

From the Department of Medicine and
Coagulation Unit, Department of Haematology, Karolinska University Hospital

Karolinska Institutet, Stockholm, Sweden

Mechanisms and effects of low plasminogen activator inhibitor type 1 activity

Anna Ågren



**Karolinska
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*Man skall vara - tror jag visst -
realist,
men i viss mån fatalist
och i grunden optimist
samt för all del först och sist
idealist.*

Nils Simonsson

ABSTRACT

Bleeding diathesis is a common reason for investigation of the haemostatic system and in about 50% of the cases an abnormality is found. Plasminogen activator inhibitor type 1 (PAI-1) is an inhibitor of tissue- and urokinase plasminogen activator (tPA/uPA). High PAI-1 levels are associated with arterial and venous thrombotic conditions but are also seen in pregnancy, inflammatory and infectious diseases, diabetes and overweight. Low levels of PAI-1 have been reported in case studies as a reason for bleeding tendency. Studies of low PAI-1 activity as a cause for bleeding in large populations have so far been lacking. The aim of this thesis was to systematically study the prevalence of low PAI-1 activity in patients with bleeding diathesis, the clinical significance, evidence for hyperfibrinolysis and regulation. The method for determination of PAI-1 activity had been improved at our laboratory to more precisely measure lower levels of PAI-1 activity.

We studied prospectively 586 patients with bleeding tendency with analysis of PAI-1 activity in addition to the routine investigation and compared with two control groups. The bleeding problems were clinically evaluated and compared between those with low PAI-1 and normal/high PAI-1. The prevalence of low PAI-1 among patients was 23% compared to 13% in blood donors and 10% in healthy controls. No specific bleeding symptom predicted for low PAI-1 activity. The 4G/5G polymorphism in the PAI-1 gene did not differ between patients and controls (paper I).

From the original 586 patients 424 were further investigated for laboratory evidence of hyperfibrinolysis with plasmin-antiplasmin complex (PAP) and D-dimer. PAP was higher among patients with low PAI-1 activity compared to normal/high PAI-1, but the coagulation activity measured by D-dimer did not differ (paper III).

In 181 samples from the same cohort we analysed C-peptide, proinsulin, high sensitivity C-reactive protein and interleukin 6 (IL-6). Body mass index (BMI), exogenous hormones, sex and age were registered. Low BMI, oral oestrogens, young age and low C-peptide were significantly associated with low PAI-1 activity. After adjustments the effect of C-peptide, IL-6 and young age disappeared and low BMI remained the strongest predictor of low PAI-1 activity (paper IV).

In 62 patients referred for transurethral resection of prostate (TURP) due to prostatic hyperplasia samples for PAI-1 were taken before surgery and analysed after hospitalisation. Haemoglobin was measured before surgery and the day of discharge, per- and postoperative bleeding was registered as well as bleeding complications, amount of resected prostate and days of hospitalisation. Bleeding complications occurred in 75% of the patients with low PAI-1 activity compared to 28% of the patients with normal/high PAI-1 activity. This became borderline significant after adjustments (paper II).

In conclusion, low PAI-1 activity seems to be more prevalent among patients with bleeding tendency than controls, but the bleeding risk in patients with low PAI-1 activity seems to be of minor clinical significance. Low BMI is the strongest predictor of low PAI-1, which is associated with higher fibrinolytic activity than in patients with normal/high PAI-1.

Key words: Bleeding, body mass index, epidemiology, plasminogen activator inhibitor, surgery, prostate, fibrinolysis, C-peptide, CRP, IL-6, proinsulin.

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LIST OF ABBREVIATIONS

ACE	angiotensin-converting enzyme
APTT	activated partial tromboplastin time
BMI	body mass index
CI	confidence interval
EDTA	ethylenediamine tetraacetic acid
FIX	factor IX
FVIII	factor VIII
Hb	haemoglobin
HDL	high density lipoprotein
HRP	horseradish peroxidase
hs-CRP	high-sensitivity C-reactive protein
IL-6	interleukin 6
IQR	interquartile range
OR	odds ratio
PAI-1	plasminogen activator inhibitor type 1
PAI-1 Ag	PAI-1 antigen
PAP	plasmin-antiplasmin complex
PT	prothrombin time
TG	triglycerides
tPA	tissue plasminogen activator
tPA-PAI-1 complex	tissue plasminogen activator- plasminogen activator inhibitor type 1 complex
TAFI	thrombin-activatable fibrinolysis inhibitor
TNF	tumor necrosis factor
TURP	transurethral resection of the prostate
uPA	urokinase plasminogen activator
VWF	von Willebrand factor
VWD	von Willebrand disease

INTRODUCTION

PAI-1 AND BLEEDING TENDENCY - GENERAL BACKGROUND

In patients with mild bleeding disorders, like bruising, menorrhagia or nose bleeding, it is possible to establish a diagnosis of a haemostatic defect in only half of the cases [1-5]. In some of the patients with no diagnosis of bleeding tendency the reason could be local factors like superficial vessels in the nose, gynaecological pathology or vascular fragility [6]. The most common aetiology today is probably medication with aspirin, which leads to an acquired platelet dysfunction. About 7% of the general population uses aspirin daily (Socialstyrelsen; drug statistics 2007). There could also be reasons for bleeding problems that have not yet been discovered.

Plasminogen activator inhibitor type 1 (PAI-1) is a protease inhibitor circulating in blood and it protects blood clots from the fibrinolytic process. After trauma the normal coagulation system should form a blood clot where the tissue damage occurs. In addition to the formation of haemostatic plugs, the human blood has another balanced system to disrupt and remove blood clots so that the integrity of the blood vessel and its lumen finally can be restored. This balanced system is called the fibrinolytic system and PAI-1 is an inhibitor of the fibrinolytic system. If PAI-1 is absent or is present at a very low concentration there may be too rapid removal of blood clots before healing has occurred. The patients could therefore continue to bleed or start re-bleeding after surgery or trauma.

Defects of the fibrinolytic system are sparsely reported [1, 3, 4]. Congenital antiplasmin deficiency is a rare disorder [7], which leads to bleeding diathesis. High concentrations of tissue plasminogen activator (tPA), urokinase plasminogen activator (uPA) and plasmin, which can lead to bleeding complications, are almost always iatrogenic due to thrombolytic treatment of venous thromboembolism, myocardial infarction or stroke.

Plasmin has earlier been used in studies concerning treatment of myocardial infarction, but overexposure can lead to degradation of normal components of the haemostatic system and predispose to bleeding. If the dose of plasmin exceeds the level of antiplasmin in plasma, it is possible for free plasmin to degrade fibrinogen, which can lead to bleeding complications [8, 9] Plasmin is able to degrade many proteins, but in the circulation its action is mainly towards fibrin. Its activity is controlled by complicated regulatory mechanisms, including the fact that the activation of plasminogen to plasmin occurs mainly on the surface of fibrin and not generally in the circulation and there is also an selective immediate inactivation of

free plasmin, that is not linked to fibrin, by its inhibitor antiplasmin [10]. In a recent study plasmin has been used in a first human phase I trial for local treatment of haemodialysis graft occlusion. The study showed promising effects with no major bleeding [11].

Since 1989 there have only been a few reports concerning patients with bleeding problems and low levels of PAI-1 [12-25]. All of these publications have been case- or family studies but systematic studies are lacking. Two of the authors have found mutations in the PAI-1 gene [12, 13]. A typical clinical bleeding pattern for these patients is bleeding complications after surgery or trauma, easy bruising and in females also menorrhagia.

The present work is an attempt to systematically characterise PAI-1 deficiency in a large population concerning bleeding patterns in general, bleeding complications at surgery, fibrinolytic parameters and possible regulation of low PAI-1 levels.

PAI-1 AND ITS FUNCTION IN THE FIBRINOLYTIC SYSTEM

The purpose of the coagulation system is to form a blood clot to prevent bleeding in case of a trauma. There is also another balancing system in the blood circulation, which is called the fibrinolytic system, where plasmin is the end product.

Plasmin is an enzyme, which binds to fibrin in the blood clot and then starts a process of breaking down the clot. Plasmin degrades fibrinogen and fibrin, but can also degrade other proteins like factor VIII (FVIII) and factor V. Fibrin split products called fragment X, Y, D and E are formed. Fibrin D-dimer is exclusively formed from degradation of fibrin that has been cross-linked by factor XIII. Fibrin degradation products compete with fibrinogen and can bind to thrombin. High levels of fibrin degradation products may also interfere with platelet aggregation. Soluble fibrin is fibrinogen where fibrinopeptide A and B have been cleaved by thrombin, but a fibrin network has not yet been formed. Soluble fibrin is present mainly in severe illnesses as in disseminated intravascular coagulation [26-29]. Free plasmin, that is not bound to fibrin, is immediately inhibited by antiplasmin, which is present in plasma in high concentration [30]. tPA and uPA can activate plasminogen to plasmin. PAI-1 is an inhibitor of tPA and uPA and through this reaction no plasmin is formed. In case of low concentration of PAI-1, the fibrinolytic process is enhanced and the patient could start to bleed again after previous surgery or trauma (figure 1).

tPA is mainly found in plasma whereas uPA essentially is found in the extracellular matrix and in exocrine glands [31]. The fibrinolytic system can also activate metalloproteinases, which have the capacity to degrade extracellular matrices in remodelling tissue such as in wound healing [32]. The system is also important for cell migration [33] and the reproductive system [34].

Thrombin-activatable fibrinolysis inhibitor (TAFI) is another inhibitor of fibrinolysis. TAFI is a procarboxypeptidase that cleaves off carboxy-terminal arginine and lysine from fibrin and limits plasminogen binding as well as plasmin formation [35-37].

The end products of fibrin are degradation products, but D-dimer could reflect activation of

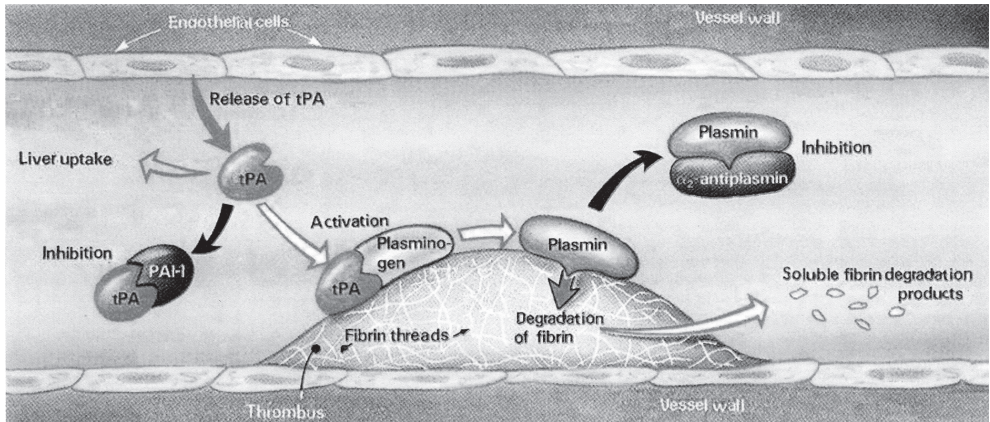


Figure 1. A simplified diagram of the fibrinolytic system (adapted from Wiman, MFR informarer, 1987).

the coagulation and fibrin formation rather than the fibrinolytic capacity [38]. Usually fibrin formation results in an increased D-dimer concentration and the analysis is used as a part of the investigation procedure in the diagnosis of deep venous thrombosis.

There is also evidence that elastase, which is released from neutrophils for instance during endotoxin shock [39], septicaemia [40] or promyelocytic leukaemia [41], is a potent proteolytic enzyme that can degrade the fibrin clot and cause other degradation products than D-dimer [42]. Elastase can also cleave and inactivate PAI-1 [43].

PAI-1 AND GENERAL CHARACTERISTICS

PAI-1 is a single-chain glycoprotein with a molecular mass of 52 kD and consists of 379 amino acids. It is a serine protease inhibitor and the gene is located on chromosome 7 and has 9 exons. PAI-1 is produced by several types of cells, such as vascular endothelial cells, adipocytes, hepatocytes, platelets, megakaryocytes, macrophages, smooth muscle cells and placenta [44]. The synthesis and secretion of PAI-1 is stimulated by several factors including corticosteroids, insulin, thrombin, endotoxin, oxidative stress, cytokines, and complement component C5a [45-47].

The plasma PAI-1 in healthy, lean individuals is suggested to come from platelets, since there are no posttranslational glycans in plasma PAI-1 or platelet PAI-1. However, in plasma PAI-1 from obese subjects there is a glycan composition similar to that of adipose tissue. PAI-1 isolated from endothelial cells, macrophages and adipose tissue expresses heterogeneous glycosylation patterns. The platelet count correlates with plasma PAI-1, which could be compatible with a constant release from platelets to plasma [48-50]. Another possible source for plasma PAI-1 could also be the liver.

PAI-1 is secreted as an active molecule and is stabilized by binding to vitronectin in plasma. Vitronectin is also an extracellular matrix protein [51]. Active PAI-1 that is not bound to tPA is cleared from the circulation within minutes [52]. In plasma samples from humans the

active PAI-1 is converted to latent PAI-1 with a half-life of 4 hours at body temperature. The half-life is longer if the temperature is lower. The functionally active PAI-1 is able to inhibit proteases, but it can be converted to a latent form under physiological conditions [53]. There is also an inactive form of PAI-1 in the α -granules in platelets [54].

