From the Clinical Experimental Research Laboratory,
Department of Emergency and Cardiovascular Medicine,
Sahlgrenska University Hospital/Östra,
Institute of Medicine, the Sahlgrenska Academy,
Göteborg University, Göteborg, Sweden

---

# Plasminogen Activator Inhibitor 1 in Platelets

## Studies of Synthesis, Activity, and Glycosylation Patterns

Helén Brogren

**Göteborg 2008**

Plasminogen Activator Inhibitor 1 in Platelets -
Studies of Synthesis, Activity, and Glycosylation Patterns
ISBN 978-91-633-2089-7

© 2008 Helén Brogren
helen.brogren@gu.se

From the Clinical Experimental Research Laboratory, Department of Emergency and Cardiovascular Medicine, Sahlgrenska University Hospital/Östra, Institute of Medicine, the Sahlgrenska Academy, Göteborg University, Göteborg, Sweden

Printed by Vasastadens Bokbinderi AB, Göteborg, Sweden, 2008

To my Family

# Plasminogen Activator Inhibitor 1 in Platelets - Studies of Synthesis, Activity, and Glycosylation Patterns

Helén Brogren

Clinical Experimental Research Laboratory,
Department of Emergency and Cardiovascular Medicine,
Sahlgrenska University Hospital/Östra,
Institute of Medicine, the Sahlgrenska Academy, Göteborg University, Göteborg, Sweden

## ABSTRACT

Plasminogen activator inhibitor 1 (PAI-1) is the main physiological inhibitor of tissue-type plasminogen activator. Thus PAI-1 plays an important role in decreasing the fibrinolytic activity in human blood. PAI-1 is present in high concentrations in platelets and also in low concentrations in plasma, but the source of plasma PAI-1 is not known. Previous studies have shown that the activity of PAI-1 in platelets is low and this finding is not in accordance with its observed role in clot stabilisation. The aim of this thesis was to investigate the role of platelets in inhibition of fibrinolysis, and in particular the physiological regulation of platelet-derived PAI-1; its synthesis, activity, and potential contribution to plasma levels.

Investigations of mRNA levels and PAI-1 protein synthesis showed that platelets, despite their lack of nucleus, have an on-going synthesis of PAI-1. The amount of PAI-1 increased on average by 25% in 24 hours and the synthesis could be further stimulated by thrombin. Importantly, the synthesized PAI-1 was active for at least 24 hours as shown by a functional assay. There were large inter-individual variations of the synthesis rate and we therefore studied if the common 4G/5G promoter polymorphism was the cause of the variations. However, the polymorphism did not influence the expression as showed by analysis of platelet PAI-1 mRNA and protein levels in 38 men homozygous for either allele.

Previous studies reporting low platelet PAI-1 activities have been performed using different preanalytical preparatory procedures potentially causing an inactivation of PAI-1 before the activity analysis. We reinvestigated the activity of platelet PAI-1 by lysis of platelets in the presence of tPA and subsequent detection of tPA-PAI-1 complex. Our results show that the choice of lysis method and preparatory procedures is critical for the result and the activity was found to be approximately 70%. This result is in better agreement with the observed role of platelet PAI-1 in clot stabilisation.

The amount of PAI-1 synthesized in 24 hours in our *in vitro* experiments suggests that a release of as little as 3% of newly synthesized PAI-1 from platelets would be sufficient to maintain normal plasma levels. We therefore wanted to elucidate if the platelets could be the source of plasma PAI-1. Investigations of the glycosylation patterns of PAI-1 synthesized by different tissues were performed to elucidate if differences in this pattern could reveal the source of plasma PAI-1. The results suggest that platelets are the source since no glycans were found on PAI-1 from neither plasma nor platelets. Conversely, PAI-1 from the other tissues studied expressed heterogeneous glycosylation patterns. Interestingly, we also found that the raised plasma PAI-1 levels found in obese subjects is due to a contribution of PAI-1 from the adipose tissue. Obese subjects had highly glycosylated plasma PAI-1 and several of the identified glycans were found on PAI-1 from adipose tissue.

In conclusion, these findings may clarify the previous irreconcilable findings of the role of platelet PAI-1 in clot stabilization. The high levels of active PAI-1 and the continuous production of large amounts of active PAI-1 in platelets could be a mechanism by which platelets contribute to stabilization of blood clots. The results also suggest that platelets may contribute to the PAI-1 plasma levels.

*Keywords*: PAI-1, platelets, plasma, fibrinolysis, synthesis, polymorphism, glycosylation, activity, platelet mRNA..

