type II. Type I heterozygous AT deficiency is defined as about 50% functional and antigen activity of AT, whereas type II is characterised by the synthesis of a variant protein with altered function. According to which function of the protein is affected, three type II subtypes can be defined: reactive site defect, pleiotropic effect (abnormal tertiary structure) and heparin binding site defect. AT deficiency is a rare disorder with a prevalence of type I of about 0.02% and type II of about 0.16% [54]. Heterozygous AT deficiency increases the risk of venous thrombosis 25-50-fold which is higher compared to any other thrombophilic state and is found in 1-4% of patients with venous thrombosis [55-57]. An exception is mutations affecting the heparin binding site which appear to be associated with a low risk of thrombosis [58,59]. Homozygous AT deficiency is probably incompatible with life except in the case of the type II defect involving the heparin binding site [60].

### *Protein C (PC) and protein S (PS) deficiencies*

PC and PS deficiencies result in defects in the PC anticoagulant system leading to decreased inactivation of cofactors Va and VIIIa. The first report of heterozygous PC deficiency was published in 1981 [61]. PC is a vitamin K dependent glycoprotein. The concentration of PC in plasma is 65 nM. PC deficiency is classified as type I: low plasma levels of both functional and antigen PC and type II: low plasma levels of functional protein with normal antigen levels. Its prevalence is about 0.2-0.3% [62]. Heterozygous PC deficiency increases the risk of venous thrombosis 10-15-fold [63]. The risk of thrombosis appears not to be different for the different types of PC deficiency [64]. PC deficiency occurs in 3-6% of patients with venous thrombosis [56,57].

PS is a vitamin K dependent glycoprotein produced mainly by hepatocytes, but also by endothelial cells and megacaryocytes [65-67]. The concentration of PS in plasma is 0.35  $\mu$ M [68]. Families with PS deficiency have been reported since 1984 [69,70]. About 40% of PS circulates in plasma as a free protein, the remainder being bound to the complement regulatory protein C4b-binding protein [71,72]. There are three types of PS deficiency. Type I is associated with low plasma levels of both total and free PS. Type II is associated with low plasma levels of functional protein and normal total and free PS levels. Type III is characterised by normal levels of total PS and low levels of free PS and is due to mutations that enhance the binding of PS to C4b-binding protein. The prevalence of PS deficiency is 0.03-0.13% [73] and occurs in 1-5% of patients with venous thrombosis [57,74].

Heterozygous PS deficiency increases the risk of venous thrombosis nearly 10-fold [75].

In persons with a deficiency of PC or PS, type I defects predominate. Homozygous PC and PS deficiency are associated with neonatal purpura fulminans, a syndrome characterised by widespread venous thromboses and acute haemorrhagic lesions in the newborn period [76].

### *Factor V (FV) Leiden mutation*

The most common inherited gene defect associated with venous thrombosis is the FV Leiden mutation [77], which results in a phenotype called APC resistance [78]. A single point mutation in the FV gene (G1691A) replaces Arg506 with Gln. This results in the loss of one of the three APC cleavage sites in FV/FVa. The PC system is affected in two ways by the mutation. The first is impaired degradation of mutant FVa by APC because one of the three cleavage sites is missing. The second is poor degradation of FVIIIa because mutant FV is a poor cofactor to APC in the degradation of FVIIIa. The mutation is predominantly found in whites and in 20-40% of patients with venous thrombosis [79,80]. The prevalence of FV

Leiden in the general population varies geographically. The highest prevalence is found in northern Europe where 7-15% carries the mutation [81]. Heterozygous FV Leiden increases the risk of thrombosis about 2-5-fold [82,83] and homozygous FV Leiden increases the risk about 18-fold [83]. Combined thrombophilias most often involves FV Leiden and another abnormality because FV Leiden is relatively common. It is suggested that persons with combined thrombophilic defects are at a higher risk of thrombosis than those with either defect alone [84,85]. The high prevalence of the FV Leiden mutation might suggest a positive selection mechanism related to decreased pregnancy bleeding complications [86].

*Prothrombin gene mutation*

A single mutation (G20210A) in the prothrombin gene is the second most common genetic risk factor for thrombosis [87]. The prothrombin function is unaffected by the mutation, but the levels of prothrombin in plasma are increased [88]. This mutation is associated with 3-5-fold increased risk of thrombosis [87]. Homozygosity for prothrombin gene mutation is much rarer and causes a higher thrombotic risk. The population prevalence of the heterozygous G20210A mutation among Caucasians is about 1-4% with considerable geographic variation [89].

**1.6.2 Acquired risk factors for venous thrombosis**

In the mid-1800s, Virchow postulated three major causes of thrombosis: changes in the vessel wall, changes in the blood flow and increased coagulability. This triad still applies. Most of the risk factors for venous thrombosis (Table 1) fall into the last two groups.

**Table 1. Inherited and acquired risk factors for VTE.**

Inherited	Acquired	Mixed
Antithrombin deficiency	Antiphospholipid antibodies	Hyperhomocysteinemia
Protein C deficiency	Cancer	Elevated FVIII, FIX, FXI
Protein S deficiency	Medical disorders	Acquired APC resistance
Factor V Leiden mutation	Immobilisation	
Prothrombin gene mutation	Surgery/trauma	
	Pregnancy/puerperium	
	Oral contraceptives	
	Hormone therapy	
	Obesity	
	Age	
	Previous VTE	



### *Antiphospholipid antibodies*

Antiphospholipid (APL) antibodies are autoantibodies directed against anionic phospholipids or protein-phospholipid complexes.  $\beta$ -2 glycoprotein I is the principal target in the antiphospholipid syndrome (APS) [90,91], but many other antigenic targets have been described including prothrombin and annexin V [92,93]. APS is a disorder that refers to recurrent arterial and venous thrombosis, and pregnancy loss associated with the presence of APL antibodies and persistently positive anticardiolipin or lupus anticoagulant (LA) positive tests. LAs are antibodies that block phospholipid surfaces important for coagulation. They reduce the coagulant potential of the plasma and prolong the clotting time in coagulation tests based on the activated partial thromboplastin time (APTT) [94]. APS may occur alone or in association with systemic lupus erythematosus (SLE). In SLE, the risk of thrombosis is increased in individuals with APS antibodies [95]. These abnormalities are found in about one-half of patients with SLE. The risk of thrombosis is also increased in patients who do not have SLE but do have a LA. This abnormality has been estimated on the average to lead to a nine-fold increase of thrombosis [96].

### *Homocysteinemia*

Homocysteine is a sulphur-containing amino acid formed during the metabolism of methionine. Mild to moderate hyperhomocysteinemia is found in individuals with genetic defects, acquired conditions, or both. Acquired causes predominate and are found in patients with dietary deficiencies of folate, vitamin B12, or vitamin B6, while inherited forms are most commonly due to mutations affecting the cystathionine  $\beta$ -synthase gene or the methylene tetrahydrofolate reductase (MTHFR) gene [97,98]. The mechanism by which hyperhomocysteinemia affects the risk of thrombosis is unknown [99]. While severe hyperhomocysteinemia is rare, mild elevations of homocysteine are present in 5-10% of the healthy population and is probably associated with 2-3-fold increased thrombotic risk [100,101].

### *High levels of factor VIII (FVIII)*

A dose-response relationship between FVIII levels and the risk of thrombosis has been observed [102,103]. Plasma FVIII levels reflect the combined influence of inherited and acquired factors. Relative to those people with levels below 1000 IU/l, those with levels above 1500 IU/l may have a 5-fold increased risk for thrombosis [102]. Increased levels of FVIII are quite common in the general population, with a prevalence of about 11%, while up

to 25% of patients with venous thrombosis have FVIII levels higher than normal [104]. FVIII is an acute phase reactant. Long term elevated FVIII levels have been demonstrated to be independent of the acute phase response [105]. Increased levels of FIX and FXI are also associated with an increased risk of thrombosis [106,107].

### *Cancer*

Venous thrombosis is a common complication in cancer patients. 10-20% of patients with venous thrombosis have cancer [108,109]. The thrombogenic effect of cancer may be the result of several factors. The tumour itself may increase the risk of thrombosis due to venous obstruction, production of procoagulants and acute phase reactions [110]. Furthermore, there may be less mobility due to the illness, as well as thrombogenic effects of treatment [110]. Cancer patients undergoing surgery have about a 2-fold increased risk of postoperative VTE compared to non-cancer controls undergoing the same procedures [111]. After middle age the prevalence of cancer in a population may be 2-3%. The incidence of symptomatic VTE in cancer patients has been described to be approximately 15% [112].

### *Immobilisation*

Thrombosis may occur in many circumstances that are associated with immobilisation such as bed rest [113] and prolonged travel [114]. These situations interfere with the function of the musculature in pumping the blood upstream through the veins. Unless accompanied by an additional risk factor the absolute risk of VTE associated with immobilisation is small.