PAI-1 inhibits tPA and uPA by forming a 1:1 stable complex. The active loop in the PAI-1 molecule has a cleavage site for tPA and uPA. By transformation of PAI-1 tPA and uPA are inactivated and they are “caught” within the inhibitor like in a mousetrap (figure 2).

The total concentration of PAI-1 in plasma is about 10-20 $\mu\text{g/L}$ [55] and about 60% is in active form. In platelets the concentration of PAI-1 is very high. It is mostly present in an inactive form (“latent”), but it has also been found that active PAI-1 is continuously formed by platelets [56]. When platelets are activated, such as during formation of serum, a large pool of PAI-1 is released from α -granules. The concentration of PAI-1 antigen in serum is approximately 100-300 $\mu\text{g/L}$ [54].

The active form of PAI-1 is measured in U/mL and the normal activity level is below 15 U/mL. Usually no lower limit of the normal range is provided, since the routine analysis is inaccurate at the low end.

The median value for tPA-PAI-complex is 4.85 $\mu\text{g/L}$ in women and 5.6 $\mu\text{g/L}$ in men in healthy controls [57].

There is a diurnal variation of PAI-1 with 2-3 times higher activity in the morning than in the afternoon. This variation is due to so called clock genes. Experiments made in knockout mice for clock genes showed no circadian variation in PAI-1 antigen or hepatic mRNA level in contrast to wild type mice [58].

PAI-1 is an acute phase protein and increases with inflammatory response (for more details see paragraph on PAI-1 and inflammation). There is a common polymorphism in the promoter

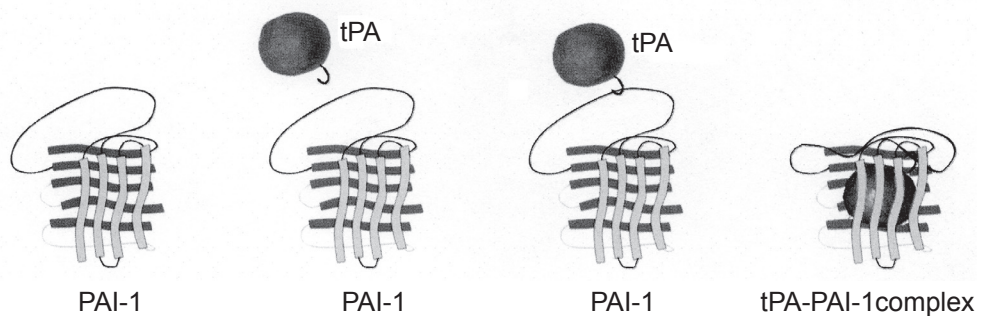


Figure 2. tPA-PAI-1 complex “the Mousetrap”.

PAI-1 has an active loop, which is very attractive for tPA. When tPA comes in contact PAI-1, the active loop is cleaved and tPA is caught within PAI-1 in a practically irreversible binding.

region, in the position -675 of the PAI-1 gene, 4G/5G, where 4G/4G has been associated with higher PAI-1 levels than 4G/5G and 5G/5G [59]. However, there is no association between the 4G/5G promoter polymorphism and platelet mRNA or protein expression [60].

PAI-1 is produced by fat tissue and people with overweight have generally higher PAI-1 levels than people with normal weight. Age does not affect PAI-1 levels to a high degree, but older have slightly higher values than younger [61]. This could be due to increasing weight with age but the association needs to be put into the perspective of the lowering effect of oestrogens on PAI-1 levels, described in fertile women [62]. Women of fertile age have lower PAI-1 levels than post-menopausal women [63].

PAI-1 activity at birth and throughout childhood is increased compared with reference values for adults [64, 65]. A recent study showed that children older than 1 year have no significant difference in PAI-1 Ag compared to older children or adults but the number of children studied was relative small [66] although adiposity in children and adolescents is associated with higher values of PAI-1 Ag [67, 68].

PAI-2

PAI-2 has a different structure from PAI-1 and consists of 415 amino acids. PAI-2 is mainly produced in placenta [69]. PAI-1 and PAI-2 have a common target for protease specificity, PAI-1 against tPA and uPA and PAI-2 towards uPA [70].

PAI-1 AND ANIMAL STUDIES

Mice with a homozygous defect of PAI-1 have a normal development *in utero* and after birth. Studies in which surgical procedures were performed on such animals have not shown more bleeding complications than in wild-type mice. PAI-1 deficient mice were less prone to develop venous thrombosis after injection of endotoxin compared to wild type [71, 72].

Mice with a combined homozygous PAI-1 deficiency, obesity and diabetes have high mortality (33%), and high morbidity compared to diabetic mice or PAI-1 deficient mice. Sixty-three percent of the male mice in the PAI-deficient, obese, diabetes group became runts, which are small and underdeveloped mice [73].

PAI-1 deficient mice have an attenuated neointima formation in the artery after arterial injury [74]. In experiments where platelet-rich thrombi were induced in PAI-1 deficient mice and wild type mice, the resistance to lysis by infusion of tPA was higher in the wild type [75].

In PAI-1 deficient knock-out mice liver injury was induced by acetaminophen. Compared to wild-type mice, the effects of liver injury was similar the first 12 hours, but then the PAI-1 deficient mice developed increased plasma levels of alanine aminotransferase (ALT) and liver necrosis with massive intrahepatic haemorrhage. Half of the PAI-1 deficient mice died compared to none of the wild- type mice. The suggested reason for the poor outcome in the PAI-1 knock out mice was that there was a premature lysis of fibrin deposits with excess plasmin formation. It was concluded that PAI-1 limits liver injury after acetaminophen overdose [76].

Transgenic mice that express a stable form of PAI-1 suffer from age-dependent coronary artery thrombosis in the absence of hypercholesterolaemia or multiple genetic mutations [77]. These mice can also develop venous occlusion [78].

In nutritionally induced obese mice a synthetic PAI-1 inhibitor, tiplaxtinin, significantly reduced weight compared to non-treated mice. Plasma triglyceride (TG) levels were reduced and low-density lipoprotein-cholesterol levels were increased. Insulin-tolerance tests revealed lower glucose levels at the end of the test in the inhibitor treated mice. Fasting glucose and insulin levels as well as glucose tolerance tests were not significantly affected by the inhibitor treatment [79].

Another PAI-1 inhibitor, TM5007, has been used in mice, where thrombosis or fibrosis was chemically induced [80, 81]. The PAI-1 inhibitor reduced the size of the blood clot and this effect was comparable with the effect of warfarin or ticlopidine and superior to tPA. In another experiment the growth of thrombi was slower in the PAI-1 inhibitor treatment group and this effect was similar to the effect of tirofiban. The PAI-1 inhibitor did not affect bleeding time, prothrombin time (PT) or activated partial thromboplastin time (APTT). The antifibrotic effect was also tested in chemically induced pulmonary fibrosis, where the PAI-1-inhibited mice had histological evidence of reduced fibrosis [82].

PAI-1 AND SEX HORMONES

Female sex hormones

Women of fertile age have lower PAI-1 levels than post-menopausal women. There is a variation in PAI-1 activity during the menstrual cycle but in most individuals it remains within the normal range and the magnitude of variation differs between women [83, 84]. Combined oral contraceptives (oestrogens and gestagens) reduce the concentration of PAI-1 [85]. Oral, but not transdermal, hormone replacement therapy (HRT) has a significant lowering effect on PAI-1 levels [62]. Exogenous gestagens are not known to exert any substantial influence on PAI-1 [86].

In pregnancy PAI-1 and PAI-2 are produced by placenta, and thus the PAI-1 activity increases until delivery about 6-fold to levels above the upper reference limit of 15 U/mL in non-pregnant women [87, 88]. After birth PAI-1 levels decrease within one day to the normal range for non-pregnant women but PAI-2 levels remain elevated for over 11 days [89, 90].

In severe preeclampsia and HELLP (Hemolysis, Elevated Liver enzymes, Low Platelets)-syndrome PAI-1 levels are higher compared to normal third-trimester women, in contrast to PAI-2 levels that are significantly lower than in normal pregnancy. After birth the levels of PAI-1 and PAI-2 show similar patterns as in normal postpartum women [91, 92]. In another study preeclamptic women with foetal growth retardation had significantly lower levels of both PAI-1 and PAI-2 compared to those without foetal growth retardation [93].

In normotensive pregnant patients with intrauterine foetal growth retardation PAI-1 levels are similar to those in normal pregnant women, but PAI-2 levels are lower. A significant correlation between PAI-2 and foetal weight has been observed [91].

Male sex hormones

Hypogonadism in males does not affect PAI-1 levels compared with controls [94]. Synthetic androgen hormones like stanozolol and danazol reduce PAI-1 [95].

PAI-1 AND INFLAMMATION

There is a close relationship between coagulation and inflammation, where the inflammatory response and the coagulation cascade form a network. The activation of coagulation can affect the inflammatory response [96]. Thrombin is chemotactic to neutrophils and monocytes and stimulates the release of interleukin-1 and interleukin-8. Monocytes and neutrophils can present tissue factor on their surface [97]. The leukocyte derived tissue-factor is important in the activation of coagulation in sepsis [98] and monocyte tissue factor is increased in patients with disseminated intravascular coagulation (DIC) [99]. Leukocytes can roll on P-selectin expressed on activated platelets and also adhere to platelet-bound fibrinogen in the haemostatic plug. The interactions between leukocytes and endothelial cells or platelets are part of host defense, but can also induce a procoagulant state [100].

High PAI-1 levels are associated with an inflammatory response [101] and with septicaemia [102, 103] and endotoxin stimulation [104]. At the site of inflammation monocytes and macrophages are attracted and they produce cytokines, which are hormone-like mediators. The cytokines send signals to the liver to produce acute-phase reactants like C-reactive protein (CRP), α_1 -antitrypsin, orosomucoid, haptoglobin, complement factors, factor VIII (FVIII) and fibrinogen. Interleukin-6 (IL-6) is the only cytokine that can stimulate the whole spectrum of acute-phase proteins [105]. IL-6 has been shown to stimulate PAI-1 production [106, 107].

CRP is a sensitive marker of acute inflammation and it rises rapidly as a part of the IL-6 mediated inflammatory response to infection. In healthy people CRP is less than 1 mg/L and it may increase 1000-fold within 24 hours in acute stress such as inflammation or tissue injury [108]. Patients with severe cardiovascular disease usually have elevated CRP-levels to 5-25 mg/L [109, 110].

CRP increases the expression of PAI-1 in human endothelial cells in cell culture experiments [111].

PAI-1 AND METABOLISM

Overweight together with the metabolic syndrome, which is characterized by large waist circumference due to fat distribution in the central part of the body, high blood pressure, dyslipidaemia with high concentration of TG and low concentration of high density lipoproteins (HDL), glucose intolerance and hyperinsulinaemia [112], have a strong association with high PAI-1 levels [113]. High levels of proinsulin [114], C-peptide and insulin [115] have also been associated with high concentration of PAI-1 (figure 3). In diabetes type 2 there is insulin resistance, which means

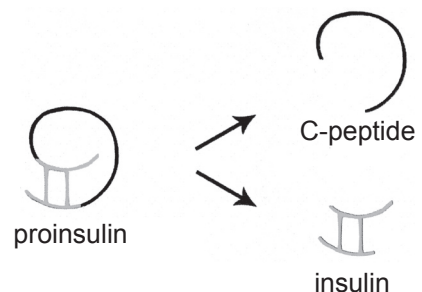


Figure 3. Proinsulin is cleaved by proteases to insulin and C-peptide in equal amounts.

that there are high levels of circulating insulin and still the plasma glucose concentration is high.

Known haemostatic changes associated with the metabolic syndrome and diabetes mellitus type 2 are platelet hyperactivity [116, 117], elevated levels of circulating tissue factor in microparticles [118] increased levels of vitamin K-dependent coagulation factors [119] elevated fibrinogen [120, 121] elevated FVIII [122] but most pronounced of all is the elevation of PAI-1.

PAI-1 levels are higher if the metabolic syndrome is severe [101]. Weight reduction, improvement of insulin resistance and treatment with anti-diabetic drugs like metformin or troglitazone decrease PAI-1 [123, 124]. It has also been reported that simvastatin significantly reduces PAI-1 after 8 weeks of treatment in patients with the metabolic syndrome [125]. PAI-1 is considered as an acute phase protein, but during obesity there is no association with the cytokine IL-6 or CRP [119].

It has been suggested that the increased level of PAI-1 is primarily derived from the adipocytes in response to cytokines like tumour necrosis factor (TNF), transforming growth factor beta or insulin [126]. Abdominal visceral fat expresses more PAI-1 than subcutaneous fat [127]. In obese mice deletion of TNF receptors or antibodies against TNF decreased plasma PAI-1 levels [128, 129]. Cultured adipocytes from PAI-1 deficient mice showed increased insulin sensitivity [130].

In cultured adipocytes and human adipose tissue, dexamethasone and cortisol are inducers of PAI-1 synthesis [131], but generally high cortisol levels have not been demonstrated in obese patients. Still there could be a local production in the adipose tissue. Proinsulin [114], insulin [132], free fatty acid [133] or very-low-density lipoproteins [134] increased the production of PAI-1 in cell culture experiments.