ISBN 978-91-633-2089-7 Göteborg 2008

## LIST OF ORIGINAL PAPERS

This thesis is based on the following papers, identified in the text by their Roman numerals:

I Brogren H, Karlsson L, Andersson M, Wang L, Erlinge D, Jern S. Platelets synthesize large amounts of active plasminogen activator inhibitor 1.
*Blood 2004;104:3943-48.*

II Brogren H, Wallmark K, Jern S, Karlsson L. Plasminogen activator inhibitor 1 expression in platelets is not influenced by the 4G/5G promoter polymorphism.
*Thrombosis Research 2007;Sep 18 [Epub ahead of print].*

III Brogren H, Wallmark K, Deinum J, Karlsson L, Jern S. Preparatory procedures may lead to underestimation of platelet PAI-1 activity.
*In manuscript.*

IV Brogren H, Sihlbom C, Wallmark K, Lönn M, Deinum J, Karlsson L, Jern S. Heterogeneous glycosylation patterns of human PAI-1 may reveal its cellular origin.
*In manuscript.*

# CONTENTS

**ABSTRACT** 5

**LIST OF ORIGINAL PAPERS** 6

**ABBREVIATIONS** 9

**HISTORICAL BACKGROUND** 11
The scope of this thesis 12

**INTRODUCTION** 13
Fibrinolysis 13
PAI-1 13
PAI-1 and thrombosis 15
Platelets 16

**AIMS** 17

**MATERIALS AND METHODS** 18
Subjects 18
Preparation of plasma and platelets 18
Cell lysis 18
Platelet and leukocyte counts 19
Preparation of cell lysates and conditioned media 20
Preparation and incubation of adipose tissue 20
Preparation and culturing of human umbilical vein endothelial cells 20
Preparation and culturing of monocytes/ macrophages 20
Preparation and incubation of hepatocytes 20
Analyzing techniques 21
Quantitative reverse transcriptase real-time PCR 21
Genotyping of the 4G/5G polymorphism 22
Enzyme-linked immunosorbent assay 22
Metabolic radio-labeling and immunoprecipitation 23
PAI-1 activity assay 23
Affinity chromatography 24
Mass spectrometry 24
Glycoprotein specific staining 27
Statistical methods 27

**RESULTS** 28
Synthesis of active PAI-1 28
Influence of the 4G/5G polymorphism on platelet PAI-1 expression 30
Analysis of platelet PAI-1 activity 31
Analysis of the effect of different lysis methods on PAI-1 activity 33
Mass spectrometry analysis of glycosylation patterns of PAI-1 34

**DISCUSSION** 38
*De novo* synthesis of PAI-1 in platelets 38
No effect of the 4G/5G polymorphism on platelet PAI-1 expression 39
Activity of platelet PAI-1 40
Underestimation of platelet PAI-1 activity due to preparatory procedures 41
Are platelets the unknown source of plasma PAI-1? 42

**CONCLUDING REMARKS** 44

**POPULÄRVETENSKAPLIG SAMMANFATTNING** 45

**ACKNOWLEDGEMENTS** 47

**REFERENCES** 49

**PAPER I-IV**

## ABBREVIATIONS

| | |
|---|---|
| ACD | acid citrate dextrose |
| BMI | body mass index |
| Bp | base pairs |
| cDNA | complementary DNA |
| CID | collision induced dissociation |
| CHO | chinese hamster ovary |
| $C_T$ | threshold cycle |
| ELISA | enzyme-linked immunosorbent assay |
| GAPDH | glyceraldehyde 3-phosphate dehydrogenase |
| GM-CSF | granulocyte monocyte colony stimulating factor |
| HUVEC | human umbilical vein endothelial cells |
| ICR | ion cyclotron resonance |
| IL-1 | interleukin 1 |
| kDa | kilo dalton |
| LTQ-FT | linear trap quadrupole - fourier transform |
| mRNA | messenger RNA |
| MS | mass spectrometry |
| MW | molecular weight |
| PAI-1 | plasminogen activator inhibitor 1 |
| PBMC | peripheral blood mononuclear cell |
| PCR | polymerase chain reaction |
| $PGE_1$ | prostaglandin $E_1$ |
| PIPES | 1,4-piperazinediethanesulfonic acid |
| ppm | parts per million |
| PRP | platelet-rich plasma |
| RT-PCR | reversed transcription polymerase chain reaction |
| SDS | sodium dodecyl sulphate |
| SERPIN | serine protease inhibitor |
| TGF-β | transforming growth factor beta |
| tPA | tissue-type plasminogen activator |
| uPA | urokinase-type plasminogen activator |

# HISTORICAL BACKGROUND

Over the last few centuries, cardiovascular disease has, together with cancer, gradually replaced infectious disease as the leading cause of death in Western societies. The majority of ischemic cardiovascular events, such as myocardial infarction and ischemic stroke, are caused by atherothrombosis. A thrombotic event is a complex process, but its key pathogenetic element is activation of intravascular clotting mechanisms that, when unopposed, progress into formation of an occluding thrombus that arrests blood flow [Fuster 1994].