### *Surgery and trauma*

Major surgery is one of the strongest risk factors for thrombosis. Without thromboprophylaxis surgery can lead to thrombosis in up to 50% of the patients, dependent on the type of surgery. VTE is often asymptomatic and high frequencies were disclosed in screening studies. In orthopaedic surgery of the hip and knee, the risk of thrombosis reaches 30-50% [115,116]. Major trauma is also an important risk factor for thrombosis and occurs in up to 50-60% of patients with head trauma, spinal injury and pelvic fractures [117]. After elective abdominal surgery, the frequency of VTE is about 20% [118]. Prophylaxis against VTE is now a routine in patients older than 40 years having a major operation [119].

### *Pregnancy*

About one in 1000 women will develop thrombosis during pregnancy [120,121]. When compared to the same age group, the risk appears about 10-fold increased. During pregnancy there is shift in the haemostatic balance towards a more hypercoagulable state, with increasing concentrations of most clotting factors and decreasing concentrations of natural anticoagulants [122,123]. These alterations protect pregnant women from severe haemorrhage during delivery. The risk of thrombosis during pregnancy is greater in women with inherited thrombophilia [124,125]. Pregnant women with AT deficiency appear to have an unusually high risk for thromboembolism, and should receive anticoagulant prophylaxis throughout pregnancy and puerperium [126]. Thrombophilia increases the risk of pregnancy loss [127,128].

### *Oral contraceptives*

The first association of oral contraceptive (OC) use and thrombosis was reported in 1961 by Jordan [129]. The increased risk of venous thrombosis was later confirmed in many reports. Early OCs contained 100 µg or more of oestrogen, but over the years the dose of oestrogen has been substantially reduced to 30 µg and even lower. The reduction in oestrogen content was accompanied by a reduction in risk of venous thrombosis, but even the low-dose OCs that are used today increase the risk of thrombosis 2-5-fold [130,131]. Oestrogens markedly affect the coagulation system, with increased levels of procoagulant factors and reduced concentrations of the anticoagulant factors PS, AT and TFPI [132,133]. Combined monophasic OCs that consist of a combination of oestrogen and progestogen are the most common birth control pills. The oestrogen content is usually ethinylestradiol, while the progestogen component has changed over the years, from first-generation which is no longer used, to second-generation (levonorgestrel), to third-generation progestogens (desogestrel, gestodone and drospirenone). The risk conferred by OCs is not restricted to the oestrogen content, but also depends on the progestogen content. Some of the effects on the coagulation system are more pronounced in OCs containing third-generation progestogens, than in those containing second-generation progestogens, leading to a more pronounced prothrombotic state [134-136]. Third-generation progestogen counteracts the prothrombotic effect of the oestrogen component less effectively compared with the second-generation progestogen. OCs containing the third-generation progestogens confers a 2-fold higher risk than the second-generation OCs [137]. OCs also greatly increases the risk of thrombosis in persons with inherited thrombophilia [138].

### *Hormone treatment*

Postmenopausal hormone therapy (HT) is used for treatment of symptoms of the menopause and to reduce the progression of osteoporosis [139]. The effective content of HT is the oestrogen, but since hormones with only oestrogens increases the risk of endometrial cancer it is common to combine oestrogen with progestogen [140]. In Scandinavia mostly oestradiol is used, which is natural oestrogen. Norethisterone acetate (NETA) is the most common progestogen used in Norway. The dose of oestrogen in HT is very low compared to OCs. Yet, several studies have demonstrated that HT increases the risk of thrombosis 2-4-fold [131,141,142]. The risk of venous thrombosis is higher shortly after therapy has started [142]. There has been a recent trend to use lower doses of oestrogen and progestogen for HT based on evidence of similar effectiveness of low-dose HT on symptoms and bone [143]. HT is associated with a significant dose-response dependent activation of coagulation [144]. Moreover HT is also associated with decreased AT, TFPI and PS concentrations and increased resistance to APC [145,146].

Raloxifene is a selective oestrogen receptor modulator (SERM). SERMs bind with high affinity to the oestrogen receptor and result in oestrogen agonist effects in bone tissue and the cardiovascular system and oestrogen antagonist effects in endometrial and breast tissue [147]. Women treated with raloxifene have an increased risk of venous thrombosis [148].

Tibolone is a synthetic steroid analogue with oestrogenic, androgenic and progestogenic properties depending on the target tissue involved [149]. It is used in the management of climacteric symptoms and bone loss. Tibolone has so far not been associated with an increased risk of venous thrombosis, but sufficient epidemiological and clinical data are missing [143]. Recently, an increased risk of stroke has been reported [150].

### *Age*

One of the strongest risk factors for thrombosis is age. The risk of thrombosis increases sharply with age, with a 1000 fold difference in risk of thrombosis between the very young and the very old [151,152]. It is not clear why there is an age gradient, but plausible reasons are a combination of decreased mobility, decreased muscular tone, increased frequency of risk enhancing diseases and acquisition of other risk factors as well as aging of the veins and the valves in the veins. Changes in the coagulation system may also contribute to an increased risk of thrombosis.

### **1.6.3 Cooperative effects on risk of venous thrombosis**

Risk factors resulting from interaction between genetics, environmental factors and behaviour may bring about thrombosis. The combination of a genetic defect with one or more environmental risk factors and the combination of two environmental risk factors result in a risk of venous thrombosis that exceeds the sum of the separate effects of the single factors. Carriers of the heterozygous FV Leiden mutation have up to 30-fold increased risk of thrombosis when they use oral contraceptives compared with nonusers with a normal genotype [153,154]. The risk is higher than expected because it is not the additive effects of the two risks. The risk factors potentiate each other.

### **1.7 Assay methods for inherited thrombophilia**

Thrombophilia was for many years investigated exclusively by means of plasma based-assays (phenotypic assays). More recently DNA-analysis has become available. Phenotype determination is more difficult to standardise and the results may be variable. For thrombophilic conditions such as AT, PC and PS deficiency the underlying defect may be attributable to several mutations and genetic testing is at present only applicable in research. AT, PC and PS deficiency states are nearly always heterozygous, which implies that the measured inhibitor activity is about 50% of normal reference.

#### *Antithrombin assays*

The laboratory diagnosis of AT deficiency is based on functional (qualitative) and immunological (quantitative) assays. Measurement of the AT antigen is not sufficient because it would leave dysfunctional AT deficiency with normal antigen level and reduced functional activity undetected. Dysfunctional AT may be investigated by running double cross-immunoelectrophoresis [155].

In commercial functional assays AT can be measured in terms of its ability to inhibit either thrombin or FXa in the presence of heparin. Residual thrombin or FXa is measured with specific chromogenic substrates. These assays are easily automated and performed on coagulation analysers. Both AT deficiency type I and type II produce pathological results. In order to distinguish between them, antigen should also be measured. AT activity may also be measured in the absence of heparin, but this type of assay is mainly used for research purposes such as to further characterise type II deficiencies.

### *Protein C assays*

Functional assays are designed to measure the inhibitory activity of APC exerted against the natural substrates FVa or FVIIIa. This is usually assessed with APTT or using synthetic chromogenic substrates. PC may be activated with the snake venom Protac or thrombin-TM [156]. These tests are commercially available and may be easily adapted to automation. Antigen PC measurements may be used for further characterisation of defects identified by functional assays.

### *Protein S assays*

The presence of both free and bound PS in plasma complicates the diagnosis of PS deficiency. Basically three types of assays can be recognised: immunological assays for free PS, immunological assays for total PS and PS functional assays.

The functional assays that are used to assess PS are based on the APC cofactor activity of PS and are not very specific. They may be affected by APC resistance [157,158]. Alternatively PS antigen can be measured. It has been debated whether total or free antigen should be measured, but it appears that the free antigen is most reliable for discriminating carriers from noncarriers of PS deficiency [159,160]. The sensitivity (and specificity) of PS assays tend to be lower than for the assays of AT and PC, and normal reference ranges accordingly larger.

### *APC resistance test*

APC resistance can be measured in plasma with APTT based methods with and without activation of PC [78]. These methods are simple and inexpensive. Heterozygous and homozygous FV Leiden mutation may be discriminated using APTT based methods. FV Leiden mutation accounts for most, but not all cases of APC resistance. An abnormal APC resistance test could also be due to a LA or other unknown causes. Improved ability to distinguish carriers from noncarriers of the FV Leiden mutation has been obtained with APTT based methods where test plasma is prediluted with FV-deficient plasma [161]. An abnormal APC resistance may be confirmed by a simple polymerase chain reaction (PCR) test [77].

### *Prothrombin gene mutation test*

PCR analysis is usually performed to detect the prothrombin G20210A mutation [87]. Plasma analysis for prothrombin measurement is seldom performed because it is unable to clearly distinguish carriers from noncarriers of the mutation.