Clock gene mutated mice with no circadian variation of PAI-1 also have metabolic disturbances in the lipid and glucose metabolism and elevated IL-6 levels, and this could support the hypothesis that there is an association between PAI-1 and metabolism [135, 136].

It is known that the PAI-1 polymorphism 4G/5G is affecting PAI-1 levels, but studies show divergent results concerning obesity, PAI-1 levels and this polymorphism in the PAI-1 gene [137-140], reflecting the fact that the polymorphism probably is not an important regulator of PAI-1 production in obese patients.

In cultured fibroblasts it was shown that PAI-1 interferes with insulin signalling by preventing binding of vitronectin to integrin $\alpha_v\beta_3$ and this can inhibit insulin-induced protein kinase B phosphorylation [141].

Some studies in PAI-1 deficient mice have demonstrated prevention of fat accumulation and improvement in insulin resistance [142, 143], whereas some studies have not shown any effect on weight gain [144, 145].

Patients with diabetes mellitus type 1 have lower PAI-1 levels than patients with diabetes type 2 [146, 147]. This could be due to lower body mass index (BMI) in the group with type 1 diabetes [148] or due to stimulation of PAI-1 secretion by high levels of proinsulin as is the case in diabetes type 2. Infusion of insulin in healthy volunteers [149] resulted in increased levels of PAI-1 within hours.

There has also been an association reported between glucagon and PAI-1 levels in men with normal glucose tolerance, when performing a lipid-glucose protein test [150].

PAI-1 AND ATHEROSCLEROSIS

It could be argued that atherosclerosis is a chronic inflammatory condition initiated in the endothelium and maintained by interactions between lipoproteins, macrophages and the arterial wall [151].

This inflammatory condition in atherosclerosis could be supported by the fact that high sensitivity CRP (hs-CRP), a marker of inflammation, has been recognized as a risk factor for cardiovascular disease [152], and that macrophages in the atherosclerotic plaque contain tissue factor, which may play a role in the initiation of coagulation following rupture of the plaque [153]. A clinical study, the Women Health Initiative Observational study, showed 40% higher risk for myocardial infarction and stroke among healthy postmenopausal women with an elevated leukocyte count (more than 6.7×10^9 white blood cells/L) [154]. In myeloproliferative diseases there is an increase in leukocyte-platelet aggregates that might play a role in the pathogenesis of the vascular complications [155].

Insulin resistance is another strong risk factor for cardiovascular disease. Patients with insulin resistance have an increased risk to develop diabetes and cardiovascular disease and also have a higher cardiovascular mortality [156]. Fasting insulin level may be an independent risk factor for coronary heart disease [157].

High PAI-1 levels have been associated with atherosclerosis [158] [159] and increased risk for recurrence of myocardial infarction [160]. In patients with type 2 diabetes mellitus, PAI-1 is increased in blood vessel walls and the incidence of acute coronary syndromes precipitated by plaque rupture is high [161]. The pathological background seems to be a paradox, since proteolysis by plasminogen activators contributes to vascular smooth muscle migration, neointimal formation, and activation of matrix metalloproteinases that precipitate plaque rupture [162] and PAI-1 would normally prevent the process. One possible theory is that PAI-1 could inhibit migration of vascular smooth muscle cells, which leads to formation of acellular atheroma plaques with lipid-laden cores and thin fibrous caps, and these plaques could be very fragile and therefore prone to rupture [163].

An association between the 4G allele of the 4G/5G polymorphism and increased plasma PAI-1 concentration in patients with cardiovascular disease has been reported [164, 165]. Patients with high proinsulin levels who were homozygous for the 4G/4G polymorphism in the PAI-1 gene were at increased risk for myocardial infarction, suggesting that PAI-1 genotype may affect vascular risk in patients with insulin resistance [164]. In the SHEEP study; a study

which compared patients with first myocardial infarction and controls, a synergistic effect between PAI-1 levels and smoking was seen in men and the 4G allele was weakly associated with an increased risk of myocardial infarction in women [166]. The risk for recurrence of myocardial infarction in the same patient material was associated with high levels PAI-1 but also with other known risk factor like diabetes [57, 167].

The renin-angiotensin system is part of the regulation of blood pressure. Inhibition of angiotensin-converting enzyme (ACE) reduces plasma PAI-1 in obese subjects [168]. The suggested mechanisms concerning the effect of ACE-inhibition on the fibrinolytic system are 1) inhibition of angiotensin II, which stimulates PAI-1 expression, 2) inhibition of degradation of bradykinin, which is a stimulus for tPA production and 3) improvement of insulin sensitivity [169].

In stroke, where tPA has been given as therapy to resolve clots, it has been observed that high PAI-1 levels are associated with clot-lysis resistance and poor outcome [170].

PAI-1 AND VENOUS THROMBOSIS

A weak but independent association between the level of PAI-1 and recurrence of venous thrombosis has earlier been reported. After 10 years of follow-up the predictive value of high PAI-1 became stronger. In one study tPA was not an independent risk factor for recurrence whereas in another study low tPA, either due to reduced synthesis or release, seemed to contribute to the pathogenesis in some of the patients [171-173].

In a prospective case control study with 208 patients who developed deep venous thrombosis and 640 controls, there was no association between PAI-1 antigen, tPA/PAI-1 complex, plasmin-antiplasmin complex and the PAI-1 -675 4G/5G polymorphism and venous thrombosis. The samples were taken before the patients were diagnosed with venous thrombosis. Therefore it is suggested that these fibrinolytic markers do not identify healthy subjects at risk for venous thrombosis [174], whereas high PAI-1 may contribute to the long-term risk of recurrent thrombosis.

PAI-1 AND CANCER

The role of PAI-1 in angiogenesis has been extensively studied but still remains controversial [32, 175].

The fibrinolytic system is involved in remodeling, wound healing and cell migration and can activate metalloproteinases (zink-dependent endopeptidases) to degrade extracellular matrix protein. The latter is a known mechanism when cancer cells invade and spread. Cathepsin D is a proteolytic enzyme, which can be released from neutrophils and is able to degrade fibrin [176] and extracellular matrix proteins. High levels of cathepsin D are associated with a poor prognosis in breast cancer and poor prognosis has also been associated with the urokinase pathway of plasminogen activation. PAI-1 and cathepsin D have been reported as independent prognostic indicators for disease free survival when measurements were done from breast tumor cytosols. The five year relapse rate among patients with low PAI-1 and low cathepsin D was 13% compared to 40% if both PAI-1 and cathepsin D were above the median [177].

Patients with high PAI-1 in extracts from solid lung tumours and low levels of uPA-PAI-1 complex had poorer survival than patients with low PAI-1 and high uPA-PAI-1 complex [178]. When PAI-1 was measured in cytosols from gliomas it was shown that there is a shorter overall survival in patients with high PAI-1 levels [179]. Endometrial cancer has been associated with high PAI-2 levels [180]. Increased levels of uPA-antigen in extract of solid tumours has been associated with poor prognosis [181, 182].

It has even been discussed that PAI-1 could be a therapeutic target in cancer, due to the evidence that high levels of PAI-1 in extracts of human primary malignant tumours is one of the most informative biochemical marker of poor prognosis in several cancer types [51].

PAI-1 AND RELATED CONDITIONS OR DISEASES

The fibrinolytic system seems to be involved in the spermatogenesis, sperm capacitation and fertilization [183] as well as in ovulation [184].

During the destruction of articular cartilage, fibrinolytic enzymes and matrix metalloproteinases may contribute to the related pathology. Patients with rheumatic arthritis and osteoarthritis have higher uPA and PAI-1 in the cartilage than controls [185].

Finally, PAI-1 is overexpressed in fibrosis in the kidney [186], lung [187] and liver [188].

LOW PAI-1 AND BLEEDING DIATHESIS

Since 1989 there have been several published case reports of PAI-1 deficiency in association with bleeding tendency [12-25] (table 1).

Schleef et al reported on the first case with severe bleeding after surgery and trauma, low level of PAI-1 activity but normal PAI-1 antigen in serum [19].

Two of the reports, described mutations in the PAI-1 gene; one was a frame shift mutation in exon 4 with production of a dysfunctional PAI-1 protein [21] and the other was a single point mutation identified in the signal peptide [13]. Their families were also investigated. Fay et al described one large kindred in the Indiana Amish population in USA where 7 people were homozygous for a frame shift mutation in the PAI-1 gene and 19 were heterozygous. The homozygous individuals had abnormal bleeding tendency after surgery or trauma including intracranial haemorrhage or haemarthrosis after mild trauma, delayed surgical bleeding, profuse menstrual bleeding and frequent bruising. Their bleeding problems were effectively prevented or treated by fibrinolysis inhibitors like tranexamic acid or epsilon-aminocaproic acid. They had no other developmental or other observed abnormality. The individuals who were heterozygous for PAI-1 deficiency had no bleeding tendency.

In the other family described by Zhang et al [13] the proband had a single point mutation in exon 2 in the PAI-1 gene with a PAI-1 activity of 10% as compared to normal. The bleeding history included life-long bleeding problems after surgery or trauma. After the diagnosis of PAI-1 deficiency was established, the patient received a fibrinolysis inhibitor before surgery

and bleeding complications were prevented. The father but no other family member of the proband had the same mutation.

It was also reported that patients with PAI-1 deficiency required higher doses of tranexamic acid than normal, to prevent oral bleeding when extracting teeth [22].

Table 1. A compilation of published cases or case series with PAI-1 deficiency

Year	Ref	Number of patients	Number of controls	PAI-1 activity U/mL (controls)	PAI-1 antigen ng/mL (controls)	Mutation found	Bleeding history
1989	19	1	5	0.36±0.12 (0.87-1.81)	31.8±5.4 (19.6-42.2)	No	Surgery and trauma, gastro-intestinal bleeding.
1991	18	1	0	<1	<6, <2	No	Surgery and trauma, epistaxis
1991	14	1	0	2	-	No	Surgery
1992	21	1	0	<0.1	< 2	2 base-pair insertion in exon 4.	Surgery and trauma
1997	12	7 homo- and 19 heterozygous in 1 kindred					See text page 19.
1993	17	1	20	0.8±0.8 (6±3.8)	1.9±1.1 (26±14)	No	Surgery, epistaxis, bruising
1996	23	3	8 family members	0.5-8.3 (9.1-20.3)	2.1-8.3 (7.8-21.9)	No	Surgery and trauma
1999	16	4	30	<1-3 (14.2±7.4)	1.6-13.8 (16.6±8.1)	No	Surgery and trauma, menorrhagia
2001	25	1	2 family members	1.02	3.62	No	Haemarthrosis subcutaneous haematoma, tooth extraction.
2004	22	3	0	1.02-3	3.68-4.9	No	Surgery, haemarthrosis, subcutaneous bleeding
2004	15	1	0	<5	11.4	No	Menorrhagia
2004	13	2	1 family member and 60 controls	0.04-0.21 (0.41)	5.6-33.24 (44)	Heterozygous missense mutation exon 2	Surgery and trauma
2005	24	1	0	0	1.6	No	Eye bleeding

A case report from 2007 describes a patient with low PAI-1 activity and bleeding tendency such as mild epistaxis, gingival bleeding and microscopic haematuria, spontaneous bleeding from the navel and the nipple [189]. Two family members also had low PAI-1 activity, one had menorrhagia and one had no bleeding tendency. However, when a normal population was examined the incidence of low PAI-1 activity (defined as below 2 U/L) was 21%.

Relative PAI-1 deficiency has been described in patients with alcohol-induced liver cirrhosis, ascites and portal hypertension, where basal tPA levels are increased and PAI-1 activity decreased [190].

In patients with severe haemophilia it has been reported that those with a more intense bleeding pattern have higher levels of tPA, PAI-1, tPA-PAI-complex and TAFI [191].

PAI-1 AND SURGERY

The fibrinolytic activity in patients undergoing elective cholecystectomy or more advanced abdominal surgery was investigated by Mellbring et al in 1985 [192].

The tPA concentration increased rapidly immediately after starting the surgery. PAI-1 started to increase at the end of surgery and reached the highest value three hours postoperatively. The plasmin-antiplasmin complex (PAP), which is a measure of fibrinolytic activity and plasmin generation, started to rise one day after surgery and continued to increase until day 6 postoperatively, when the study ended. This study shows that the fibrinolytic system is activated at the time of surgery and the activation process is partly inhibited by PAI-1, but the plasmin generation continues for several days after surgery. This could be a reason why families with inherited PAI-1 deficiency suffered from delayed surgical bleeding and it could also be reason why treatment with antifibrinolytic drugs should start before surgery. The PAI-1 increase related to surgery had no relationship with increased levels of cortisone [193].

When surgery was performed on patients with abdominal aortic aneurysm it was shown that the fibrinolytic system was more intensely activated in patients with ruptured than non-ruptured abdominal aortic aneurysms [194].