Already in the 1850s, the German pathologist Rudolf Virchow hypothesized that thrombus formation resulted from an untoward combination of three predisposing conditions: vessel wall abnormalities, blood flow, and coagulability of blood [Virchow 1856, Owen 2001]. Remarkably, the significance of Virchow's insights is now 150 years later still valid and his classical triad covers the broad range of pathophysiological processes leading to thrombogenesis [Chung and Lip 2003, Lowe 2003]. As noted by pathologists for centuries, the appearance of thrombi forming in arteries and veins are distinctly different; whereas thrombi in vessels with rapidly flowing blood are firm and palish (so-called white thrombi), those appearing in vessels with slow or stagnant blood flow are redish and gelatinous (red thrombi). These differences are due to variations in clot composition caused by differences in the relative contribution of the three Virchow components.

Thrombus formation in the arterial circulation, which is the scope of this thesis, preferentially occurs on sites of structural vascular injury due to atherosclerotic plaques. In Virchow's wording, such "*vessel wall abnormalities*" provide stimuli for activation of "*blood coagulation*" by exposing the blood to clotting-activating factors. However, as predicted by Virchow, the "*blood flow*" component was also important. We now know that although a rapid blood flow may prevent the stagnant type of blood coagulation, it may instead promote formation of platelet-rich white thrombi by imposing high shear forces and turbulence phenomena of the blood that cause platelets to aggregate and become activated.

However, the existence of the platelet was not known by Virchow by that time, and it was not until 1882 that the Italian researcher Giulio Bizzozero described "a constant blood particle, differing from red and white blood cells" [Bizzozero 1882]. Bizzozero recognized that the platelet played a role in thrombogenesis, and was the first to describe that white thrombi mainly consisted of platelets in contrast to red ones whose content was dominated by red blood cells and fibrin. However, the significance of the platelet was largely neglected until the 1960s, when the beneficial antiaggregatory effects of aspirin were demonstrated [Quick 1966, Weiss and Aledort 1967].

The "coagulability" of blood was not discovered by Virchow, but the actions of blood coagulation had been described already by Hippocrates and Aristotle who observed that freshly drawn blood usually clots within a few minutes. The "modern" history of coagulation begun during the $19^{th}$ century when some enzymes involved in coagulation were identified. However, it was not until 1905 when the German physiologist

Paul Morawitz described four coagulation factors that the "classic" theory of blood coagulation was formulated. The remainders of the biochemical factors in the complex cascade reactions of the coagulation system were discovered in the 20th century by the concerted work of many scientists.

However, it had been known for many years that human blood also possesses lytic activity to spontaneously resolve clots. In 1794, the British surgeon John Hunter reported that in "animals killed by lightning or by electricity" or in animals "who are run very hard, and killed in such a state" the blood does not clot [Hunter 1794]. One hundred years later (1893), the phenomenon of spontaneous dissolution of blood clots was named "fibrinolysis" by the French physiologist Jules Dastre, who also discovered a fibrin-degrading proteolytic enzyme in serum, plasminogen [Owen 2001]. The major discoveries of the other specific components of the fibrinolytic system were made in the 1940s and 50s, and the key fibrinolytic activator tissue-type plasminogen activator (tPA) was discovered in 1947 [Owen 2001]. Not until 40 years later, in 1983, a specific inhibitor of tPA was described and the inhibitor was subsequently named plasminogen activator inhibitor 1 (PAI-1).

## The scope of this thesis

Despite the fact that some 8,000 papers have been published since its discovery 25 years ago, the role of the fibrinolysis inhibitor PAI-1 still remains partly unclear. In particular, two areas of controversy still exist; the first concerns the origin and function of PAI-1 in plasma, and the second the role of the large and supposedly inactive pool of PAI-1 in platelets.

Of the total blood pool of PAI-1, 90% is stored in the platelets and only a small fraction is present in plasma [Booth 1999]. The origin of plasma PAI-1 is unknown and its role in counteracting vascular fibrinolysis is uncertain. Furthermore, a substantial number of arterial thrombi undergo spontaneous lysis before they cause ischemic tissue injury, probably as an effect of endogenous t-PA release. White platelet-rich clots are relatively resistant to degradation both by endogenous fibrinolysis and pharmacological thrombolysis [Kucia and Zeitz 2002]. The amount of platelets in clots and their content of the fibrinolytic inhibitor PAI-1 determine their resistance to thrombolysis [Potter van Loon, *et al* 1992]. The enigma is that most studies have shown that the vast majority (approximately 95%) of platelet PAI-1 is inactive and unable to inhibit fibrinolysis [Booth, *et al* 1988, Declerck, *et al* 1988a, Booth, *et al* 1990, Lang and Schleef 1996]. This observation has of course been very difficult to reconcile with its putative role in clot stabilization.