## 1.8 Global coagulation assays

Global coagulation tests reflect in various ways the formation of thrombin. Screening assays are performed in order to get an overview of the entire coagulation system including the interactions of enzymes, cofactors and inhibitors. Thrombin activity may be recorded most simply in clot-based assays, where the end point is the formation of a fibrin clot. The clotting time assays prothrombin time (PT) [162] and APTT [163] are clinically useful to detect hypocoagulability. It has so far been difficult to develop laboratory methods that globally measure the existence and the degree of thrombophilia in a simple way. Thrombosis is often multifactorial, caused by an acquired risk factor in an individual with a thrombophilic condition. A method that measures not only the presence of a single thrombophilic condition, but the combined effects of the pro and anticoagulant activity might limit the use for specific assays. A dynamic monitoring of the coagulation process may be obtained by measuring fibrin aggregation [164,165]. Another approach is to assess thrombin generation through the use of chromogenic or fluorescent peptide substrates [166].

### 1.8.1 Clotting time assays

#### *Prothrombin time (PT)*

The PT assay is performed by adding a thromboplastin or TF reagent and calcium to test plasma and measuring the clotting time [162]. Thromboplastins are complex mixtures prepared from extracts of brain or placenta. Newer thromboplastins most often contain recombinant TF relipidated into phospholipids. PT is prolonged with deficiencies of FVII, FX, FV, prothrombin and fibrinogen. It is most frequently used to monitor warfarin therapy. The International Normalised Ratio (INR) is used to standardise anticoagulant monitoring. It is used to compensate for different sensitivities of thromboplastins to factor deficiencies. The INR system is also used in patients with liver failure. The Owren PT procedure can be used to measure FVII, FX and prothrombin [167]. Adsorbed plasma is used as a source of fibrinogen and FV.

#### *Activated partial thromboplastin time (APTT)*

Originally the APTT was the coagulation time of the test plasma added exogenous phospholipids (cephalin) as platelet substitutes and calcium chloride [168]. It has been modified by the addition of activators of the contact phase of coagulation such as kaolin and ellagic acid [163] which shorten the clotting times. The phospholipid in this assay is called

partially thromboplastin because TF is absent. APTT became the test of choice to screen patients for congenital and acquired hemorrhagic diseases. Automated assays have replaced the old manual methods. The clotting times varies according to the reagent and coagulometer used [169]. The APTT is a global test sensitive to low levels of all the coagulation factors except factors FVII and FXIII. It is the test most often used to monitor unfractionated heparin [170]. The APTT has been extensively used to search for LA in patients with a history of thrombosis or fetal loss [171]. Recently it was found an association between shortened APTT and the risk of VTE [172]. Hypercoagulability due to high coagulation FVIII, FIX, FXI, prothrombin and fibrinogen may possibly be detected by the APTT.

#### *ProC global (PCG)*

PCG is a coagulation assay which tests the global function of the PC pathway [173]. It is a one stage-clotting time assay that is sensitive to deficiencies in the PC system. The anticoagulant response in plasma is measured by an APTT and clotting times in plasma are measured with and without APC. Endogenous APC is activated by the addition of the snake venom Protac [174] which leads to prolonged clotting times in normal plasma. The assay is sensitive to the FV Leiden mutation and PC deficiency. The sensitivity towards PS deficiency is lower, and it is not suited for the detection of AT deficiency. An advantage is that the assay can be performed with any automated coagulation instrument.

### **1.8.2 Thrombin generation assays**

The original concept of a thrombin generation (TG) test was described in 1953 [175]. TG was triggered in the primary reaction tube containing plasma and clotting times were measured by subsampling at regular intervals into secondary indicator tubes containing fibrinogen solution. Measurement of TG provided a method for quantifying the effect of the procoagulant and anticoagulant factors that determine coagulation capacity.

#### *Endogenous thrombin potential (ETP):*

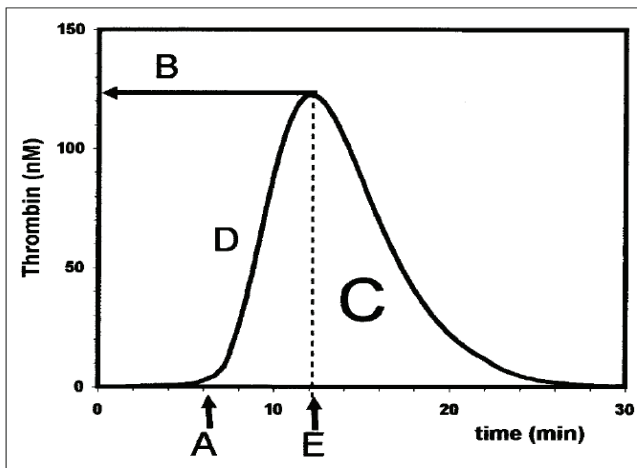
The principle of the TG assay was developed further by Hemker et al [176]. The fibrinogen solution was replaced by a thrombin specific chromogenic substrate and the primary plasma sample was defibrinated. A time-recording pipette linked to computerised data capture was added to plot generated thrombin against time. From this TG curve or thrombogram, the area under the curve named the ETP was calculated in addition to lag time, peak height and time to



peak (Figure 4). The calculation of thrombin activity was complicated by the use of a chromogenic substrate, which in itself influences the thrombin-decay process. Some of the thrombin in plasma reacts with  $\alpha 2$ -macroglobulin to form a complex that, though biologically inactive, still is capable to convert small substrates. A further development of measurement of the ETP was the use of a slow reacting substrate, which permitted continuous registration of thrombin activity in the primary reaction tube [177]. With this modification, the test was fully automated.

*Calibrated automated thrombogram (CAT):*

The replacement of the chromogenic substrate with a slow reacting fluorogenic substrate [178] enabled continuous measurement of TG without the need for defibrination, as the signal from the fluorophore is not quenched by turbidity. This made it possible to measure TG also in platelet rich plasma. The absence of a direct linear relation between thrombin activity and the fluorescent signal is overcome in CAT by monitoring the splitting of a fluorogenic substrate and comparing it to a constant known thrombin activity in a parallel non-clotting sample. Even though this modified method in combination with a microtiter plate fluorimeter was an improvement, it seems that the assay system is still technically complicated.



**Figure 4. The parameters of the thrombogram.** A: Lag time, B: Peak height, C: Endogenous thrombin potential (ETP), D: Maximal rising slope, E: Time to peak. Adopted with permission from Hemker et al [166]

### 1.8.3 Specialised assays

#### *Clot waveform analysis*

Standard clotting assays such as APTT or PT can be elaborated to provide more information than just the clotting time. In clot waveform analysis fibrin formation is monitored photo-optically by measurement of the changes in light transmittance that occur as plasma clots. Thus, clot waveforms can be performed at the same time as the clotting end point is determined for the APTT and PT assays. Information on the velocity and acceleration may then be derived from the data. The APTT waveform analysis was found to be sensitive to very low levels of FVIII [179]. The degree of abnormal biphasic APTT waveforms are directly correlated to haemostatic dysfunction in DIC [180]. The PT waveform analysis may be promising for the detection of LA [181].

#### *Thromboelastography (TEG)*

TEG records the viscosity and the elastic changes that occur during coagulation and provides a graphical representation of the fibrin polymerisation process using a specialised instrument [182]. The viscoelastic properties of whole blood are measured. Fibrin formation results in a pictorial haemostasis profile, the time taken for initiation and the kinetics of clot formation, the strength of the clot and its dissolution are recorded. Unlike clotting assays, TEG monitors haemostasis as a dynamic process. This makes it possible to study the interaction between platelets and the coagulation cascade and can provide information on interactions with the fibrinolytic systems. Hence, this method may more closely reflect the *in vivo* clotting situation.

TEG was originally designed as a bedside monitor using native whole blood. The main uses of TEG have been to monitor blood component therapy during surgery, primarily to detect haemostatic failure earlier than by conventional assays. The use of the TEG in the laboratory setting represents a significant change of use for the instrument. To perform tests within the laboratory plasma samples are used.

In a study of pregnant women there was a correlation between TEG parameters and AT level. There was no relationship between TEG variables and levels of PS and PC, or the FV Leiden mutation, prothrombin and MTHFR genetic mutations [183].

Recently, a modified TEG analysis principle with the use of whole-blood and a very low TF concentration to trigger the reaction was used to study hypercoagulation. A certain degree of hypercoagulation was detected in a group of unselected thrombosis prone patients [184]. In an evaluation of whole blood clotting, the thrombus generation curve as measured by the TEG system and the thrombin generation curve as measured by a thrombin-AT reference method were found to be inter-related [185].