It has been suggested that activation of the fibrinolytic system could be a reason for excessive blood loss during surgery of the prostate [195, 196]. The constant flow of urine containing urokinase over the resected prostatic cavity, in the combination with the high concentration of plasminogen activators in the prostate gland, are possible explanations. There have been several trials to examine the efficacy of antifibrinolytic drugs in prostatic surgery with conflicting results [197, 198]. Nielsen et al have suggested that systemic fibrinolysis does not influence the persistent and delayed haemorrhage after transurethral resection of the prostate (TURP), but postoperative blood loss could be dependent on *in situ* fibrinolysis in the urine of patients undergoing TURP [199].

There are mainly three types of antifibrinolytic drugs that have been used in mostly cardiac and orthopaedic surgery. Epsilon-amino caproic acid and tranexamic acid block the interaction of plasminogen and fibrin. Tranexamic acid is the most potent drug of these two. Aprotinin reduces fibrinolysis by direct inhibition of plasmin.

In one randomized study of different types of cardiac surgery, where the patient either received tranexamic acid or saline, it was shown that the release of tPA and enhancement of D-dimer was effectively inhibited by tranexamic acid. Levels of PAI-1 and PAP were not different in the two groups of patients. There was a significant reduction in intraoperative and total blood loss in the tranexamic acid group and no patients suffered from thrombotic complications [200]. In another randomized trial comparing tranexamic acid with placebo in 300 patients with aortic valve replacement it was concluded that 24.5% of those treated with tranexamic acid received blood products versus 45% of control patients [201].

It has been reported that the postoperative bleeding pattern in patients receiving tranexamic acid or placebo in cardiopulmonary surgery is depending on the PAI-1 polymorphism 4G/5G, where patients with 5G/5G polymorphism were prone to higher amount of postoperative bleeding and tranexamic acid more effectively prevented excessive blood loss [202].

Aprotinin has been utilized for several years in cardiac surgery but a recent study was stopped due to a higher death rate in the aprotinin group [203]. A former study from 1993 by Sundt et al showed increased risk of postoperative renal dysfunction and failure and death in elderly patients [204].

BLEEDING TENDENCY AND COAGULATION DISORDERS

The most common severe inherited forms of bleeding disorders are haemophilia A (FVIII-deficiency), haemophilia B (factor IX [FIX]-deficiency) and von Willebrand disease (VWD; deficiency of von Willebrand factor, VWF) with a prevalence in the severe form of about 12/100,000, 2/100,000 and 1/200,000 respectively. The prevalence of von Willebrand's disease in any form (mild, moderate and severe) is about 2/100. Patients with VWD can also have FVIII deficiency, since VWF is a carrier protein for FVIII. These coagulation disorders are often diagnosed in early childhood.

Haemophilia A and B in the severe form, affect mostly boys, since the mutated gene for FVIII and for FIX is located on the X chromosome and it is a recessive inherited disease. VWD has autosomal inheritance and affects boys and girls equally. Common symptoms in haemophilia A and B are muscle and joint bleedings and in VWD also mucosal bleedings. In the moderate and mild forms of these bleeding disorders the diagnosis is made later in life, for instance after investigation for prolonged bleeding after tooth extraction, extended traumatic bleeding after sport activity or profuse menstrual bleeding at menarche. Other causes of bleeding diathesis could be specific deficiencies or defects in coagulation factors II, V, VII, X, XI, XIII or fibrinogen.

There are rare inherited disorders of platelet dysfunction like Bernard-Soulier's disease and Glanzmann's thrombasthenia, which lead to a life long bleeding tendency with symptoms like bruises, epistaxis and mucosal bleedings. More common are unspecific, inherited platelet dysfunction or acquired platelet disorders, secondary to medication, including acetylsalicylic acid (Aspirin), clopidogrel, non-steroidal anti-inflammatory drugs, selective serotonin reuptake inhibitors or Omega-3-fatty acids.

Other medications that can lead to bleeding symptoms are warfarin, heparin or low-molecular-weight-heparins. Warfarin is a drug that affects the synthesis of all the K-vitamin dependent coagulation factors (II, VII, IX and X), as well as the natural inhibitors protein C and S. Warfarin blocks the formation of active forms of these factors and can therefore cause spontaneous bleeding symptoms. Heparin and low-molecular-weight- heparins mainly inhibit factor IIa (thrombin) and Xa, at the central point in the coagulation cascade where thrombin generation and thrombin-induced fibrin formation occur.

The role of defects in the fibrinolytic system as a cause for bleeding symptoms is less clear. There are a few reported cases of antiplasmin deficiency, which in the heterozygous form has been associated with bleeding tendency after trauma, surgery and tooth extractions and in homozygous form with more severe bleeding tendency [205]. There are only iatrogenic cases with thrombolytic treatment with excess amount of uPA, tPA and plasmin as a cause of bleeding tendency. There are no reported cases of bleeding due to deficiency of thrombin activatable fibrinolysis inhibitor (TAFI).

Low PAI-1 levels have, as mentioned above, been reported in case- and family studies as a cause of bleeding diatheses, especially after surgery and trauma. Systematic studies concerning fibrinolytic defects as a reason for bleeding tendency are lacking.

AIMS OF THE STUDY

The aims of the present study were:

- To investigate if there is an association between low PAI-1 activity and bleeding symptoms
- To study if there is an association between low PAI-1 activity and the 4G/5G-polymorphism or perhaps other defects in the PAI-1 gene
- To evaluate the regulation of PAI-1 activity in relation to sex, age, BMI, inflammatory and metabolic parameters and in women the impact of combined oral contraceptives or hormone replacement therapy.
- To explore if there is laboratory evidence for enhanced fibrinolytic activity in patients with low PAI-1 activity

PATIENTS AND METHODS

THE PATIENT- AND CONTROL POPULATIONS

A total of 648 patients and 200 controls were investigated. Study I included 586 patients referred to the Coagulation Unit at Karolinska University Hospital, Solna, due to bleeding diatheses, and two different control populations with 100 blood-donors and 100 healthy controls in each. In study II we recruited 62 men planned for transurethral resection of prostate due to benign prostate hyperplasia.

In study III we evaluated 424 patients, with samples available, from the original 586 patients (study I) and study IV was based on samples from 181 patients of the original population (figure 4).

In **study I**, 586 consecutive patients, referred because of bleeding symptoms during the period of October 1998-October 2002, were included. Analyses of PAI-1 activity and tPA-PAI-1 complex were added to the routine investigation of bleeding disorders. Bleeding symptoms were classified as clinically significant or not by two of the investigators, who were blinded to the laboratory results. Clinically significant bleeding included bleeding complications after surgery, tooth extraction or child delivery, bleeding in the central nervous system, gastrointestinal canal, joints or muscles as well as referral for menorrhagia, confirmed as

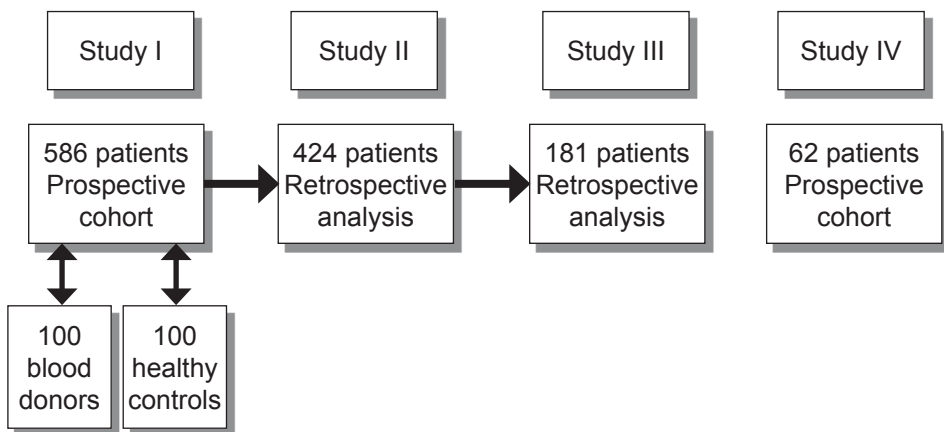


Figure 4. Relation of patient populations in the different studies.

menstrual bleeding requiring change of pads or tampons at least every 2 h. Not clinically significant bleeding were for example subcutaneous haematoma, epistaxis, gingival and subconjunctival bleeding. In some patients also PAI-1 antigen in serum and the 4G/5G polymorphism in the PAI-1 gene were analysed.

The routine investigation consisted of platelet count, APTT, PT, template bleeding time, fibrinogen, FVIII activity, VWF activity (ristocetin co-factor) and VWF antigen. In case of prolonged APTT or PT further factor analyses were done as appropriate. In case of normal results in the routine investigation, thrombin time, factor XIII and antiplasmin were added.

A control group consisted initially of 100 anonymous blood donors, equally distributed in age and sex. Height and weight were noted, but no other information was possible to obtain for the integrity of the donors. As it was found that the distribution of sex was different in the patient population compared with the blood donors and that it was impossible to get information on bleeding history from the blood donors since they were anonymous, another control group was recruited. This control group consisted of 100 friends or non-blood relatives to the patients or hospital staff without any known haemostatic defect. This control group was age and sex matched with the patients and they filled out a questionnaire on bleeding history, height and weight, use of oral oestrogens or combined contraceptive pills. Their bleeding symptoms were, as in the patients, classified as clinically significant or not. This control group is called the "healthy controls".

In **study III** we analysed plasma samples that were still available from 424 of these patients. The patients with insufficient plasma material were randomly distributed over this period. PAP and D-dimer were analysed and compared with PAI-1 levels.

In **study IV** we included all 63 patients for whom plasma samples were still available and with PAI-1 activity below 1 U/ml as well as from 118 randomly distributed patients with PAI-1 levels ≥ 1 U/ml. Totally 181 plasma samples were therefore analysed for C-peptide, proinsulin, CRP and IL-6, and they were compared with PAI-1 levels.

In **study II**, consecutive patients with benign prostatic hyperplasia and planned for TURP were asked for participation in this study during the period of September 2001 to June 2005. A total of 62 patients were included in the study. Haemoglobin was measured before surgery and on the day of discharge. We registered perioperative bleeding amount, haematuria, days with Foley catheter and irrigation of the bladder postoperatively, days of hospitalisation, amount of blood products and plasma expanders given during hospitalisation, anaesthesia, surgeon, operative time, weight of prostatic tissue, blood pressure, medication, height and weight of the patient. The intention was to have a single surgeon, which was possible in 51 of 62 patients. The sample for PAI-1 was taken before surgery and analysed after discharge.

The primary end point was a bleeding complication, which was defined as 1) reoperation due to bleeding, 2) more than 40 mL intraoperative bleeding/g resected prostate, 3) postoperative blood loss corresponding to more than 100 g haemoglobin. Secondary endpoints were number of days with irrigation and length of hospitalisation. The patients with bleeding complication were referred to the coagulation unit for elective investigation of haemostatic defects.

Blood donors and coagulation

The effect on coagulation by plasmapheresis in blood donors has been investigated by Wood et al and Grgicevic [206, 207]. The coagulation proteins seem to be stable in plasma and returning to normal within 72 hours after the plasmapheresis.

The blood donors are asked before their first donation if they have a haematological disorder, a coagulation disorder or if they have had blood transfusions before. If the answer is yes, this is further discussed with a doctor or nurse. Repeated blood transfusions or bleeding episodes without a known diagnosis of coagulation- or blood disorder does, however, not exclude the patient from being a blood or plasma donor (Standard Operating Procedures, Department of Immunology and Blood Transfusion, Karolinska University Hospital).

ASSESSMENT OF BLEEDING AND BLOOD LOSS

Evaluation of bleeding tendency

The clinical diagnosis of mild bleeding disorders is sometimes difficult, since it usually requires an evaluation of the lifelong bleeding history. In addition, for VWD and platelet function disorders there are limitations of the laboratory tests [208]. The interpretation of clinical data is subject to bias. Although bleeding symptoms may be more frequent in certain haemorrhagic disorders, there is no single symptom that could be considered pathognomonic for a specific bleeding disease. Therefore it is the “haemorrhagic picture” of the patient rather than a particular symptom that may be helpful for the diagnosis. Definitions and standardised questionnaires are nowadays more frequently used, but were not common when we started the studies. However, even normal subjects report bleeding symptoms [209].

Measurements of blood loss in surgery

Blood loss during transurethral resection of the prostate can be assessed by using the HemoCue photometer (HemoCue AB, Ängelholm, Sweden). All the blood lost during transurethral surgery is mixed with the irrigating fluid that can be collected in buckets. The haemoglobin concentration can then be measured in the irrigating fluid. To prevent clots in the collection bucket 1000 U of heparin is added. The system has been tested by using known amounts of bank blood that was run into buckets containing known volumes and weights of irrigating fluid. The solution was stirred and duplicate samples were drawn from the periphery and the centre of surface and from the bottom of the buckets. The mean accuracy of the measurement with the HemoCue was 98.2 % (SD 3.7) at the periphery, 98.1 % (SD 5.1) at the centre and 88.8 % (SD 14.8) at the bottom of the bucket. The coefficient of variation in serial analyses ranged from 2 % to 3 % . The system has been used in the clinical routine for several years and provides an opportunity to measure peri-operative blood loss very precisely [210].

In other types of surgery, when the blood cannot be collected in a single container, there is a possibility to visually assess the amount of blood in swabs or weigh the swabs. The problems with these methods are that visual assessment has poor validity and the swabs can absorb exudates or irrigation fluids or there could be errors due to evaporation or dilution [209].