The scope of the present work is to address these controvercies by investigating the hypotheses a) that there is a continuous production of PAI-1 in the platelet, b) that the platelet is the source of plasma PAI-1, and c) that the majority of platelet PAI-1 is stored in an active configuration that make clots resistant to lysis but that its activity state has been underestimated in previous studies.

# INTRODUCTION

The fluidity of blood and integrity of the circulatory system is maintained by the hemostatic system comprising platelet aggregation, coagulation, and fibrinolysis also termed primary, secondary and tertiary hemostasis. Platelet aggregation and coagulation function to prevent excessive bleeding after vessel injury, whereas fibrinolysis maintains a viable circulation by keeping the blood in an uncoagulated state. The functions of these physiological processes are tightly regulated by the endothelium, the platelets, and the coagulation and fibrinolytic plasma proteins.

To prevent blood loss when a vessel is injured, platelets adhere to collagen in the exposed subendothelial matrix [Siljander, *et al* 2004]. They become activated and release substances that cause propagation of the plug by recruiting more platelets, and a concurrent activation of the coagulation cascade results in a stabilizing fibrin network. Eventually, when the injury is restored, the blood clot is dissolved by the fibrinolytic system. There is a delicate balance between these counteracting systems; too much fibrinolysis will cause bleeding whereas too much coagulation and platelet aggregation will cause thrombosis.

## Fibrinolysis

The fibrinolytic system is involved in the lysis of clots and also acts to restrict thrombus propagation beyond the site of injury, as a counterregulatory mechanism to the coagulation cascade. The efficacy of fibrinolysis is demonstrated by the spontaneous reperfusion that occurs in about 30% of patients with myocardial infarction [DeWood, *et al* 1980, DeWood, *et al* 1983, Rentrop, *et al* 1989, Stone, *et al* 2001]. The key enzyme in fibrin lysis is the serine protease plasmin which circulates in plasma as an inactive precursor, plasminogen. Plasminogen is converted to plasmin by two different activators, tissue-type (tPA) and urokinase-type (uPA) [Collen 1980, Lijnen and Collen 1995]. tPA is the most important activator of intravascular fibrinolysis [Collen 1980, Brommer 1984, Fox, *et al* 1985, Lijnen and Collen 1997], whereas uPA appears to mainly be involved in cell movement and tissue remodelling [Dano, *et al* 1985, Lijnen and Collen 1997]. To prevent excessive and/or premature degradation, the blood clot is stabilized by different mechanisms. One level of regulation is the efficiency of the activation of plasminogen by tPA; in plasma this activation is very inefficient but when they both are bound to fibrin the activation rate increases several hundredfold [Collen 1980]. Plasmin and tPA are also regulated by specific serine protease inhibitors (serpins). The main inhibitor of plasmin is $\alpha_2$-antiplasmin [Lijnen and Collen 1995] and the main inhibitor of tPA is plasminogen activator inhibitor 1 (PAI-1) [Chmielewska, *et a*l 1983, Kruithof, *et al* 1984, Verheijen, *et al* 1984].

## PAI-1

PAI-1 was first identified in 1983 as the principal inhibitor of tPA [Chmielewska, *et al* 1983, Loskutoff, *et al* 1983]. It occurs in low concentrations in plasma (20 ng/ml) [Declerck, *et al* 1988a, Booth 1999], but the platelets represent the major pool and contains approximately 90% of the circulating PAI-1. [Kruithof, *et al* 1987, Booth, *et*

*al* 1988, Declerck, *et al* 1988b, Urden, *et al* 1987]. PAI-1 is produced by a variety of cells in culture and is widely distributed in many tissues [Lucore, *et al* 1988, Sawdey and Loskutoff 1991, Simpson, *et al* 1991]. These findings raise the possibility that under normal conditions PAI-1 in plasma reflects the output from several sources. According to the present view, liver, endothelial cells, platelets, macrophages, and adipocytes, are considered to be the most likely sources of PAI-1 in plasma [Dellas and Loskutoff 2005]. Beside its role in intravascular fibrinolysis, PAI-1 is also involved in cell associated proteolysis, cell migration, and tissue remodelling. Thereby it plays a role in pathological processes such as cancer invasion, metastasis and inflammation [Myohanen and Vaheri 2004, Dano, *et al* 2005].