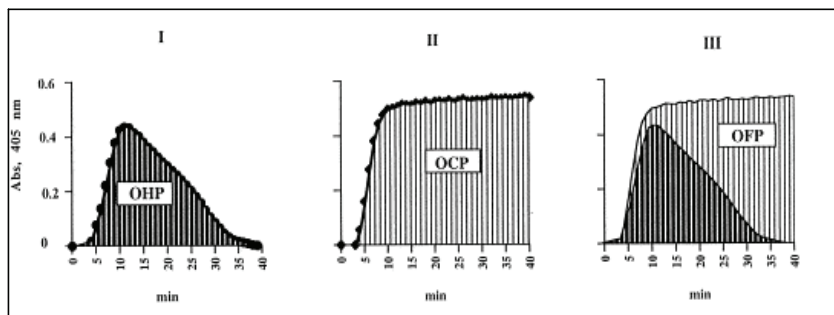
#### *Overall haemostasis potential (OHP)*

He et al developed a laboratory method in order to identify imbalances in the haemostatic system [164,165]. OHP in plasma was established to detect a hypercoagulable or a hypocoagulable state. The principle of the assay was spectrophotometric monitoring of opacity caused by fibrin aggregation and lysis in plasma activated by small amounts of thrombin, tissue-type plasminogen activator (t-PA) and calcium chloride. OHP was determined by calculating the area under the opacity curve (AUC).

In their original assay the AUC reflected variations according to the concentration of added purified pro- and anticoagulants to examined plasma *in vitro* activated by 0.2 IU/ml thrombin, 700 ng/l t-PA and 17 mM calcium chloride. Addition of AT decreased the AUC in a dose-dependent way.

To prevent initiation of clotting by the exogenous added thrombin the assay was modified. Thrombin in a decreased dose (0.04 IU/ml) with or without t-PA was added to plasma for initiation of fibrinogen clotting. In addition to OHP, the overall coagulation potential (OCP) curve, which is coagulation of plasma without t-PA was monitored. The relative difference between the areas under the OHP and OCP reflects the overall fibrinolysis potential (OFP) and is calculated as  $((\text{OCP}-\text{OHP})/\text{OHP}) \times 100\%$  (Figure 5).

The assay detected elevated OHP levels in normal pregnancy, more elevated in preeclampsia and in coronary heart disease [186]. Increased OHP levels were found in patients with previous DVT and in patients with FV Leiden mutation [186]. To obtain an assay system more similar to the haemostasis balance *in vivo* OHP was further modified by introducing a platelet reagent containing TF and phospholipids. The authors reported extremely low or undetectable levels of OHP and OCP in samples of F VIII-, IX-, VII-, V-, X- or II-deficient plasma, showing an improved power to identify hypocoagulability [187].



**Figure 5. Overall haemostasis potential (OHP), overall coagulation potential (OCP) and overall fibrinolysis potential (OFP) assayed in normal plasma.** Adopted with permission from He et al [165].

Recently a modification of the global assay described by He et al [164,165] and Smith et al [188] was presented by Goldenberg et al [189]. The Clot Formation and Lysis assay (CloFAL) simultaneously measures coagulation and fibrinolytic capacities in plasma after addition of trace amounts of calcium, TF and t-PA. Absorbance of clot formation and lysis are monitored for three hours and the AUC is calculated in addition to time to and amplitude of maximal clot formation.

## **2. Selected methods and materials**

### **Patients**

#### *Paper I*

Blood samples were collected from 18 persons with hereditary thrombophilia who had attended the Thrombosis Clinic, Aker University Hospital. Six were on warfarin treatment, (18-87 years, mean age 53). All of the patients on warfarin treatment had a history of VTE. Of the twelve persons not taking warfarin (25-69 years, mean age 41), six persons had previously sustained VTE. One person spontaneously developed VTE three months after this blood sampling.

Blood samples were also obtained from 23 healthy controls (31-68 years, mean age 43) (health personnel at Aker University Hospital).

Specific tests for AT, PC, PS and FV Leiden mutation were performed in all these plasma samples.

#### *Paper II*

Blood samples were collected from 24 persons with thrombophilia (14-71 years, mean age 35) who had attended the Thrombosis Clinic, Aker University Hospital. Seven of them had previously sustained VTE. None of them were on warfarin medication.

Blood samples were also collected from 24 normal controls (25-70 years, mean age 44) (health personnel and medical students at Aker University Hospital).

Specific tests for AT, PC, PS and FV Leiden mutation were performed in all these plasma samples.

#### *Paper III*

Blood samples were obtained from 23 persons with thrombophilia. These persons were identical to the persons in Paper II. Blood samples were also obtained from 23 healthy controls. 16 of these persons were identical to the donors in Paper II.

Specific tests for AT, PC, PS and FV Leiden mutation were performed in plasma from the additional controls included in the study.

#### *Paper IV*

Blood samples were collected from 202 healthy postmenopausal women (45-65 years). None of them had a history of VTE.

Specific tests for AT, PC, PS, FV Leiden and prothrombin gene mutation were performed in plasma from the persons included in the study. Twelve of them had heterozygous FV Leiden mutation and two had heterozygous prothrombin gene mutation.

#### **The CIP assay**

For each plasma sample analysed runs A and B were performed in triplicate. In both runs aliquots of 100 µl of citrated plasma were diluted with buffer. Run A is based on the OHP assays described by He et al [165]. In run B the snake venom Protac was added 5 min prior to and pentasaccharide immediately prior to recalcification, making their concentrations 0.15 IU/ml and 1.1 µg/ml respectively. The reactions were started by adding TF (Innovin) (1.5 pM in Paper III and 0.67 pM in Paper IV), CaCl<sub>2</sub> (17mM). Buffer was 66.0 mM Tris, 130.0 mM NaCl, pH 7.4.

When thrombin 0.4 nM was used to trigger the reaction instead of TF (Paper I, II and III) 0.16 IU/ml Protac and 1.2 µg/ml pentasaccharide were used.

t-PA (440 ng/ml) was included in the assay in Paper I, II and III.

The absorbance was measured at 405 nm in a Dynex MRX microplate reader at 37 °C every minute for 20 min. When t-PA was added the recording was 40 min to follow fibrinolysis. A curve was obtained by plotting absorbance against time. The AUC was calculated as the sum of these absorbance values.

Inhibition was calculated by using the reduction of the AUC in run B relative to the result obtained in run A:  $((A-B)/A) \times 100$ , expressed in Units (U).

The coefficient of variation (CV) for curves A and B in Paper II ranged 8.8 to 9.0% (intra-assay) (n = 8) and 7.2 to 6.7% (inter-assay) (n = 8).

In Paper III CV for curves A and B ranged 0.8 to 6.6% (intra-assay) (n = 8) and 3.6 to 12.4% (inter-assay) (n = 8) for normal controls. For persons with thrombophilia, the intra-assay CV ranged 2.0 to 2.3% (n = 8) and inter-assay CV ranged 4.2 to 5.4% (n = 8).

In Paper IV CV for curves A and B ranged 1.8 to 6.9% (intra-assay) (n = 28) and 2.9 to 9.3% (inter-assay) (n = 28).

## **Statistical analysis**

*P* values < 0.05 were chosen as the level of statistical significance.

### *Paper I*

Differences between the individual groups were analysed using the Mann-Whitney test. Data analysis was performed using the Minitab for Windows statistical software.

### *Paper II*

Differences between two individual groups were analysed using the Mann-Whitney test.

Receiver operating characteristic (ROC) curves were used for sensitivity and specificity analysis. The Pearson correlation coefficient was applied for correlation analysis.

The statistical program package of social science (SPSS) version 11.0 was used for Mann-Whitney and correlation analysis. SPSS, Stata/SE 8.0 and S-Plus statistical software were used for ROC curve analysis.

### *Paper III*

Differences between two independent groups were analysed using the Mann-Whitney test.

The Pearson correlation coefficient was used for correlation analysis. ROC curves were used for sensitivity and specificity analysis. SPSS version 11.0 was used for Mann-Whitney and correlation analysis. SPSS, Stata/SE 8.0 and S-Plus statistical software were used for ROC curve analysis.

### *Paper IV*

Nonparametric tests were chosen because of markedly skewed efficacy variables. At baseline the individual groups were compared using the Kruskal-Wallis test. For each group the comparisons between baseline values and last visit were analysed using Wilcoxon signed rank test. Differences between two individual groups were analysed using the Mann-Whitney test. Correlation between two variables was expressed as Spearman rank correlation coefficient. Data analyses were performed using the SPSS version 13.0 and the Minitab for Windows statistical software.

### **3. Aims of the study**

The main aims of this study were

1. To develop a global coagulation assay for use in routine laboratories that can detect the presence of hereditary thrombophilia.
2. To evaluate our assay by testing different patient materials, including other conditions with an increased tendency to thrombosis.

Specifically

- Investigate to what extent addition of substances that potentiate endogenous coagulation inhibitors have on blood coagulation. These substances are reagents that mimic the effect of the normal endothelial cell.
- Evaluate our assay by testing plasma samples with severe thrombophilia and compare it with other related published assays.
- Analyse plasma samples from postmenopausal women before and after treatment with hormone therapy.



## **4. List of papers**

### **I.**

Andresen MS, Iversen N, Abildgaard U.