Measurement of blood in suction devices during surgery can be inaccurate since the volumes collected are often small. Dilution by exudates or irrigation fluids and blood remaining in the tubes could be a problem.

Blood loss in post-operative drainage could be underestimated due to compartments of blood or large subcutaneous haematomas in the operation area and the blood in the drainage tubes may also clot.

Another way of measuring blood loss is to use indirect methods by analysing the drop in haemoglobin. Consideration must then be taken to the initial blood volume of the patient and changes in blood volume during surgery due to transfusions of fluids or blood. Different formulas have been suggested for calculation of blood loss [211, 212]. The haemoglobin drop may not only reflect actual blood loss or dilution of blood but also haemolysis.

Measurements of blood volume

Several formulas have been used to calculate blood volume. The formula of Allen et al [213] states that blood volume = $0.417 \times \text{cube of height (m)} + 0.045 \times \text{body weight (kg)} - 0.03$.

The formula of Nadler et al [214] states that blood volume (L) for men = $\text{body height (m)}^3 \times 0.3669 + \text{body weight(kg)} \times 0.03219 + 0.6041$ and blood volume (L) for women = $\text{body height (m)}^3 \times 0.3561 + \text{body weight(kg)} \times 0.03308 + 0.1833$.

Accurate formulas are required for blood volume measurement to be useful in a clinical setting. The primary physiological determinant of normal blood volume is body composition and formulas have been developed based on weight and body surface area, but these can have systematic errors arising from variations in the body composition or body size [215].

Calculation of blood loss in the study on TURP surgery

The intraoperative blood loss was measured by analysing haemoglobin in the irrigating fluid using HemoCue Low Hemoglobin Photometer (HemoCue AB, Ängelholm, Sweden). The total amount of haemoglobin in a bucket is the product of the measured haemoglobin concentration and fluid volume [210]. The intraoperative volume of lost blood equals the sum of the haemoglobin contents in all buckets divided by the preoperative haemoglobin concentration in blood.

The postoperative blood loss was calculated by estimating the haemoglobin mass, which is the product of haemoglobin concentration and blood volume. The formula of Nadler et al [214] was used for calculating the blood volume; blood volume (L) = $\text{body height}^3 \text{ (m)} \times 0.3669 + \text{body weight (kg)} \times 0.03219 + 0.6041$.

The preoperative Hb mass plus transfused Hb mass minus intraoperative blood loss was compared to the postoperative Hb mass, which was calculated from the haemoglobin concentration on the day of discharge. The difference should correspond to the postoperative blood loss.

If the patient had transfusions of red packed cells the Hb mass was calculated based on a mean volume of 288 ml per transfused unit with a haemoglobin concentration of 198 g/L corresponding to a Hb mass of 57 g.

Withdrawal of medication before surgery

When to withdraw aspirin before surgery is a subject of debate in Sweden [216] and in other countries [217]. A study in healthy volunteers showed that the defect in primary haemostasis (changes in bleeding time) had largely disappeared 48 h after the last dose [218]. Early initiation of aspirin after urological surgery did not appear to carry an increased risk of postoperative bleeding [219]. Discontinuation of aspirin before invasive therapy or surgery is also related to higher risk of major cardiovascular events in cardiac patient [220]. ACCP guidelines recommend withdrawal 7 to 10 days before surgery, but for patients at high risk for cardiac events continuing aspirin up to and beyond the time of surgery is suggested [221].

Other medications that could possible lead to a higher bleeding risk are non-steroidal anti-inflammatory drugs (NSAID), selective serotonin reuptake inhibitors (SSRI) and medication from nature like Omega-3-fatty acids from fish-oil, Ginkgo biloba and garlic, which could affect platelet aggregation and bleeding time [222].

ANALYSIS OF FIBRINOLYTIC PARAMETERS IN THE STUDY

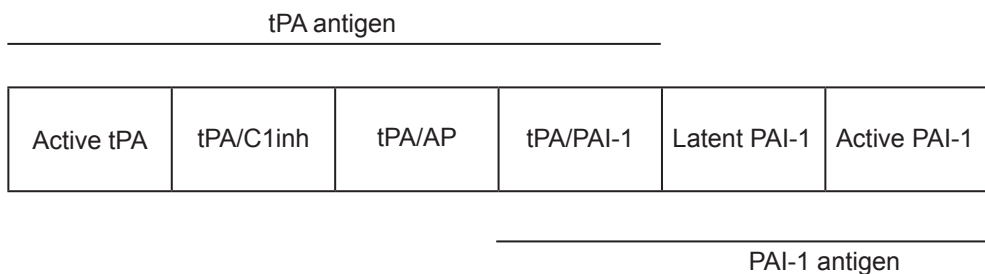


Figure 5. A schematic picture on what is measured with tPA antigen and PAI-1 antigen methods. C1inh= C1 inhibitor; AP=antiplasmin. (courtesy of B. Wiman, *Klinisk Biokemi i Norden* 2008).

Method of determination of PAI-1 activity

The PAI-1 sample should be taken between 7 and 10 a.m. on a fasting stomach or after a fat-free breakfast and after at least 15 minutes of rest. The timing is necessary to standardise the activity according to the diurnal variation and the peak activity of PAI-1. Furthermore, it is crucial to avoid sampling from patients with acute infection or during pregnancy, when PAI-1 activity is increased. Nine volumes of blood are collected in tubes with one volume of 0.129 M trisodium citrate. The tubes are centrifuged within 30 minutes at 2000 g for 15 min after which plasma is separated, immediately frozen and kept at -70 °C until analysis.

We chose to use Chromolize™ PAI-1 kit from Biopool, since it was the routine method at Karolinska University Hospital, used mainly for investigation of thrombosis patients and for research concerning venous and arterial thrombosis.

Chromolize™ PAI-1 kit from Biopool is an immunoactivity assay. Active tPA is lyophilized while immobilised on the surface of the microtiter plate well. After reconstitution plasma

samples containing PAI-1 are added and active PAI-1 reacts with tPA. Simultaneously, a horseradish-peroxidase (HRP)-conjugated monoclonal antibody to PAI-1 is added. After incubation, unbound sample and conjugated antibody are washed away and the bound PAI-1 is quantified with an HRP-sensitive substrate. The amount of colour developed is directly proportional to the concentration of active PAI-1 in the sample.

The manufacturer states that the analysis of PAI-1 activity has a detection range of 2.0–50 U/mL. Our local laboratory has so far provided a reference range of <15 U/mL. In order to improve the precision at low PAI-1 levels, more measuring points were added to the low end of the calibration curve, which was read using a point-to-point approach instead of linear regression. An in-house control with a PAI-1 activity of 0.9 U/mL was also added. The intra-assay coefficient of variation (Cv) was 11.5% for the low control (n = 108) and 5.7% for the high control (PAI-1 19.4 U/mL; n = 106), and the inter-assay Cv was 4.5% and 3.6%, respectively.

After log-transformation the PAI-1 levels attained a normal distribution. In the PAI-1 analysis, the values of absorbance at <1 U/mL did not differ significantly from the 0-line, and thus it was not meaningful to set the lower limit of normal below 1.0 U/mL. PAI-1 activity less than 1 U/mL was considered as low PAI-1 activity.

PAI-1 is labile and at pH 7.3 and 37° C in plasma, conversion to latent PAI-1 will occur with a half-life of 4 hours. In addition loss of activity will occur at every thawing/freezing cycle. It is therefore very important that the preanalytical conditions are optimal.

Other PAI-1 activity methods

Zymutest (Aniara©) PAI-1 activity. An ELISA plate is coated with active tPA, which binds active PAI-1 present in plasma or tested sample. This is then revealed with a specific PAI-1 murine monoclonal antibody and labelled with HRP. Detection threshold is ≤ 0.1 ng/mL.

Spectrolyse® PAI-1 (American Diagnostica Inc.) is a two-stage, indirect assay for measuring PAI-1 levels. The lower limit of detection has been determined to be 4.6 U/mL.

Method of determination of PAI-1 antigen

TintElize® PAI-1 test from Biopool is based on a double antibody principle with a coat antibody. One type of the microtest well contains monoclonal antibodies against PAI-1 immobilized on the well surface and soluble antibodies against PAI-1. The other type of well also contains monoclonal antibodies against PAI-1 immobilized on the well, but with soluble non-immune antibodies. HRP-conjugated PAI-1 antibodies are added and will also bind to PAI-1. This forms a sandwich of 1) coat antibody, 2) PAI-1 and 3) HRP-conjugated antibody. A chromogenic substrate is then added and converted to a yellow coloured product at a rate proportional to the amount of HRP bound. The difference between the wells is the specific PAI-1 response.

Normal values of PAI-1 antigen in human platelet-poor plasma have been reported in the range 4-43 $\mu\text{g/L}$ (18 ± 10 $\mu\text{g/L}$ with a correlation to PAI-1 activity of $r = 80$) [223]. In serum, when the platelets have been activated the concentration of PAI-1 is 100-300 $\mu\text{g/L}$, which reflects the release of PAI-1 from activated platelets [54].

For comparison, the immunochemical assays measure active PAI-1, latent PAI-1 and PAI-1 in complex with tPA or uPA (figure 5). The preanalytical conditions have to be very precise to avoid that latent PAI-1 can leak from the platelets in case of inadequate anticoagulation in the tube, in which the sample is collected [224].

Method of determination of tPA-PAI-1 complex

TintElize® tPA-PAI-1 from Biopool is an enzyme immunoassay. Polyclonal antibodies against human tPA are lyophilised while immobilised on the surface of the microtest well wall. After reconstitution, plasma samples containing tPA-PAI-1 are added and the complex reacts with the immobilised antibodies. Simultaneously an HRP-conjugated monoclonal antibody to PAI-1 is added. After incubation, unbound sample and antibody are washed away and the bound tPA-PAI-1 complex quantified with an HRP-sensitive substrate. The amount of colour developed is directly proportional to the concentration of active tPA-PAI-1 complex in the sample.

The manufacturer states that plasma concentration of tPA-PAI-1 complex in citrate samples from 40 healthy blood donors was found to be in the range of 0.6-6.7 µg/L (2.5 ± 1.5 µg/L).

Determination of plasmin-antiplasmin complex

Determination of PAP was performed by a classical two-site enzyme-linked immunosorbent assay. The microtiter wells were coated with a goat antibody raised against PAP and were suitably adsorbed. The antibodies were reacting towards a neoantigen in the antiplasmin moiety of the complex and the affinity was about 200 times higher for the complex as compared with the free antiplasmin [225]. The measuring antibody was an HRP-conjugated goat antiplasminogen immunoglobulin G. Purified PAP was used as a standard (0–12 mg/L). By diluting the plasma samples 1/200 in phosphate–EDTA–Tween buffer (0.04 mmol/L sodium phosphate buffer, pH 7.3, containing 0.1 mol/L sodium chloride, 0.005 mol/l EDTA and 0.05% Tween 20), the influence on the assay of free antiplasmin present in plasma was negligible. The minimal detectable concentration using this procedure is about 0.1 mg/L. The accuracy measured as the recovery of pure PAP added to several different plasma samples was close to 100%. The coefficient of variation, measured at 1.5 mg/L, was less than 10%. The reference range, based on a large control sample from another study, is approximately 1–3 mg/L (unpublished data).

Determination of fibrin D-dimer

Fibrin D-dimer was analysed in all patients using a commercially available kit (TintElize D-dimer; Biopool) with a detection range from 0 to 1000 µg/L.

One of the end products of fibrin is the degradation product D-dimer, but this test could reflect activation of the coagulation and fibrin formation rather than the fibrinolytic capacity [38]. Usually fibrin formation results in an increased D-dimer level, and the analysis is used as a part of the investigation procedure in the diagnosis of deep venous thrombosis.

tPA, uPA and tPA-PAI-1 complex

tPA tests immunologically and functionally are very different. Active tPA is only a small part of the total tPA antigen present in plasma. tPA easily forms complex with inhibitors

such as PAI-1, antiplasmin and C1-inhibitor [226]. Increased tPA antigen in plasma could therefore reflect an inhibition of the fibrinolytic activity, rather than an increased fibrinolysis. The correlation between tPA antigen and PAI-1 activity is quite good and high levels of tPA antigen can indicate a risk for thrombosis [57]. Assessment of tPA activity requires caution against possible inactivation when handling the sample, which should be taken in acid buffer vacuum tubes. tPA activity is inversely correlated to PAI-1 activity. However there is no correlation between tPA activity and tPA antigen. tPA antigen correlates better to PAI-1 activity [10].

Active tPA has been given intravenously to healthy volunteers, who were recruited according to their PAI-1 levels. tPA antigen was rapidly cleared from the circulation; in subjects with low PAI-1 $t_{1/2}$ was 3.5 ± 0.7 min and in objects with high PAI-1 $t_{1/2}$ was 5.3 ± 0.9 min and the clearance was faster for active tPA than tPA-PAI-1 complex. In patients with high PAI-1 more active tPA was converted to tPA-PAI-1 complex and this was slowing the clearance [52].

For detection of uPA in plasma, an ELISA method is typically used. Determination of uPA-antigen has mainly been used in extracts of solid tumour and not for detection of uPA in plasma [181].