PAI-1 is a single chain protein with a molecular weight of ~45 kDa. The mature protein consists of 379 amino acids and is encoded on chromosome 7. The gene spans approximately 12 kb and is composed of nine exons and eight introns [Strandberg, *et al* 1988]. The PAI-1 promoter has been extensively characterized and many important regulatory elements have been identified [Chen, *et a*l 1998, Eriksson, *et al* 1998, Du, *et al* 2000, Hou, *et al* 2004]. The promoter also contains a common polymorphism which appears to be of importance for its transcriptional activity and possibly also partly determines plasma PAI-1 levels. This polymorphism consists of a single basepair insertion/deletion (4G or 5G) located -675 base pairs upstream of the transcription start site [Dawson, *et al* 1993]. It has been shown in expression experiments in HepG2 cells by Dawson *et al* that, when stimulated with IL-1, the 4G allele produces six times more PAI-1 mRNA [Dawson, *et al* 1993]. An association between the 4G/5G polymorphism and cardiovascular disease has been observed in some studies [Eriksson, *et al* 1995, Juhan-Vague, *et al* 2003]. However, although previous studies have suggested 42 to 60% heritability rates of PAI-1 levels [Hong, *et al* 1997, de Lange, *et al* 2001], a large number of the clinical studies that have evaluated the influence of the 4G/5G polymorphism on plasma PAI-1 concentration have shown divergent results [Doggen, *et al* 1999, Jeng 2003, Nordt, *et al* 2003, Zietz, *et al* 2004, Martinez-Calatrava, *et al* 2007].

PAI-1 has three potential sites for N-linked glycosylation N232, N288 and N352 [Xue, *et al* 1998]. It has been shown that human PAI-1 expressed naturally, or recombinant PAI-1 expressed by human cell lines, has a heterogeneous glycosylation pattern on two of the three sites (N232 and N288) [Gils, *et al* 2003]. However, the glycan composition of PAI-1 synthesized by human tissues *in vivo* is not known.

PAI-1 is a member of the serine protease inhibitor (serpin) superfamily. The proteins of the serpin family share a common tertiary structure and they serve as pseudo-substrate for their target serine protease by a reactive centre that mimics the natural substrate. They form very stable 1:1 inactive complexes with their protease by complex mechanisms [Rau, *et al* 2007] and the complicated reaction cascade of binding and inhibition of tPA by PAI-1 is not completely understood [Lindahl, *et al* 1990, Bjorquist, *et al* 1994, Stromqvist, *et al* 1996, Komissarov, *et al* 2007]. The binding and inactivation of tPA by PAI-1 is very rapid with second-order rate constants in the magnitude of $10^7$ $M^{-1}$ $s^{-1}$ [Chmielewska, *et al* 1988, Thorsen, *et al* 1988, Lawrence, *et al* 1989, Lijnen, *et al* 1991]. The ~110 kDa complex is remarkably stable under physiological conditions [Bjorquist, *et al* 1994] but dissociation can be accomplished with $NH_4OH$ [Lindahl, *et al* 1990] or SDS [Gaussem, *et al* 1993].

PAI-1 exists *in vivo* in at least two different forms; active and latent or inactive [Sancho, *et al* 1995]. Only the active form of PAI-1 is able to complex bind and inhibit tPA [Kooistra, *et al* 1986]. Regardless of tissue origin, PAI-1 is synthesized in an active configuration but spontaneously converts to the thermodynamically more stable inactive form [Hekman and Loskutoff 1985, Levin 1986, Sprengers, *et al* 1986, Wagner, *et al* 1986]. The half-life of active PAI-1 is approximately 1-2 h at 37°C and pH 7,4 but is slower at lower temperature and pH [Levin and Santell 1987, Lindahl, *et al* 1989, Loskutoff, *et al* 1989]. In plasma the active form of PAI-1 is stabilized by binding to vitronectin which increases its half-life several-fold [Declerck, *et al* 1988b, Wiman, *et al* 1988, Mimuro and Loskutoff 1989, Seiffert and Loskutoff 1991]. PAI-1 can be reactivated *in vitro* by treatment with denaturants such as SDS, guanidine HCl, and urea [Hekman and Loskutoff 1985]. Whether PAI-1 also can be reactivated *in vivo* is uncertain, although a possible reactivation has been reported in a study of human recombinant PAI-1 in rabbits [Vaughan, *et al* 1990] and it has also been suggested that negatively charged phospholipids exposed on the surface of activated platelets could reactivate PAI-1 [Lambers, *et al* 1987].

## PAI-1 and thrombosis

The association of decreased fibrinolytic activity with thrombotic events has been recognized for decades, but it was not until the 1990s, ten years after the discovery of PAI-1 that the essential function of PAI-1 in intravascular fibrinolysis was demonstrated [Dieval, *et al* 1991, Fay, *et al* 1992, Carmeliet, *et al* 1993]. The importance is now evident as shown by numerous studies. It has been shown both in human studies and in studies on PAI-1 knockout mice that deficiencies or absence of PAI-1 will cause accelerated fibrinolysis and bleeding [Dieval, *et al* 1991, Fay, *et al* 1997]. On the contrary, spontaneous thrombus formation is observed in mice over-expressing active PAI-1 [Erickson, *et al* 1990, Eren, *et al* 2002], and high levels of PAI-1 are commonly observed in conditions with increased risk of thrombotic disease such as obesity, metabolic syndrome, and type 2 diabetes [Alessi, *et al* 2007, Aso 2007]. Furthermore, studies in transgenic mice have shown that PAI-1 not only influences the resistance to thrombolysis but also the rate of progression of thrombus formation following vascular injury [Konstantinides, *et al* 2001].