Overall haemostasis potential assays performed in thrombophilic plasma: the effect of preactivating protein C and antithrombin.

*Thromb Res. 2002 Dec 15;108(5-6):323-8.*

### **II.**

Andresen MS, Abildgaard U, Liestol S, Sandset PM, Mowinckel MC, Odegaard OR, Larsen ML, Diep LM.

The ability of three global plasma assays to recognize thrombophilia.

*Thromb Res. 2004;113(6):411-7.*

### **III.**

Andresen MS, Abildgaard U.

Coagulation Inhibitor Potential: a study of assay variables.

*Thromb Res. 2005;115(6):519-26.*

### **IV.**

Andresen MS, Eilertsen AL, Abildgaard U, Sandset PM.

Hormone therapy and raloxifene reduce the coagulation inhibitor potential.

*Blood Coagul Fibrinolysis. 2007;18:455-60.*

## **5. Brief summary of results**

### **Paper I**

#### **Overall haemostasis potential assays performed in thrombophilic plasma: the effect of preactivating protein C and antithrombin.**

The “Overall Haemostasis Potential” (OHP I) assay for determination of the fibrin aggregation curve in plasma developed by He et al was tested to see if inheritable thrombophilia could be detected, particularly combined PS deficiency and heterozygous FV Leiden mutation.

Plasma from persons with inherited thrombophilia and normal controls were analysed. The median OHP I values for the area under the opacity curve were very similar in thrombophilic samples and normal controls. The assay was therefore modified by enhancing inhibition of coagulation by adding Protac to activate PC and pentasaccharide to activate AT. Whereas the AUC in normal controls was reduced by the addition of Protac and pentasaccharide, the thrombophilic samples were not significantly affected by the addition of coagulation inhibitors. In the modified version of the assay the median value for AUC was significantly lower in normal controls compared with the group with thrombophilia ( $P = 0.035$ ).

The assay was also performed on plasmas from a small group of thrombophilia patients on warfarin treatment. Protac and pentasaccharide had little effect on samples from the warfarin treated thrombophilias.

In the original OHP I assay, He et al had reported high AUC values in pregnancy and preeclampsia. Since PS is lowered and APC resistance is increased in these conditions, seven of the thrombophilic samples with combined PS deficiency and FV Leiden mutation were compared with normal controls. Their median AUC value in the OHP I assay was, however, not significantly different from the median level of the controls.

### **Paper II**

#### **The ability of three global plasma assays to recognize thrombophilia.**

“Coagulation Inhibitor Potential” (CIP) is a modified version of the OHP assay developed by He et al which is based on spectrophotometric registration of fibrin aggregation in plasma. We increased the sensitivity towards thrombophilia by adding reagents that potentiate the coagulation inhibitors in plasma. CIP measures the effect of adding Protac and pentasaccharide relative to the result obtained with the unmodified OHP assay.

Three different global clotting assays were compared in order to test their ability to detect different types of inherited thrombophilia. “Calibrated Automated Thrombogram” (CAT), “ProC Global” (PCG) and CIP were performed on plasma from normal controls and persons with inherited thrombophilia.

The CAT monitors the generation of thrombin in clotting plasma using a fluorometer. The assay separated all plasma samples with AT deficiency, but was less sensitive to abnormalities in the PC system. In the PCG assay APTT clotting times are measured with and without APC which is activated by preincubation with Protac. PCG was more sensitive to abnormalities in the PC pathway. The median PCG value in thrombophilic samples was significantly lower compared with the median value of the healthy controls ( $P < 0.0001$ ), but the assay did not detect AT deficiency and plasma from persons with PS deficiency had median value close to that of the healthy controls. The CIP assay was approximately equally sensitive to AT deficiency and defects in the PC system. The median CIP value in the thrombophilic group was significantly lower than in the control group ( $P < 0.0001$ ). With the CIP assay ROC analysis showed that with a sensitivity of 100% the specificity was 87.5%.

### **Paper III**

#### **Coagulation Inhibitor Potential: a study of assay variables.**

The purpose of this study was to optimise assay conditions in the CIP assay. TF was compared with thrombin as a trigger of coagulation in plasma samples from 20 persons with severe thrombophilia and 20 normal controls. The CIP assay detected different thrombophilic conditions equally well with TF and thrombin ( $r = 0.89$ ,  $P < 0.0001$ ). The addition of Protac, TM and pentasaccharide as accelerators of the inhibitors of coagulation was evaluated. The tested concentrations of Protac and TM increased the anticoagulant effect, but only in the presence of pentasaccharide. TM also reduced fibrinolysis. With pentasaccharide alone, coagulation was delayed in a dose-dependent manner. Pentasaccharide and Protac had a synergistic effect resulting in reduced coagulation in normal controls, but less in persons with thrombophilia. CIP was tested as a pure coagulation assay by omitting t-PA in attempt to reduce analysis time. Plasma samples from 23 thrombophilias were compared with 23 normal controls. The performance of the CIP assay was not decreased by omitting t-PA and CIP could therefore be used without t-PA in future analysis.

## **Paper IV**

### **Hormone therapy and raloxifene reduce the coagulation inhibitor potential.**

The effect on CIP in plasma samples from postmenopausal women treated with conventional-dose hormone therapy (HT), low-dose HT, the alternative drugs raloxifene, and tibolone was compared. HT is a combination of oestrogen and progestogen (see page 18). Use of HT and raloxifene are associated with an increased risk of venous thrombosis. The CIP assay was performed on plasma samples from 202 healthy women who were randomly assigned to receive treatment for twelve weeks treated with one of four different therapeutic regimens. Twelve women were heterozygous carriers of the FV Leiden mutation. Major thrombophilias were excluded. Fibrin aggregation in plasma was monitored after activation of coagulation with TF, and the effect of potentiated inhibition of coagulation was measured.

Compared with baseline the median CIP value was reduced by 64% in the conventional-dose HT group, by 38% in the low-dose HT group, 31% in the raloxifene group, whereas for those treated with tibolone median CIP increased by 9%. These changes were significant for the two HT groups ( $P < 0.0001$ ) and the raloxifene group ( $P = 0.003$ ). Tibolone treatment did not significantly change the median CIP value. Women with heterozygous FV Leiden mutation had significantly reduced median CIP level ( $P < 0.0001$ ) at baseline.

## 6. General discussion

### 6.1 Development of the CIP assay

#### *Methodological considerations*

The major aim of this project was to develop a global screening test that could predict the presence of thrombophilia. Two existing assay methods were considered, as they were explicitly designed to reflect not only the activation, but also the control of coagulation.

The CAT assay is the latest version of TG assay developed by HC Hemker [178]. It is technically advanced and requires specialised equipment. Thrombin is measured in plasma by the use of a fluorogenic substrate without subsampling. As thrombin is so central in the haemostatic process it could be of interest to use this enzymatic method to study conditions associated with thrombotic tendency. Two different versions of this assay with added APC and TM have been developed [190], but no single version could be used for the detection of the different forms of inherited thrombophilia. We considered the possibility to manipulate the assay principle in order to increase the sensitivity of the assay. This would be difficult, as the CAT assay employs advanced software required to compensate for the influence of low molecular weight substrates on the inhibitory reactions.

We therefore decided to explore and to evaluate the OHP assay introduced by He et al [164,165]. This assay was described as a simple method to demonstrate a hypercoagulable or hypocoagulable state. In addition, the OHP assay can be performed with a temperature controlled ELISA reader. Results in plasma from persons with hereditary thrombophilia had not been reported at that time, but addition of AT had been shown to reduce the global activity [164].

#### *Evaluation of the OHP method*

We tested plasma samples from normal controls and thrombophilic persons, but neither the OHP I nor II assays were sensitive to severe thrombophilia [Paper I]. In plasma from normal controls and also from thrombophilic persons the median AUC values were higher in the OHP I assay compared with the OHP II assay. This probably reflected that the higher thrombin concentration in OHP I contributed to fibrinogen concentration. Using the lower thrombin

concentration coagulation was delayed and this reduced the AUC value. This modification, however, did not increase the sensitivity towards thrombophilia [Paper I].

In the OHP assay the conversion of plasma fibrinogen into a fibrin gel resulting in increasing absorbance values is continuously monitored over time. Each absorbance value represents a fibrin level at the corresponding time point. The resulting AUC, expressed as the absorbance sum gives information about fibrin generation throughout the measurement period.

We compared the AUC to the fibrinogen level and found a significant positive correlation in normal controls ( $r = 0.75$ ,  $P = 0.001$ ) and in thrombophilic persons ( $r = 0.805$ ,  $P < 0.0001$ ) (unpublished results). The median fibrinogen level was not significantly different in the two groups. The AUC values within the two groups varied considerably.

He et al had reported that the median AUC level was increased in women with heart failure compared with the controls and that fibrinogen concentration correlated to the AUC values [165]. In a later study women with previous VTE with or without APC resistance had higher AUC values compared with the controls, but the AUC values were unrelated to the fibrinogen concentration [186].