Other tests of fibrinolysis

Euglobin clot lysis time, fibrin plate clot lysis and whole blood clot lysis have been used historically for global evaluation of fibrinolysis, but these tests are laborious and difficult to automate. The relation between euglobin clot lysis time (ELT) and tPA and PAI-1, has showed positive correlation and the data suggested that PAI-1 has a strong influence on the ELT [227, 228].

Global coagulation methods like overall haemostatic potential (OHP) and overall fibrinolytic potential (OFP) have been developed by He et al [229] and have been used in evaluation of TAFI generation [230] or for assessing patients with hypercoagulable states [231].

Another global method is thromboelastography (TEG or ROTEM) and it has been used in a model of systemic activation of fibrinolysis and coagulation in healthy volunteers who were randomised to endotoxin or saline infusions [232].

The importance of measuring TAFI in clinical studies has been controversial. One recent study could not find any association between increased TAFI and absolute risk for venous and arterial thrombosis in thrombophilic families [233].

Analysis of 4G/5G polymorphism

The analyses of 4G/5G polymorphism were done by pyrosequencing technique [249].

Storage of frozen samples

Earlier findings have documented a longitudinal stability in the biochemical properties of frozen plasma in coagulation, fibrinolytic and inflammatory parameters, when the plasma had been frozen at -70° C, for 7-59 months [234].

STATISTICAL ANALYSIS

The skewed distribution of PAI-1 activity levels was normalised after log-transformation. Student's *t*-test (two-tailed) for equal variances was used. Adjusted odds ratios were calculated by logistic function regression.

Binomial or dichotomised data were analysed with chi-squared test with Yate's correction. In the TURP-study binomial data were analysed with Fisher's exact test.

Relations between continuous data were assessed by linear regression analysis or Mann-Whitney for skewed distribution. Distribution was characterised by standard deviation, if normal distribution or inter-quartile range (IQR) if appropriate.

A *P* value less than 0.05 was considered statistically significant.

Confounding

The issue of confounding and independent versus dependent variables was often discussed during the analysis of the results from our studies, particularly Study IV. In the case of PAI-1 this dilemma is probably more complex than in many other situations. Although the reviewers of the submitted manuscripts agreed with our statements and conclusions in this respect, it is still worth pondering over the relationships.

Confounding can be caused by variables that are associated with both outcome and exposure and are not on the causal pathway between exposure and outcome [235, 236]. There are different concepts of relationships of variables in research; confounding, mediation and effect modification (synonyms: interaction or moderation). While an exposure may cause disease it is also possible that exposure and disease may be related if both variables are caused by another factor and this factor is called the confounder. A confounder is a true cause of a disease, while bias is an artefact. A mediator is also associated with the exposure and the outcome, but is part of the causal chain. A confounder and a mediator cannot be distinguished on statistical ground; on the contrary they can only be separated from each other based on an understanding of the total disease process.

An effect modifier is a variable that moderates or modifies the way in which the exposure and the disease are related. When an exposure has different effects on the disease at different values of a variable, that variable is called an effect modifier. That means for instance that the exposure could have different effects in men and in women.

It has been suggested that PAI-1 represents a possible causal link in the development of obesity [237] due to the fact that PAI-1 is synthesised by adipose tissue, that plasma levels are increased in obesity and reduced with weight loss. Administration of tiplaxtinin (PAI-039), an orally active synthetic inhibitor of PAI-1, has resulted in weight reduction in a diet-induced obesity mouse model [79]. Infusion of insulin in healthy volunteers resulted in increased levels of PAI-1 within hours [149].

If there is a causal link between PAI-1 and obesity, it is also possible that there could be a causal link between PAI-1 and C-peptide and proinsulin, since these substances are closely

related to insulin resistance in the metabolic syndrome, which is associated with overweight. If obesity is a mediator and part of the pathway between PAI-1 and C-peptide, we should not consider obesity as a confounder, since this could in fact lead to a risk for misinterpretation of the study results. Therefore it may be argued that BMI is not a confounder and that we should not adjust for this parameter in our study.

Power calculation for study II

In the initial sample size calculation we considered the prevalence of low PAI-1 activity to be 5%, based on preliminary data from the epidemiological study (study I). We estimated the incidence of bleeding complications at 10%, based on literature data ranging from 5% to 7% for transfusion, readmission or re-operation due to bleeding and additional 5% for the intra- and postoperative blood loss measured as described above. With an alpha of 0.05 and a power of 80% we would need 150 patients to exclude that low PAI-1 is not a cause of at least half of the bleeding complications. Assuming a quantitative, inverse relationship between PAI-1 activity and blood loss, we planned to also use linear regression analysis for this correlation and to reduce the number of patients needed. A relocation of the TURP procedure to another hospital and a concomitant change of surgical technique occurred in 2005 and obviated further inclusion of patients to reach the planned number.

ETHICAL CONSIDERATIONS

The studies were approved by the Ethics Committee at Karolinska University Hospital, and all patients and normal controls gave oral informed consent after having read the consent form, which was specifically designed for each population group.

All patients had initially provided consent for the investigation of several fibrinolytic parameters. Study IV with the additional analyses was subsequently approved by the Ethics Committee of the Karolinska University Hospital.

RESULTS AND DISCUSSIONS

EVALUATION OF LOW PAI- 1 ACTIVITY AS A RISK FACTOR FOR HAEMORRHAGIC DIATHESIS (STUDY I)

Results

The main reasons for referral for investigation of bleeding tendency were perioperative bleeding complication (25%), multiple bleeding symptoms (21%) and menorrhagia (20%). Other reasons were subcutaneous haematomas (13%), peripartum bleeding complications (5%) and bleeding complications after tooth extraction (2.5%) and to a smaller degree (below 1.5% for each) intracranial bleeding, abnormal haemostatic screening test, gingival bleeding, gastro-intestinal bleeding, haematuria, haemarthrosis, muscle haematoma, abnormal bleeding during antihemostatic therapy, liver disease, family investigation, posttraumatic bleeding complication, bleeding after venipuncture, petechiae, haemoptysis, renal disease, bleeding into cyst, subconjunctival bleeding, renal haematoma, haematospermia and iron deficiency.

Clinically significant bleeding, fulfilling our criteria, had occurred in 75% of the patients and in 18% of the healthy controls.

In the routine investigation of bleeding tendency an abnormal result was found in 43% of the patients and there was a normal investigation in 57%. Among the patients with abnormal investigation there were 143 patients with a platelet function defect (57 %), 59 patients with VWD (24%), 14 with capillary fragility (6%), 7 with thrombocytopenia (3%), 7 with carriership of haemophilia A (3%), 5 with factor VII-deficiency (2%), 4 with factor XI-deficiency (1.6%), 3 with hypofibrinogenaemia (1.2%), 2 with isolated prolonged APTT (0.8%) and one of each with mild haemophilia A, factor V-deficiency, prothrombin deficiency, deficiency of all vitamin K-dependent coagulation factors, Ehlers-Danlos syndrome and Osler-Rendu-Weber's syndrome.

PAI-1 levels

The PAI-1 levels were skewed and obtained a normal distribution after log-transformation (figure 6). In the improved PAI-1 analysis for the lower levels, the values of absorbance at <1 U/mL did not differ significantly from the 0-line and therefore the limit of low PAI-1 was set to <1 U/mL.

Among the 586 patients 23% had PAI-1 levels below 1 U/mL. In the control group with blood

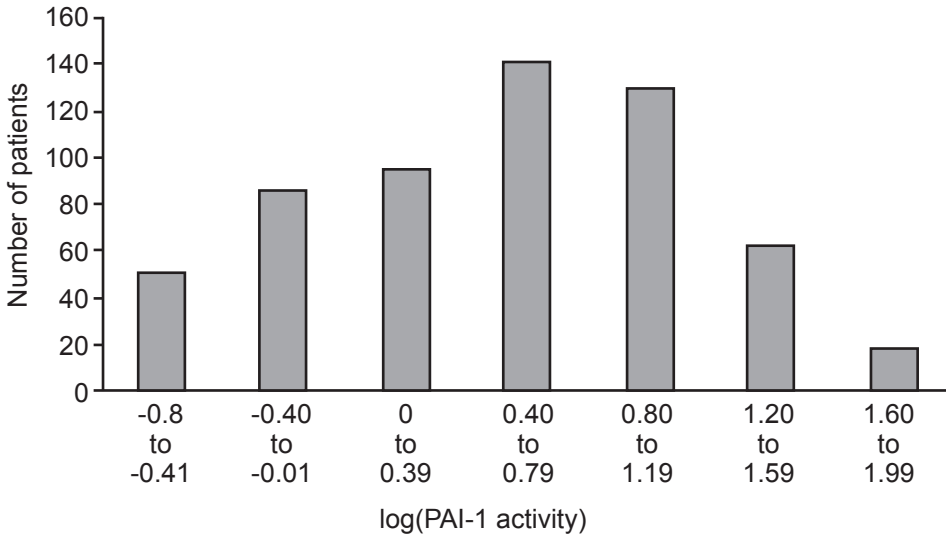


Fig 6. PAI-1 activity - distribution in patients.

donors 13% had PAI-1 levels <1 U/mL and among the healthy controls the proportion was 10%. The odds ratio (OR) for a PAI-1 level of <1 U/mL in the patients compared with the control group of blood donors was 2.04 (95% confidence interval [CI], 1.11-3.77) and compared with the healthy controls it was 2.75 (95% CI, 1.39-5.42). After adjustments for sex, age, BMI and use of oestrogens the OR was 3.23 (95% CI, 1.22-8.56) for a low PAI-1 activity among patients compared with healthy controls. Due to the above-mentioned lack of information such adjustments were not possible for the blood donor population (figure 7).

Females had a higher proportion of PAI-1 levels below 1 U/mL than males in all populations. There was a statistically significant difference in the proportion of females with low PAI-1 between healthy controls (11%; 95% CI, 4.3-17.7) and patients (25%; 95% CI 21.2-28.8) ($P=0.007$). The absolute number of males in these groups was small and did not provide the

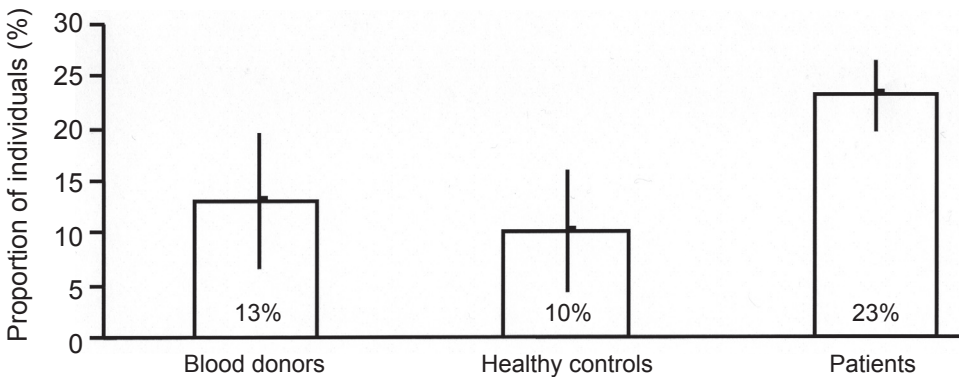


Fig 7. Low PAI-1 activity per population.

power for assessment of difference in the proportion of low PAI-1 levels. In the women who were on oral contraceptives the PAI-1 levels were lower (median 1.25 U/mL in patients and 1.6 U/mL in healthy controls) than among the women without exogenous oestrogens (median 4 U/mL in patients and 3.35 U/mL in healthy controls). The median PAI-1 level was slightly higher in all populations over 43 years of age compared to those younger than 43 years of age. This effect seemed to be related to the effect of oral contraceptives in younger ages but was only significant in patients ($P= 0.002$) (figure 8).

There was an association between BMI and PAI-1 levels (in blood donors, coefficient of determination $R^2= 0.2$, $P<0.001$). In the 586 patients 14 had BMI over 25 and PAI-1 levels < 1 U/mL but none with BMI >25 in the two control groups had low PAI-1. The BMI did not affect the differences seen in the females on exogenous oestrogens.

The total number of patients with low PAI-1 activity was 137 (23 %) and out of these 97 (71 %) had a clinically significant bleeding tendency, according to preset criteria. Among the patients with normal PAI-1 levels, $n = 449$, 77% had clinically significant bleeding tendency. Comparison of PAI-1 levels and the proportion of clinically significant bleeding tendency according to the final diagnosis showed that 73% of those with low PAI-1 and otherwise normal investigation had clinically significant bleeding versus 80% of those with $\text{PAI-1} \geq 1$ U/mL and no other diagnosis, 78% in those with platelet function defect, 58% with platelet function defect and low PAI-1, 80% in VWD, and 79% in VWD and low PAI-1 (not statistically significant differences).