Arterial clots have been shown to contain 2-3-fold more PAI-1 than venous clots [Booth, *et al* 1992, Robbie, *et al* 1996], and there is a close correlation between the relative PAI-1 content of a clot and its resistance to thrombolysis [Potter van Loon, *et al* 1992]. It is likely that the major part of the PAI-1 in thrombi has been released from activated platelets, since platelets contain large amounts of the inhibitor in their α-granules [Erickson, *et al* 1984]. The importance of platelet PAI-1 is further supported by *in vitro* clot assays on platelets from a patient with complete loss of PAI-1 expression [Fay, *et al* 1994], as well as by studies on thrombi generated in the Chandler loop experimental thrombosis model [Torr-Brown and Sobel 1993, Stringer, *et al* 1994].

On the other hand, the pathophysiological importance of platelet PAI-1 for inhibition of fibrinolysis has been difficult to reconcile with the fact that 95% of PAI-1 in platelets is inactive [Schleef, *et al* 1985, Booth, *et al* 1988, Declerck, *et al* 1988a]. This enigmatic relation would seem to suggest that either data on the physiological role of platelet-derived PAI-1 are incorrectly interpreted, or that the activity of the platelet PAI-1 pool has been underestimated.

## Platelets

Platelets are anucleate cytoplasts mainly produced in the bone marrow by fragmentation of the cytoplasm of megakaryocytes [Chang, *et al* 2007]. They circulate in blood for approximately 10 days [Dale 1997], normally in a concentration of 150-400 x $10^9$ /L (inferring that approximately 1.5 million platelets are formed every second). The most abundant organelles in the platelet cytoplasm are the three different types of granules; α, dense, and lysosomal, that contain a large number of biologically active molecules crucial for platelet function. The most numerous ones are the α-granules which contain a large number of adhesion proteins, growth factors, cytokine-like proteins and components of coagulation and fibrinolysis (and among them PAI-1) [Rendu and Brohard-Bohn 2001]. Upon activation, platelets expel the contents of the secretory granules. The release of PAI-1 may theoretically protect the developing thrombus from premature lysis, at least if it is released in an active configuration.

Because platelets lack nucleus, they have traditionally not been considered to synthesize proteins. Even though Booyse *et al* showed already in 1967 that platelets retain megakaryocyte-derived translationally active mRNAs [Booyse and Rafelson 1967a, Booyse and Rafelson 1967b], it was not until the late 1990s that the interest of this mRNA was revived. Electron microscopy studies of platelets have revealed the presence of rough endoplasmic reticulum and polyribosomes and some studies reported protein synthesis in platelets [Belloc, *et al* 1987, Kieffer, *et al* 1987, Newman, *et al* 1988] but it was suggested that the synthesis was just a remnant from the megakaryocyte stage. However, in 1998 Weyrich *et al* identified regulated synthesis of a specific protein (BCL-3) [Weyrich, *et al* 1998]. During the last few years platelet mRNA have been extensively studied by quantitative PCR, microarray [Bugert, *et al* 2003, Gnatenko, *et al* 2003, McRedmond, *et al* 2004] and SAGE (serial analysis of gene expression) [Gnatenko, *et al* 2003, Dittrich, *et al* 2006]. These studies have revealed a platelet specific transcriptome with some 2500 different transcripts. Furthermore, recent studies have revealed synthesis of a number of specific proteins, some of which with a highly sophisticated regulation [Lindemann, *et al* 2001, Brogren, *et al* 2004, Denis, *et al* 2005, Evangelista, *et al* 2006, Panes, *et al* 2007, Thon and Devine 2007].