### *Enhancing the inhibition*

Fluidity of the blood in the vascular system depends *inter alia* on the interaction between plasmatic inhibitors of coagulation and their accelerators present on the endothelial surface. Heparan sulphate accelerates the inhibitory activity of AT. TM binds thrombin which now activates PC. In an attempt to increase the sensitivity of the fibrin aggregation assay we added reagents that mimic these endothelial accelerators.

The snake venom Protac is a fast-acting PC activator. The pentasaccharide is a copy of the heparin sequence which binds to and activates AT. Binding of the pentasaccharide to AT induces a conformational change which results in an accelerated inhibition of FXa by AT. The pentasaccharide is not a thrombin inhibitor because it is too short to bridge AT to thrombin. This implies that only activity of TG will be enhanced. In contrast to heparin, pentasaccharide does not react with platelet factor 4 which may be present in different concentrations in plasma samples. For these reasons, pentasaccharide was chosen as an accelerator of AT. Protac and pentasaccharide both exerted anticoagulant effects when added to plasma in our test system. This effect was more pronounced in normal plasma than in thrombophilic plasma. With the addition of Protac and pentasaccharide we obtained markedly lower AUC median levels in the normal controls, but only slightly lower in the thrombophilic group [Paper I].

The OHP and modified OHP assay was later developed into the Coagulation Inhibitor Potential (CIP) assay. In the CIP assay the effect of added Protac and pentasaccharide was measured relative to the result obtained with the unmodified assay. With the CIP assay an improved separation between normal controls and thrombophilias could thus be obtained. A weaker inhibitory response in thrombophilic plasma samples contrasted with results in normal controls. The inhibitory effect in the control group varied considerably [Paper II] and the reason for this is as yet unknown.

We described the effect of enhancing the coagulation inhibitors in Paper III. Pentasaccharide alone delayed the coagulation in a dose dependent manner. In the CIP assay we used 1.2 µg pentasaccharide per ml plasma when thrombin was used as a trigger. When Protac was added alone (0.16 IU/ml) this unexpectedly caused an increase in AUC. The reason for this is probably due to an effect on fibrin polymerisation. Additions of higher doses Protac however, resulted in decreased AUC (unpublished results). Together Protac and pentasaccharide had a synergistic effect, even with lower Protac concentrations, demonstrated by reduced AUC levels in normal plasma samples. The concentrations of Protac and pentasaccharide were chosen to obtain a balanced effect of both inhibitory pathways.

As an alternative to Protac we added TM. Human TM alone did not affect the coagulation in our assay [Paper III]. Rabbit TM, which may be a better activator of human PC [191] and pentasaccharide together increased delay more than pentasaccharide alone. Rabbit TM also inhibited fibrinolysis in reactions where t-PA was added. This may be due to the activation of thrombin activable fibrinolysis inhibitor (TAFI) [192]. TM was more expensive and apparently Protac was as useful in the assay.

The CIP assay was performed at 37 °C to ensure optimal conditions for the various enzymatic reactions. We have later tested plasma samples from normal controls and persons with thrombophilia at 27 °C, but this did not change the results (unpublished results). This suggested that the CIP assay may be performed without a temperature controlled ELISA reader.

#### *The choice of trigger*

We compared TF with thrombin as a trigger of coagulation [Paper III]. The addition of a small amount of exogenous thrombin sufficient to trigger the activation of coagulation via a feedback effect was originally used in the CIP assay. With TF (1.5 pM) we obtained similar reaction curves as with thrombin (0.04 IU/ml). The results from 20 normal controls and 20 persons with thrombophilia indicated that TF was equally suited as thrombin as a trigger of

coagulation. Theoretically this is an advantage because TF is the biological trigger of coagulation which is initiated when TF binds to FVIIa in plasma.

### *Fibrinolysis*

The CIP assay was initially used as a combined assay that reflected both fibrin generation and fibrinolysis by the addition of t-PA, in the same way as the OHP assays. After addition of t-PA, plasminogen is converted into plasmin, which digests fibrin into soluble fragments. Polymeric fibrin increases the catalytic property of t-PA [193]. During fibrin generation and accumulation of the fibrinolytic breakdown products the accelerated fibrinolytic process led to a decline in the absorbance values.

We found that the sensitivity of the CIP assay to thrombophilia was not decreased by omitting t-PA as a reagent [Paper II]. Although plasminogen deficiency has been described as a rare cause of thrombophilia, abnormal fibrinolysis is probably seldom the cause of inherited thrombophilia. Omitting t-PA had the practical advantage that analysis time was reduced from 40 to 20 minutes.

In other situations, e.g. postoperatively, low fibrinolytic activation may be important for development of thrombosis. In some clinical situations, it might be interesting to include fibrinolysis in the assay. Although the CIP assay routinely can be used as a pure coagulation assay, alternatively, the fibrinolytic process can be included by the addition of t-PA.

### *Expression of results*

For each plasma sample tested the CIP assay compares run A and run B. The area under the A curve (A value) did not separate thrombophilias from normal controls (Table 2). In run B coagulation was inhibited by the addition of Protac and pentasaccharide. Thus, the area under the B curve (B value) was decreased compared with the A value. This was distinct in normal plasma samples but not in thrombophilic plasma samples.

**Table 2. Ways of expressing the results with the CIP assay.** Median values for 24 normal controls and 24 thrombophilias [Paper II]. Inhibition:  $((A-B)/A) \times 100$

	A	B	(A-B)	Inhibition
Normal controls	162.7	68.2	101.4	55.5
Thrombophilia	190.5	143.4*	34.8*	17.3*
Overlapping	46/48	11/48	10/48	3/48

\*( $P < 0.0001$ ) as compared with healthy controls



Three different possibilities for expressing the results existed. The simplest way would be to report the B value. Although the median B value for normal controls was about half of the median B value in the thrombophilia group, almost half of the normal controls had values that overlapped with the thrombophilic group (Table 2).

The difference between A and B values obtained by the subtraction of A-B separated normal controls and thrombophilias better (Table 2). Yet, the overlap between the normal controls and the thrombophilia group was considerable. Ten of the 24 normal controls had values within the range of the thrombophilic group.

The inhibition was calculated as  $((A-B)/A) \times 100$  where A is the AUC in the absence and B the AUC in the presence of potentiated inhibition. This separated the normal controls and the thrombophilia group better than by using the subtraction of A-B or the B curve. Now, three out of 24 normal controls had CIP values that were within the thrombophilic area (Table 2).

We thus concluded that inhibition was the best parameter to separate severe thrombophilias from normal controls. The A value is influenced both by the fibrinogen level and the level of the coagulation factors in the plasma samples. Expressing the results as inhibition, where value A is present both in the nominator and denominator reduces the influences of varying coagulation factor levels.

In Paper I, II and III degree of inhibition was given in percent. In Paper IV we introduced units (U) to avoid confusion with other figures given in percent. In future work we will stick to reporting inhibition in Units.

## **6.2 Comparison of three global assays**

### *Performance of the CIP assay*

Our results with the CIP assay suggested relatively high sensitivity with an apparently acceptable specificity. Of the 24 normal controls, three of them had values within the range of the 24 thrombophilic persons [Paper II]. Setting sensitivity to 100% the specificity was 87.5%. High sensitivity in a global screening test for thrombophilia is important. In case of a pathological CIP value, specific tests for the different hereditary thrombophilias can then be used for further testing to decide a thrombophilic state.

In paper III five of the 23 controls had values within the range of the 23 thrombophilias both in the absence and presence of t-PA. With a sensitivity of 100% we thus obtained a specificity of 78%.

The reason for low inhibition in a normal control may be due to unexplained thrombophilia. Clinically, even familial thrombophilia may exist even though this has not been confirmed by any of the specific tests available.

Our results so far indicated that with the CIP assay we may obtain low inhibition in plasma samples from the majority of persons with AT, PC and PS deficiency and combined thrombophilias [Paper I, II and III].

We decided to compare CIP with the other global assays that are sensitive to hereditary thrombophilia.

#### *Performance of the PCG assay*

The PCG assay was designed to evaluate the functionality of the PC pathway. The assay is based on the ability of Protac to prolong the APTT in plasma by the activation of endogenous PC.

We confirmed that the PCG assay was sensitive to the homozygous FV Leiden mutation, and heterozygous FV Leiden in combination with AT or PS deficiency. PC deficiency was also detected by this assay, whereas persons with PS and AT deficiencies were not identified [Paper II]. The median CIP values obtained for PS and AT deficiencies were very similar.

Other larger studies have confirmed that PCG is a reliable method for the detection of PC and combined deficiencies [194,195]. Also low sensitivity for PS by this test has been reported in other studies [194,195]. A further limitation is that AT is not detected by this method. Heterozygous FV Leiden mutation has been detected with 100% sensitivity in other studies [194-196], but was not tested in our study.