When listing the bleeding symptoms separately (postoperative bleeding, bleeding after tooth extraction, peri- or postpartum bleeding, subcutaneous haematoma, menorrhagia, gingival bleeding, epistaxis, gastro-intestinal bleeding, haematuria, abnormal post-traumatic bleeding, muscle haematoma and haemarthrosis) the prevalence of most bleeding manifestations was slightly lower with a low PAI-1 activity alone than combined with a defined haemostatic defect and there was no significant difference between bleeding manifestations in those with

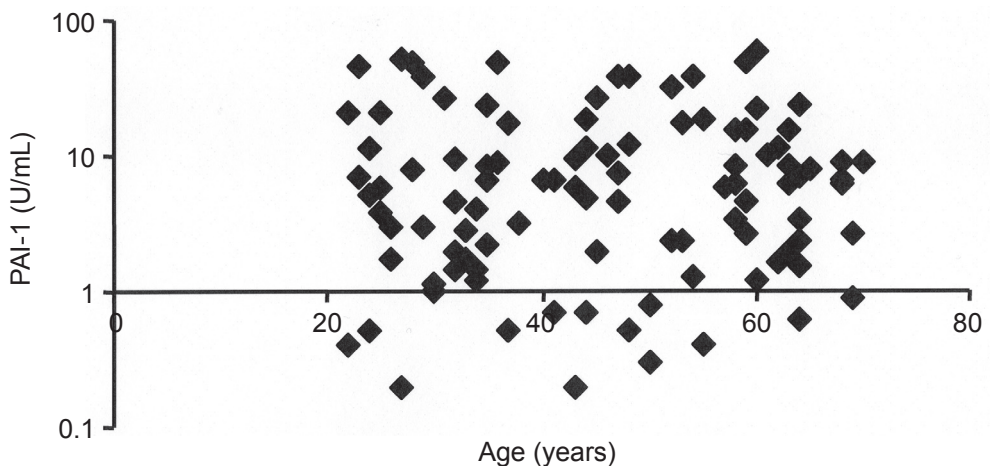


Fig 8. PAI-1 activity according to age in 100 blood donors.

low or normal/high PAI-1 levels and otherwise negative investigation. The only exception was a higher risk of bleeding complication after delivery in patients with low PAI-1 than those with normal PAI-1 in otherwise negative investigation ($P=0.02$).

In the healthy control population 18 (18%) had a history of clinically significant bleeding and 3 (17%) of these had low PAI-1 compared with 7 (8.5%) of 82 without such history (no statistically significant differences) (table 2).

Table 2. PAI-1 levels according to demographic characteristics

Characteristic	Blood donors		Healthy controls		Referred patients	
	median	%<1 U/mL	median	%<1 U/mL	median	%<1 U/mL
N	100		100		586	
Sex						
Males	7.3	6%	9.0	6%	4.8	15%
Females	3.6	20%	2.8	11%	3.6	25%
Body mass index						
<25	3.1	24%	2.4	14%	3.2	25%
≥25	8.9	0%	7.0	0%	9.0	13%
Age						
<43	4.8	12%	2.7	13%	3.0	26%
≥43	6.4	14%	3.8	7%	5.0	20%

tPA-PAI-complex

There was a positive correlation between tPA-PAI-1 levels and PAI-1 activity ($R^2=0.71$, $P<0.001$). The tPA-PAI-1 antigen levels were similar in patients and blood donors with a median tPA-PAI-1 level in patients of 2.6 ng/mL (range 0.02-27.8) and 2.6 ng/mL (range 0-14.3) in blood donors. There was no association between low tPA-PAI-1 levels and increased bleeding tendency (table 3).

Table 3. tPA•PAI-1 levels in ng/mL according to demographic characteristics

Characteristic	Blood donors	Referred patients
N	100	512
Median (range)	2.6 (0-14.3)	2.6 (0.02-27.8)
Males, median	3.4	4.3
Females, median	2.4	2.5
Age <43, median	2.3	1.9
Age ≥43, median	3.6	4.2
BMI <25, median	2.1	1.3
BMI ≥25, median	4.1	8.4

PAI-1 antigen

In 52 patients with low PAI-1 activity (<1 U/mL) PAI-1 antigen was analysed in serum. The concentration was 199 ± 71 ng/mL (range 62-362 ng/mL). In one patient there was no measurable PAI-1 antigen in serum. This patient also had thrombocytopenia, mild factor V deficiency and suspected Wiscott-Aldrich syndrome.

4G/5G polymorphism

Analysis of the 4G/5G polymorphism was performed in 133 patients with low PAI-1 and in 48 healthy controls. The results were compared with results from 1614 controls from another study- the SHEEP study [166]. Homozygous form of 4G was seen in 24.8% of the patients, 27.1 % of the healthy controls and 28.6 % of the SHEEP-controls. Homozygous form of 5G was found in 21.1 % of the patients, 27.1 % of the healthy controls and 20.4 % of the SHEEP-controls. There was no statistical difference.

Discussion

In the earlier case studies it had been reported that PAI-1 deficiency is associated with bleeding tendency especially after surgery and trauma and in two of the studies mutations in the PAI-1 gene were found. No systematic studies have been performed earlier.

We found that low PAI-1 activity, below 1 U/mL, is more common among 586 patients with bleeding tendency (23%), than in two control groups of 100 people each (13 and 10% respectively).

One of the difficulties with defining low PAI-1 activity has been that the commercially available reagent kit, has been designed primarily for detection of normal or high PAI-1 levels, as part of assessment of thrombotic risk. The lower reference limit in the commercially available kit (Chromolize™ PAI-1, Biopool, Umeå, Sweden) was set to 2 U/mL, whereas values below this limit were considered uncertain.

In our study we were able to improve the low detection limit from 2 U/mL to 1 U/ml with adding more reference points at the lower end of the calibration curve, using a point-to-point approach instead of linear regression and adding a low in-house control.

Still we could not differ between the patients that may have had a true zero level of PAI-1, from those with for example 0.5 U/ml. It is possible that patients with low PAI-1 levels, but above the zero-line, can increase their PAI-1 levels when necessary, for instance in inflammatory diseases or surgery. To further investigate if there were patients with no PAI-1 production, we performed PAI-1 antigen analyses of serum, where PAI-1 from the platelets is released. This analysis was made in 52 patients with PAI-1 activity below 1 U/mL. Only one of these 52 patients had an unmeasurable PAI-1 antigen level, and this patient was found to have a low platelet count, which made it difficult to draw any conclusion.

It is known that PAI-1 activity can be affected by the 4G/5G polymorphism, but among the patients with low PAI-1 activity there was no increase in the proportion of 5G/5G, which has been associated with lower PAI-1 levels in thrombotic studies, compared with our control populations.

One possible reason for low PAI-1 activity is that most of PAI-1 could be bound to tPA in the tPA-PAI-1 complex, but we found a positive correlation between PAI-1 activity and tPA-PAI-1 complex ($R^2 = 0.71$, $P < 0.001$) and therefore low PAI-1 activity was instead associated with low tPA-PAI-1 complex. We could not find any association between the levels of tPA-PAI-1 complex and bleeding tendency, which could be the case if there were unbound tPA, which we did not measure.

The bleeding tendency with different bleeding manifestations was carefully examined in the patients and the healthy controls. We could not find any typical feature of bleeding among the patients with low PAI-1 activity except that the risk of bleeding complication after delivery was higher and often was the only abnormality in the investigation (32%), compared to a completely negative investigation (21%). Due to a large number of comparisons this may be spurious. In normal pregnancy PAI-1 levels increase above the upper reference limit (>15 U/mL) and we have also seen the same pattern in ten patients with previously diagnosed low PAI-1 activity, who were followed during pregnancy (unpublished data). In normal women after child birth PAI-1 decreases to non-pregnant levels within a day after delivery, which means that post-partum haemorrhage due to PAI-1 deficiency, could be expected.

The bleeding tendency for patients with low PAI-1 activity in combination with other haemostatic defects did not worsen compared to patients with the same haemostatic defect but without PAI-1 deficiency.

The bleeding pattern was classified as clinically significant or not. We had a control group with blood donors, but we were not allowed to interview them regarding their bleeding history, since they were anonymous. Blood donors are not rejected because of a bleeding propensity as long as their haemoglobin value is within acceptable limits. We decided then to recruit another control group, with sex and age matched individuals, who filled out a standard questionnaire on their bleeding history. In this control group 18% fulfilled the criteria for clinically significant bleeding. In these healthy controls there was a non-significant difference in this type of bleeding among those with low PAI-1 activity (17%) versus those with higher activity (8.5%) (OR 2.1, 95% CI 0.5-9.2).

Some of the patients were young and had not yet experienced tooth extractions, pregnancy or surgery, why the risk of post-operative haemorrhage could not be fully evaluated.

PAI-1 activity is affected by many factors. There is a diurnal variation with the highest levels in the morning and our samples were therefore taken in the morning between 7 and 10 am. Infections or inflammation may also affect the levels and in patients or controls with an infectious disease the sampling was postponed until they had recovered. Exogenous oestrogens or pregnancy would increase the PAI-1 levels and such medications or condition were registered. Metabolic syndrome and obesity increase PAI-1 levels and therefore data on height and weight were collected. We did not measure waist-hip ratio since we were not aware of the possible influence on PAI-1 levels at this point. The 4G/5G polymorphism was examined in the study. The healthy controls were matched for age, gender and they had a similar proportion of females on oestrogen therapy and a similar BMI.

In our study there was a high proportion of women (83.4%) why any conclusion for populations with a higher proportion of males may be uncertain. Subsequent to the publication of Study I a study on PAI-1 in a Spanish blood donor population was published[189]. They also found a high prevalence of low PAI-1 activity, since 21% of the 66 donors had <2 IU/mL, but they questioned any association between low PAI-1 activity and bleeding diathesis. The small sample size and the same inability to obtain a full medical history from the blood donors as in our study are obvious limitations.

LABORATORY EVIDENCE OF HYPERFIBRINOLYSIS IN ASSOCIATION WITH LOW PLASMINOGEN ACTIVATOR INHIBITOR TYPE 1 ACTIVITY (STUDY III)

Results

The patient population consisted of 350 (83 %) women and 74 (17%) men. The median age was 41 years (range 18-81 years).

There was a weak negative correlation between the PAI-1 activity and the PAP-level ($r = -0.16$, 95% CI, -0.25 to -0.06; two tailed P value 0.0011). When the PAI-1 value was dichotomised as low or normal/high values with the cut-off PAI-1 level at <1 U/mL the effect on PAP was more pronounced. In patients with low PAI-1 the median PAP-level was 1.73 mg/L with IQR 1.53-2.30 mg/L. In patients with normal or high PAI-1 levels the median PAP-level was 1.54 with IQR 1.37-1.83 mg/L. The difference was highly significant with a P value of < 0.0001. The best cut-off value for PAI-1 in the correlation with PAP levels was at PAI-1 of 1.2 U/mL, which is close to our previous definition of low PAI-1 activity below 1.0 U/mL.

There was no statistically significant association between the PAI-1 activity and the D-dimer level.

No correlation was observed between PAP and age, sex or clinically significant bleeding, but there was a weak negative correlation between PAP and BMI ($R^2 = 0.047$) when analyses were made on the whole population ($n = 424$). In the patients with low PAI-1 activity ($n = 95$) there was also a weak negative correlation between PAP and BMI ($R^2 = 0.07$) and no correlation between PAP and age or history of clinically significant bleeding.

Discussion

We found that low PAI-1 activity is associated with higher levels of plasmin formation measured by PAP, but not with increased formation of fibrin degradation products measured as D-dimer. This suggests that plasmin activation occurs without fibrin formation. This could support the concept that patients with low PAI-1 activity have increased bleeding risk, due to the fact that there is not enough PAI-1 to suppress the tPA activity, which stimulates the formation of plasmin. However, we could not see any association between PAP-levels and the severity of bleeding tendency in patients with low PAI-1 activity, but in this group of 424 patients (from originally 586) 26% had not yet experienced haemostatic challenges like surgery and 31 % of the patients had not undergone tooth extraction. Both surgery and tooth extraction are the kind of occasions where PAI-1 deficient patients typically have presented with bleeding complications in earlier case studies [22].

Patients with normal PAI-1 activity have the ability to induce a higher production of PAI-1 at surgery, in inflammatory or infectious diseases. In patients with low PAI-1 activity, perhaps this reaction is not fast enough to prevent excessive fibrinolysis in the post-operative and post-partum period and this could result in a higher risk for bleeding complications. There have been several studies in hip and knee surgery [238], where the use of the fibrinolytic inhibitor tranexamic acid has reduced the need for allogenic blood transfusions. In some hospitals it is a routine to give a single dose of tranexamic acid at hip or knee surgery. Tranexamic acid in a high single dose is also used before cardiopulmonary surgery. Theoretically the positive effect of tranexamic acid could be even more pronounced in patients with low PAI-1 activity.

LOW PAI-1 ACTIVITY IN RELATION TO INFLAMMATORY PARAMETERS, INSULIN PROFILE AND BODY MASS INDEX (STUDY IV)

Results

The patient population consisted of 155 (86 %) women and 26 (14 %) men. The median age was 42 years (range 18-80 years). In the univariate analysis, patients with low PAI-1 activity were significantly younger and had a lower BMI and a higher proportion of the females used oral oestrogens. In the group with low PAI-1 activity ($n=63$) 21 patients used combined oral contraceptives and 3 patients had hormone replacement therapy. In the group with normal/high PAI-1 activity ($n=118$) 8 patients had oral contraceptives and 7 patients used hormone replacement therapy.

In linear regression analysis there was an association between PAI-1 activity and C-peptide, proinsulin ($P < 0.001$ for both) or IL-6 ($P = 0.03$) but not with hs-CRP. When the PAI-1 values were dichotomised to low versus normal/high values, there was a significant association with C-peptide and a trend for association with IL-6.