# AIMS

The overall objective of this thesis was to study the physiological importance of platelet PAI-1, and the specific aims were:

- to investigate if platelets contain mRNA for PAI-1 and, if so, if there is an on going synthesis of active PAI-1 in platelets
- to investigate if the 4G/5G promoter polymorphism is influencing the PAI-1 expression in platelets
- to investigate the activity of platelet PAI-1
- to investigate if platelets are the source of plasma PAI-1 and to elucidate if tissue-specific glycosylation patterns can reveal the origin of plasma PAI-1

# MATERIAL AND METHODS

## Subjects

Blood samples for isolation of plasma and platelets were collected from apparently healthy male and female subjects with platelet counts of 150-350 x $10^9$/L. Samples for studies of 4G/5G genotype and PAI-1 levels were only collected from male subjects due to the known variations of plasma PAI-1 levels during the menstrual cycle [Siegbahn, *et al* 1989, Chung, *et al* 1998, Giardina, *et al* 2004]. All samples for investigations of levels of PAI-1 were collected between 09:00 and 10:00 a.m. because of the diurnal variation of plasma PAI-1 [van der Bom, *et al* 2003]. Blood from four obese subjects with body mass indexes from 36.6 to 40.7 kg/$m^2$ (three males and one female, 47-74 years of age) was collected for analysis of the glycosylation pattern of PAI-1.

All test subjects had been advised not to take aspirin or non-steroid anti-inflammatory drugs 10 days prior to the blood sampling. They had also been asked to abstain from extreme physical activity and alcohol intake, and avoid high fat diet at least one day prior to the sampling. The protocols were approved by the Ethics committee of Göteborg University.

## Preparation of plasma and platelets

To minimize platelet activation during sampling, no stasis was used and blood was drawn through butterfly needles into syringes containing acid citrate dextrose (ACD) and prostaglandin $E_1$ ($PGE_1$). Platelet-rich plasma (PRP) was prepared by centrifugation at 150 x g for 20 min. The PRP was re-centrifuged at 150 x g for 10 min and then pelleted at 800 x g for 15 min. Plasma was removed and the platelet pellet was resuspended in Pipes/saline/glucose buffer containing $PGE_1$. Finally, platelets were pelleted (800 x g, 15 min) and the supernatant was discarded. Platelets were immediately used for subsequent preparations and analysis. For isolation of PAI-1 for studies of glycosylation pattern, plasma and platelets were collected concurrently. Plasma was removed after pelleting of the platelets and was re-centrifuged at 2000 x g for 20 min at 4°C to remove residual platelets.

For analysis of plasma PAI-1 concentrations, blood was collected in 0.129 M citrate and immediately put on ice. Samples were centrifuged at 2000 x g at 4°C for 15 min, and plasma was then collected and stored at -80°C until analysis.

### *Cell lysis*

In general, cell lysis was performed on ice by addition of lysis buffer to a final Triton X-100 concentration of 0.1%. After 30 min, cell debris was removed by centrifugation at 10 000 x g for 10 min at 4°C. Experiments of platelet lysis in the presence of tPA were performed at room temperature (RT).

Three separate experiments were performed to evaluate the effect of different platelet lysis methods on the activity of PAI-1, and platelets were lysed in accordance with

different previously described protocols. The platelets were lysed either by freezing and thawing [Declerck, *et al* 1988a, Lang, *et al* 1992], or by sonication [Booth, *et al* 1988, Booth, *et al* 1990, Lang and Schleef 1996]. Sonication was performed using a Branson Sonifier® 250 (Branson Ultrasonic Corporation) equipped with a microtip. The instrument frequency was 20 kHz and sonication was performed on ice 5 or 10 x 5 s in one min intervals with the instrument on setting 2 or 7.

### *Platelet and leukocyte counts*

Platelets were manually counted in all studies after dilution in Stromatol solution, using a Bürker chamber. In Study II, platelets were also counted automatically by flow cytometry (Celldyn 2000, Abbot). However, all the calculated results presented are based on manual counts. Analysis of leukocyte contamination was made in all preparations for subsequent platelet RNA analysis. In Study I, leukocytes were automatically counted by flow cytometry on a FACSCalibur (Becton Dickinson) using LeucoCOUNT™ (Becton Dickinson) and the contamination was less than 3 leukocytes per $10^6$ platelets (Figure 1). In Study II, leukocytes were manually counted in a Nageotte chamber after dilution of samples in Türk's reagent and the number of leukocytes was less than 3 per $10^5$ platelets in all samples.

*Methodological comment*: Evaluation of leukocyte contamination is especially important for studies of platelet mRNA, since the relatively high amount of RNA in leukocytes potentially could interfere with the results. Hence, the number of leukocytes has to be kept at a minimum to ensure that the RNA studied only represents platelet RNA. By analyzing the presence of leukocyte specific transcripts, a previous study has evaluated the influence of contamination and found that less than 3-5 leukocytes in $10^5$ platelets could be considered to be below interfering leukocyte RNA levels [Gnatenko, *et al* 2003]. Also, since monocytes are known to synthesize PAI-1 [Hamilton, *et al* 1993, Tipping, *et al* 1993, Ishibashi, *et al* 2002], it was important to rule out the possibility that contaminating monocytes could be the source of the newly synthesized PAI-1 in Study I.