A main advantage of PCG is that it is a simple method that also can be performed with an automated coagulation analyser available in any laboratory.

#### *Performance of the CAT assay*

In the CAT assay the TG in clotting plasma was measured by monitoring the splitting of a fluorogenic thrombin substrate. In our study we showed that with the CAT assay TG was increased in AT deficiency and thus separated from the control group even though the variability in the control group was large [Paper II]. The assay was much less sensitive to

deficiencies in the PC pathway. The TG in samples from persons with FV Leiden mutation, PS deficiency and PS deficiency in combination with FV Leiden mutation varied considerably.

In addition to high TG in AT deficiency [197], carriers of the prothrombin gene mutation are also found to have increased TG [198].

A modified version of the TG assay with added APC made the assay sensitive to FV Leiden mutation in addition to PS deficiency, but did not reflect AT and PC deficiency [199].

Recently, a version of CAT with added TM to activate PC decreased TG in plasma samples from persons with thrombophilia. A significant resistance to the inhibitory effect of TM was found in plasma samples from persons with PC and PS deficiency as well as FV Leiden mutation and combined abnormalities [200]. Large variation in the control group resulted in overlap with the patient groups and made the assay in its present form not suited as a screening test for prothrombotic risk factors.

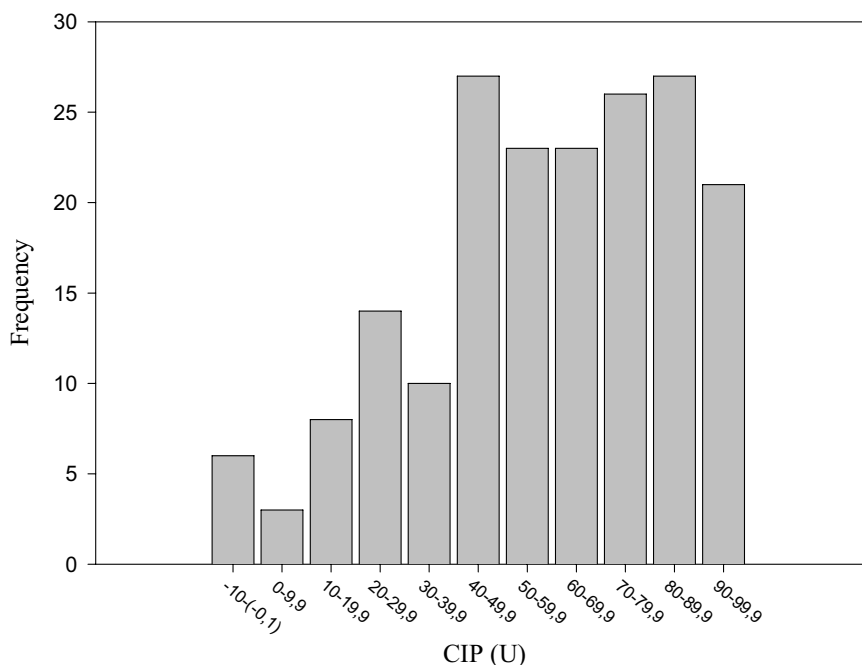
The CAT method is technically demanding and software is required to convert fluorescence changes into thrombin concentration. The advantage of a fluorescent substrate compared with chromogenic substrate was that the optical turbidity due to fibrin formation did not disturb the fluorescent signal and the need for defibrination was abolished. This influenced the TG since thrombin is formed in a different manner in the presence and absence of fibrin. Moreover, the presence of fibrinogen attenuates the formation of  $\alpha$ 2-macroglobulin-thrombin.

### **6.3 Further studies on thrombophilia**

Although the CIP was superior in the comparison of the three global assays, the thrombophilic subgroups examined were too small for definite conclusions to be drawn and a further evaluation in larger materials was needed. Also the control group was small.

In two ongoing studies we have performed the CIP assay in plasma samples from persons with defined thrombophilia. In collaboration with Professor Dr. Hajna Losonczy and Dr. Orsolya Toth, University of Pecs, Hungary we have analysed plasma samples from 71 persons with hereditary thrombophilia. We have also analysed 21 samples from persons with AT deficiency, twelve of them from persons on oral anticoagulants (OAC). These samples were obtained from Professor Dr. Jacqueline Conard, Hôtel Dieu hospital in Paris, France.

The so far the largest normal material analysed with the CIP assay was samples from 188 normal postmenopausal women included in the RET study [Paper IV] (Figure 6). That study additionally included samples from twelve women with heterozygous FV Leiden mutation.



**Figure 6. Distribution of CIP (U) values obtained in samples from 188 normal postmenopausal women included in the RET study.**

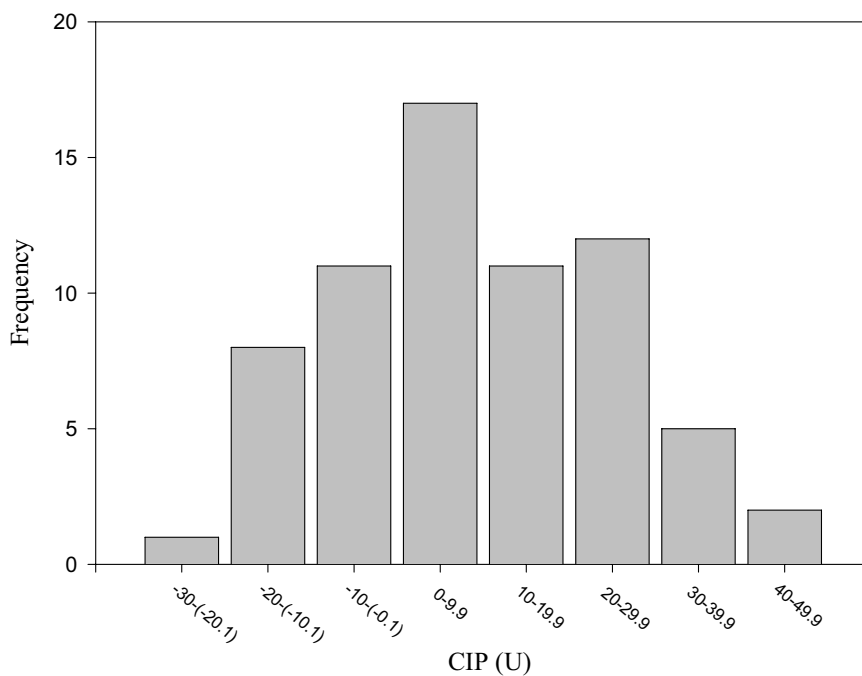
The results so far can be summarised as follows (Figure 7):

The median CIP levels in the AT, PC and PS deficiency groups were significantly lower compared with the RET control group ( $P < 0.0001$ ,  $P = 0.001$  and  $P < 0.0001$ , respectively) (Table 4).

The CIP values obtained in samples from persons with heterozygous FV Leiden were within the range obtained in the heterozygous FV Leiden group in Paper IV.

We obtained a median CIP value in the heterozygous G20210A mutation group that was very similar to the median CIP level obtained in the RET controls (61 U).

Even though LA is associated with an increased risk for thrombosis the median CIP level was higher compared with the RET control group.



**Figure 7. Distribution of CIP (U) values obtained in samples from persons with thrombophilia:** AT deficiency (n = 9), PC deficiency (n = 5), FV Leiden, heterozygous (n = 15), PC + other thrombophilic condition (n = 9), PS + other thrombophilic condition (n = 7), FV Leiden, heterozygous + G20210A, heterozygous (n = 9).

**Table 4. Median (range) CIP values obtained in samples from normal women, persons with single thrombophilia states and LA.**

	N	Previous VTE	Median (range) (Units)
Controls, (RET)	188	0	61 (-10 – 100)
AT deficiency	9	2	15 (-5 – 38)
PC deficiency	5	5	15 (3 – 23)
PS deficiency	13	8	21 (-11 – 43)
FV Leiden, heteroz	3	3	3 (3 – 29)
FV Leiden, heteroz, (RET)	12	0	-6 (-19 - 36)
G20210A, heteroz	14	11	59 (6 – 98)
LA	11	7	72 (34 – 98)

The median CIP level in persons with a single thrombophilia (AT, PS, PC deficiency and heterozygous FV Leiden) (14 U), were higher compared with the persons with combined thrombophilias (4 U), but the difference were not significant ( $P = 0.058$ ) (Table 5). These results suggested that combined thrombophilias are well detected with the CIP assay.

**Table 5. Median (range) CIP values obtained in samples from persons with combined thrombophilias.**

Thrombophilia	N	Previous VTE	Median (range) (Units)
PC deficiency + other	9	7	1 (-17 – 13)
PS deficiency+ other	7	7	-2 (-23 – 25)
FV Leiden heteroz + G20210A, heteroz	9	8	14 (-9 – 40)

*Sensitivity and specificity:*

Based on the results from the RET study, Pecs study and the collaboration with Paris ROC analyses could be used to find sensitivity and specificity (Table 6).