However, after adjustments for BMI, age and the use of oral oestrogens as possible confounders, none of the associations remained significant. BMI had the strongest relationship with C-peptide, proinsulin and hs-CRP. Age was the variable with the strongest relationship with IL-6, but age had no influence on the PAI-1 activity among patients without oral oestrogens.

Low BMI remained the most important determinant of low PAI-1 activity.

Discussion

This study was designed as an attempt to explain the regulation of low PAI-1 activity. We have earlier, in study I, analysed the polymorphism 4G/5G without finding any differences among the patients with low PAI-1 compared to normal population. Since high PAI-1 levels have been associated with metabolic syndrome, obesity and inflammatory or infectious diseases, it could be speculated that patients with low PAI-1 activity have a lower level of inflammatory or metabolic response. Tissue expression of PAI-1 is essentially inducible and not constitutive. Main inducers seem to be mediators of oxidative stress and inflammation.

Exogenous oestrogens have in contrast to inflammation and metabolic syndrome, a lowering

influence on PAI-1 levels. Pregnancy increases PAI-1 activity, but patients with known pregnancy were excluded from the study.

As markers for inflammation we decided to analyse IL-6 and hs-CRP. IL-6 is produced at the site of inflammation and mediates the acute phase reaction [239]. CRP is produced by the liver and is part of the acute phase reaction and changes rapidly with inflammatory state. High-sensitivity CRP is more suitable for detection of levels in the lower range and this could be of importance since cardiovascular studies have found that hs-CRP levels of 1.0-3.0 mg/L indicate an average risk for cardiovascular events and values above 3 mg/L a high risk [240]. Since high levels of PAI-1 and CRP are associated with increased risk for cardiovascular events, it could be expected that low PAI-1 activity and low CRP are associated with each other.

As markers for the metabolic syndrome we decided to analyse C-peptide and proinsulin. Insulin is formed from proinsulin, which is secreted by beta-cells in the pancreas. Proinsulin is cleaved by proteases to insulin and C-peptide in equal amounts [241]. Since insulin rapidly is cleared from the circulation and C-peptide is eliminated more slowly it is possible to analyse the amount of C-peptide as a measure of the production of insulin [241]. C-peptide provides a better estimate of the synthesis of insulin, as insulin but not C-peptide is rapidly metabolised in the liver [242]. Studies on patients with coronary artery disease indicate that C-peptide has a stronger correlation than insulin with high PAI-1 [115]. In patients with non-insulin dependent diabetes mellitus elevated PAI-1 is better correlated with proinsulin than with insulin [243].

Only plasma samples from 63 of initially 137 patients with low PAI-1 activity (<1 U/ml) were still available, but this was most likely a random selection, since the proportion of females was identical and the age was quite similar. When the analyses were done, PAI-1 activity appeared, in linear regression, to be associated with C-peptide and proinsulin and to a minor degree also with IL-6 but not at all with hs-CRP. After further adjustments for possible confounders as BMI, age and use of oral oestrogens, the associations disappeared. A low BMI seemed to be the strongest factor influencing low PAI-1 activity and use of oral oestrogens was an additional explanation for some of the cases. Age had no influence on the PAI-1 activity among patients without oral oestrogens.

The strong influence of BMI on PAI-1 level is congruent with the results in an earlier study where the predictive effect of elevated PAI-1 on the occurrence of myocardial infarction disappeared after adjustment for other components of the metabolic syndrome [244].

Still these results do not exclude that there is an direct influence by fat tissue on PAI-1 levels as well as on the C-peptide. Adipocytes are known to produce PAI-1 [245], the metabolic syndrome is associated with insulin resistance and high levels of C-peptide [113] and PAI-1 levels increase in healthy volunteers after insulin infusion [149]. If there is a direct influence on PAI-1 and on the mechanisms of insulin resistance, it could be a mistake to consider BMI as a confounder in this aspect.

LOW PAI-1 ACTIVITY IN RELATION TO THE RISK FOR PERIOPERATIVE BLEEDING COMPLICATIONS IN TRANSURETHRAL RESECTION OF THE PROSTATE (STUDY II)

Results

82 patients were screened for inclusion in the study, but seven of these were not eligible due to exclusion criteria. Thus 75 patients were included and blood samples were taken for analysis of PAI-1, but 13 of these 75 patients were not evaluable due to change of surgical procedure, diagnosed cancer before surgery or they did not have surgery at all.

The evaluable patients were finally 62 and of these 4 had low PAI-1 activity (<1 U/mL). The baseline characteristics for the patients with low or normal/high PAI-1 activity showed no statistical differences. The mean tPA-PAI-1 complex was 2.35 µg/L in the patients with low PAI-1 activity and 6.12 µg/L in patients with normal/high PAI-1 activity ($P = 0.05$) and the mean PAI-1 antigen level was 8.4 µg/L and 21.9 µg/L respectively ($P=0.01$). In linear regression analysis there was no correlation between intra- or postoperative blood loss and PAI-1 activity ($R^2 = 0.0$).

Bleeding complications, fulfilling the predefined criteria, occurred in 19 (31 %) of the patients. There was a statistical trend to more bleeding complications among the patients with low PAI-1 activity (OR 7.9, 95% CI 0.76-81.2, $P = 0.082$). After adjustments for resection time, resected amount of prostate tissue and systolic blood pressure, which are all known variables that correlate with increased bleeding, the risk for bleeding complications in patients with low PAI-1 activity, the difference became borderline significant (OR 11.8, 95% CI 1.00-139; $P=0.05$).

Table 4. Bleeding complications in the patients with PAI-1 activity <1U/mL, compared to patients with normal-high PAI-1, in the TURP-study.

Case number	Bleeding complication (primary end point*)	Perioperative bleeding (ml blood/g resected prostatic tissue)	Post-operative blood loss (g Hb)	Reoperation	Other
1	Yes	6	261	Yes	
2	Yes	9	147	No	
3	Yes	45	65	No	
4	No	28	8	No	Haematuria day 9
Patients with PAI-1 \geq 1U/mL (mean values) n=58	28%	22	51	5%	

*The primary end point was a composite of perioperative bleeding >40 mL/g, postoperative loss >100 g Hb and reoperation due to bleeding.

Three of the four patients with low PAI-1 activity had bleeding complications due to the preset criteria. Two of the three patients with the largest blood loss had low PAI-1 activity and one patient needed a reoperation due to bleeding (table 4).

One of the patients with low PAI-1 activity did not meet the criteria for bleeding complications, but he actually contacted the surgeon on day 9 postoperatively due to macroscopic haematuria.

The proportion of patients with bleeding complications was the same among those with or without prostate volume reducing drugs, with or without previous treatment with acetylsalicylic acid, with normal or mildly to moderately elevated creatinine (111-306 $\mu\text{mol/L}$), with general or spinal anaesthesia, with or without additional minimal surgical procedure (urethrotomy, circumcision, cystolithectomy, urinary bladder biopsy) and with the intended surgeon or another surgeon.

Linear regression analysis showed a statistically significant correlation between intraoperative but not postoperative blood loss and systolic blood pressure, resected mass and resection time.

All the patients with bleeding complications were referred to the coagulation unit for investigation of haemostatic defects and this was done several months after the surgery, but only 13 of 19 patients were eventually investigated. Six patients declined due to old age and concomitant diseases. All the three patients with low PAI-1 activity and bleeding complications had normal results in this screening. Of 10 investigated patients with normal PAI-1 activity, 9 patients had normal results and 1 had prolonged APTT (42 s on two different occasions, normal range 28-40 s), but with normal factor levels and negative lupus anticoagulant and no reason was found to the prolonged APTT.

Discussion

The definition as bleeding complication in this study can be discussed. We chose to not consider transfusions as a criterion for bleeding complication, since giving transfusions is often a decision based on a number of criteria including patient age and cardiovascular status and this decision process varies between hospitals, countries and types of surgery [246].

The blood loss in prostate surgery depends on the amount of resected prostatic tissue, resection time, systolic pressure and type of anesthesia [210], and therefore the absolute volume of blood loss was not considered to be a criterion for bleeding complication. The limit for excessive intra-operative blood loss was set at 40 mL blood/g resected prostate, based on oral communication with R.Hahn, who has published articles on this topic [210]. In the present study the 90th percentile was indeed 41 g/L. The limit for the postoperative blood loss during hospitalisation was set to 100 g haemoglobin, which was not based on literature data, but was the approximate amount of two units of red packed cells (114 g of haemoglobin). In a post-hoc analysis we set the upper limit for the blood loss at the 90th percentile, 124.4 g/L, which eliminated 3 bleeding complications in the group with normal PAI-1 activity. The rate of bleeding complications remaining was then 75% with patients with low PAI-1 activity compared to 22% in the group with normal/high PAI-1 activity, compared to 75% versus 28% when 100 g/L was used.

It is also difficult to select the optimal time point for post-operative haemoglobin. We decided to use the day of discharge, since it was an objective decision taken by the surgeon, when the patients was ready for discharge. Sometimes this haemoglobin sample was taken a day before the patient actually left the hospital, due to the fact that the patient left early in the morning. This could have lead to false low haemoglobin due to incomplete restoration of the fluid balance, since the patients during surgery were given crystalloids or colloids as intravenous infusions.

When the study was planned, we thought that bleeding complications may be related to the absolute PAI-1 level and that we could reduce the sample size by adding linear regression analysis, but it seems that only low PAI-1 activity (<1 U/mL) may have any influence on the risk of bleeding. We were unable to continue this study due to the fact that the referral pattern for these patients changed to another hospital for TURP. Our intention was to have a single surgeon, but this was not always possible. However we could not find any difference in bleeding complications between the surgeons.

Some patients were on medication with platelet inhibitors and this was discontinued several days before surgery as a routine at the clinic. We could not see any difference in bleeding complications between the patients with or without previous treatment with antiaggregants. Eight percent of the patients had general anaesthesia instead of spinal anaesthesia, but there was no difference in primary endpoints.

GENERAL DISCUSSION

Since the discovery of plasma PAI-1 in 1983 [247, 248] many attempts have been made to characterise and understand the pathology behind the increase of PAI-1 that is associated with so many diseases. Only a few case-reports have focused on the conditions that are connected with low PAI-1 activity, which mainly results in bleeding problems after surgery or trauma.

We have now performed a large study in which we showed that low PAI-1 activity is not of major importance for the bleeding diathesis, since low PAI-1 activity is not associated with any specific bleeding manifestations and the combination of low PAI-1 activity and other haemostatic disorders does not aggravate the clinical symptoms. Low PAI-1 activity is also present in a substantial proportion of the normal population. We did, however, find that low PAI-1 activity is more prevalent among patients with bleeding problems (23 %) than in control groups (13 % and 10%) and that the fibrinolytic activity measured by PAP was increased among the patients with low PAI-1 activity.

Since the fibrinolytic activity is higher among patients with low PAI-1 activity; they could be vulnerable to bleeding complications after surgery. Our TURP-study provided indications for this possibility, but a larger study would be necessary to confirm these findings. Anti-fibrinolytics like tranexamic acid are effective medications to prevent bleeding at surgery in PAI-1 deficient patients [20, 22].

The main problem with the analysis of PAI-1 activity is that the method is not sensitive enough to differ absolute zero from a very low, but still present, level of PAI-1 activity. In our studies we have improved the method for detection of a lower activity (from <2 U/mL to <1 U/mL). We also performed analyses of PAI-1 antigen in serum, tPA-PAI-1 complex and polymorphisms in the PAI-1 gene, in an attempt to characterise patients with very low PAI-1. Still it is problematic to discern the pathological PAI-1 deficient patient from the substantial proportion of individuals with low PAI-activity that probably is harmless.

PAI-1 activity is affected by many conditions, like overweight, use of oral combined contraceptives, pregnancy, infectious and inflammatory diseases, and these parameters must always be taken into consideration when evaluating and interpreting studies on PAI-1. We made all efforts to control and adjust for these variables.

Since high PAI-1 activity is related to many diseases, it is possible that there could be an

advantage in life to have naturally low PAI-1 activity, with the exception of situations like trauma or surgery, where the bleeding risk may be enhanced.

Analysis of PAI-1 activity as a screening investigation for bleeding disorders does not seem to be meaningful, but could be included in selective cases, and then perhaps with additional analyses of for instance PAI-1 antigen in serum to obtain more information on the nature of the deficiency.

More research should be done concerning the effects of low PAI-1 activity in a wider perspective, including possible long-term benefits thereof.

CONCLUSIONS

- Low PAI-1 activity is common in patients with a bleeding diathesis, but it is a risk factor of minor clinical importance and not associated with specific bleeding manifestations
- Low PAI-1 activity may contribute to the risk of bleeding after transurethral resection of the prostate.
- The activation of plasminogen measured as PAP was higher in patients with bleeding symptoms in combination with PAI-1 activity less than 1.0 U/ml than in those with PAI-1 activity of at least 1.0 U/ml. The coagulation activity under normal conditions, as measured with D-dimer, did not differ between the two patient subsets.
- Patients with bleeding tendency and low PAI-1 activity have inflammatory and insulin profiles similar to those with normal or high PAI-1, whereas low BMI seems to be the most important determinant.

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