The highest sensitivity and specificity were obtained in the groups with PC deficiency and combined PC and PS deficiencies followed by the group with AT deficiency.

**Table 6. Sensitivity, specificity and cut off values obtained with the CIP assay in different thrombophilic states.**

Thrombophilia	N	Sens %	Spec %	Cut off (U)
AT	9	100	79	38
PC, PC + other, PS + other	21	100	88	25
PS, FV Leiden, FV Leiden + G20210A	25	100	75	43
PS, FV Leiden, FV Leiden + G20210A	25	84	80	34

#### 6.4 Influence of warfarin on CIP

Warfarin therapy reduces vitamin K dependent coagulation factors and changes the haemostatic balance. Anticoagulant therapy with warfarin protects persons with severe thrombophilia as it decreases the risk for VTE.

Findings in Paper I suggested that abnormal results with the CIP test may be obtained in persons with AT and PC deficiency even when on warfarin therapy. This topic has now been explored in more detail.

We obtained plasma samples from persons with AT deficiency on OAC and samples from persons without thrombophilia but on OAC (Table 7) from J. Conard in Paris. Increasing INR values correlated significantly with delayed initiation of coagulation in samples from persons taking OAC ( $r = 0.86$ ,  $P < 0.0001$ ). The median CIP value in the AT deficiency group on OAC were higher compared with the AT deficiency group not taking OAC, but the difference were not significant ( $P = 0.382$ ).

**Table 7. Median (range) CIP values obtained in samples from persons on OAC.**

Thrombophilia	N	Previous VTE	Median (range) (Units)
Controls	4		51 (35 – 61)
AT	12	12	23 (0 – 90)

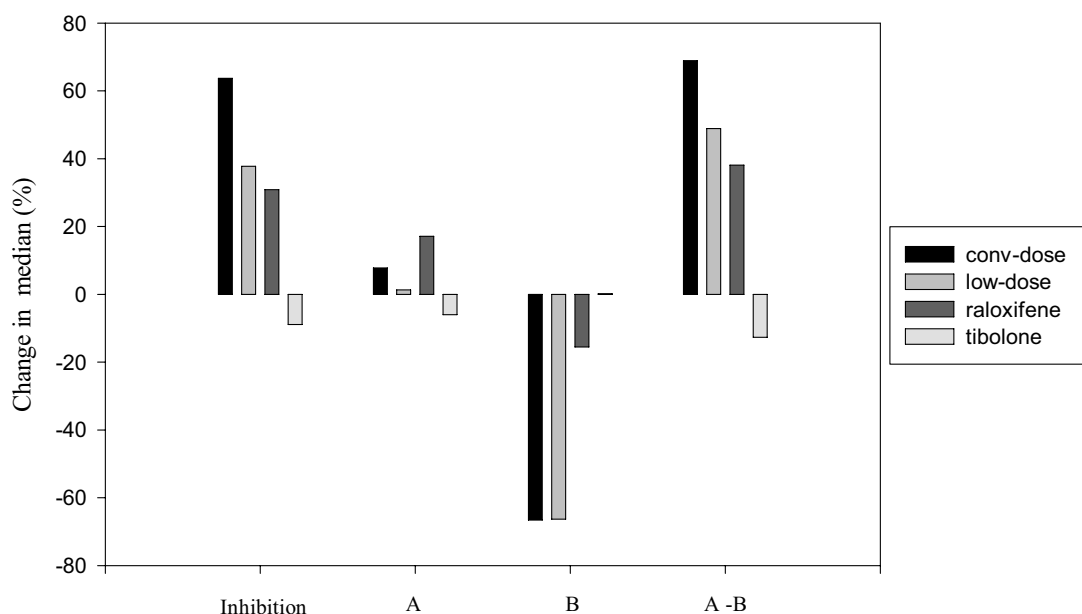
## **6.5 Influence of oestrogen-progesterone, raloxifene and tibolone on CIP**

In Paper IV we wanted to see if CIP could reflect changes in coagulation related to the use of medication that may increase the risk of VTE. Plasma samples from postmenopausal women treated with four different therapeutic regimens used to prevent osteoporosis and other ill effects of the menopause were compared.

The most distinct reduction in median CIP was found in the women treated with conventional-dose oestrogen-progestogen (2 mg 17 $\beta$ -estradiol + 1 mg norethisterone acetate (NETA)) and low-dose oestrogen-progestogen (1 mg 17 $\beta$ -estradiol + 0.5 mg NETA). A lower median CIP value in the conventional-dose oestrogen-progestogen group compared with the women treated with low-dose oestrogen-progestogen suggested a dose-response effect, but this difference was not statistically significant ( $P = 0.111$ ). The changes in the median CIP values were caused mainly by increased B values, although a moderate decrease in the A value in the conventional-dose oestrogen-progestogen group was also found. The A-B median values were thus reduced (Figure 8). This confirmed that the AT and PC pathways are affected by this treatment [146]. In a study based on the same women material and in other studies the use of oestrogen-progestogen reduced the sensitivity to APC [145,201,202], which is a known risk factor for VTE [131].

Raloxifene also reduced median CIP, but less than oestrogen-progestogen. The median A-B value was also lowered. The change in CIP was caused both by an increase in the B values and by a decrease in the A values. The reduction in the A values may be explained by reduced level of fibrinogen. Fibrinogen concentration was reduced after use of raloxifene in a study based on the same women material [146]. In that study it was also shown that AT, PC and TFPI levels decreased, whereas FVIII activity levels increased after raloxifene treatment. Additionally, raloxifene also reduced sensitivity to APC [202]. Previous studies have reported decreased AT activity and increased FVIII levels [203-205] as well as reduced sensitivity to APC [206] after treatment with raloxifene.





**Figure 8. Change in median values (%) from baseline to last visit for A and B values, A-B values and Inhibition:  $((A - B)/A) \times 100$  in the conventional-dose (conv-dose) and low-dose oestrogen-progestogen group, raloxifene and tibolone group.**

We found that tibolone did not appear to affect inhibition of coagulation significantly. In a previous study based on the same women material it has been suggested that tibolone does not affect the sensitivity to APC [202]. In another previous study, AT and PC levels decreased, whereas PS levels and TFPI activity increased after tibolone treatment [146]. So far tibolone has not been found to increase the risk of VTE, but more data are needed. An increased incidence of stroke, however, has recently been reported [150].

These results suggested that in this study expression of the results as subtraction of A-B could be an alternative to inhibition (Figure 8). The B value did not separate the change in median CIP in the two oestrogen-progestogen groups. A change in A value caused by the treatment may be of importance for the result, but would not be reflected when only the B value is used.

We have analysed plasma samples from six women who used oral contraceptives (OC) (unpublished results). The CIP assay, with thrombin as a trigger and added t-PA resulted in low inhibition between 3 U and 17 U. The median CIP value in the thrombophilia group tested in Paper II was 17.3 (Table 2). OC use is associated with a decrease in PS and AT [132].

In conclusion, conventional oestrogen-progestogen and raloxifene treatment as well as use of OC increase the risk of VTE. These results suggest that also the presence of acquired risk factors for venous thrombosis might be detected with the CIP assay.

## **7. Main conclusions**

1. An assay method described by He et al was tested to see if it could be used to detect inherited thrombophilia. As the median levels for the area under the fibrin polymerisation curve in plasma samples from normal controls and persons with thrombophilia were similar, this method did not detect inherited thrombophilia.
2. The original assay was modified by adding Protac to activate protein C and pentasaccharide to activate antithrombin. Inhibition of coagulation was enhanced in normal plasma samples, but only slightly in samples from persons with inherited thrombophilia.
3. We developed the Coagulation Inhibitor Potential (CIP) assay. Inhibition is measured in Units as  $((A-B)/A) \times 100$  where A is the area under curve in the absence, and B in the presence of Protac and pentasaccharide.
4. Three global plasma assays were compared to test their ability to recognise inherited thrombophilia. The CIP assay performed better than the Calibrated Automated Thrombogram and the ProC Global assay.
5. The CIP assay performed equally well with tissue factor and thrombin as triggers of coagulation.
6. The performance of the CIP assay was not decreased by omitting fibrinolysis.
7. Median CIP levels were reduced in plasma samples from healthy postmenopausal women treated with oestrogen-progesterone and raloxifene. Oestrogen-progestogen and raloxifene use are associated with increased risk of venous thrombosis.
8. Plasma samples from postmenopausal women taking tibolone did not have reduced median CIP level.

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## **Errata**

Paper I, page 326, Table 4: Median controls, Difference should read: 34.0.

Paper II, page 414, legend Fig. 1. First line should read: Individual values of the CAT assay.

Paper III, page 522, Figure 2 A: Two experimental values in the control group are missing. These are: 90% and 99% inhibition. (The median value (horizontal line) is correct).

Paper IV, page 456, Statistics, bottom line should read: ...(five cases) were used instead.