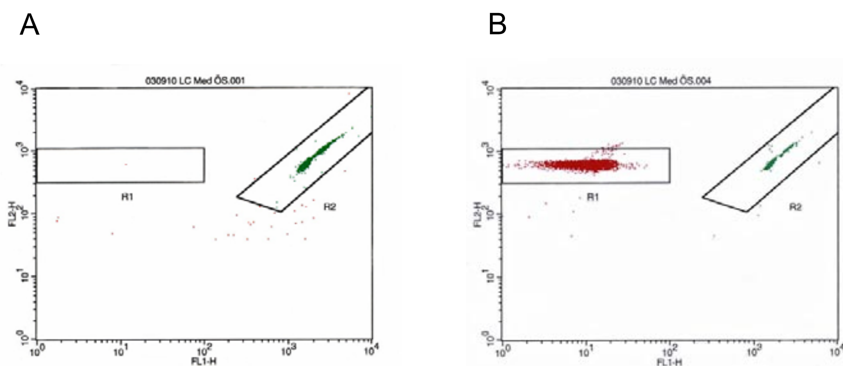


**Figure 1.** Detection and enumeration of residual white blood cells in platelet-rich plasma using LeucoCOUNT™ and flow cytometry. A. Platelet-rich plasma (PRP) prepared by repeated centrifugation contained 2-3 contaminating leukocytes per 10 million platelets. B. Buffy coat contaminated PRP used as control.

## Preparation of cell lysates and conditioned media

### *Preparation and incubation of adipose tissue*

Omental adipose tissue for studies of adipose tissue PAI-1 glycosylation was obtained from three obese women (20, 25, and 46 years old) undergoing laparoscopic gastric by-pass. Their BMIs were 31.7, 41.5, and 34.9 kg/m$^2$, respectively. Connective tissue and blood vessels were removed and 500 mg tissue was incubated in Medium 199 with Hank's salts (Gibco), supplemented with 30 mM $NaHCO_3$, 1% human serum albumin (Immuno AG), 150 nM adenosine, 7175 pM insulin (Novo Nordisk), and 0.1 mg/mL cephalothin (Lilly France), during 1-3 days and the medium was changed daily [Ottosson, *et al* 1994]. In total, 150-300 ml conditioned medium from each of the three incubations were collected.

### *Preparation and culturing of human umbilical vein endothelial cells*

Fresh umbilical cords were obtained from the maternity ward, Sahlgrenska University Hospital/Östra. Human umbilical vein endothelial cells (HUVEC) were isolated by collagenase digestion according to the method of Jaffe *et al* [Jaffe, *et al* 1973]. In brief, the umbilical vein was catheterized under sterile conditions and the blood was removed by infusion of phosphate buffered saline (PBS pH 7.4) at 37°C. Endothelial cells were explanted by incubation with 0.1% collagenase following gentle manipulation of the umbilical cord. Isolated cells were maintained in EGM-2 complete culture medium (Cambrex/Clonetics) at 37°C in a humidified 5% $CO_2$ incubator. Medium was collected after two days.

### *Preparation and culturing of monocytes/macrophages*

Buffy coat prepared from 500 ml whole blood was obtained from 3 healthy blood donors at the hospital blood centre. Preparation of mononuclear cells (PBMCs) was performed using Ficoll-Paque™ PLUS (GE Healthcare). After separation and washing, the PBMCs were resuspended in RPMI 1640 (Invitrogen) containing PEST (penicillin 100 U/ml and streptomycin 100 µg/ml), 2 mM non-essential amino acids, 20 mM sodium pyruvate, and 2 mM glutamine. The cells were allowed to adhere for 1 h at 37°C in a humidified 5% $CO_2$ incubator, and after removal of the non-adherent cells, macrophage-specific medium (Invitrogen) containing 5 µg/ml human GM-CSF (R&D Systems) and PEST was added. The cells were washed and macrophage-specific medium without GM-CSF was added every three days. On day seven, macrophages were stimulated with 10 ng/ml TGF-β (Sigma-Aldrich), and medium was collected after 3 days for PAI-1 isolation. In one of the three preparations, macrophages were stimulated with both 10 ng/ml TGF-β and 400 ng/ml dexamethasone (Sigma-Aldrich).

### *Preparation and incubation of hepatocytes*

Human primary hepatocytes and conditioned media were a kind gift from Annika Janefeldt and Sara Leandersson at AstraZeneca R&D Mölndal. In brief, hepatocytes were cultured in suspension (Williams E+, supplemented with 2 mM L-glutamine and 25 mM HEPES) for 4 hours, subsequently cells were pelleted by centrifugation, medium was collected and hepatocytes were lysed in lysis buffer as described above.

## Analyzing techniques

### *Quantitative reverse transcriptase real-time PCR*

Total RNA was extracted using Trizol® and the total RNA concentrations were determined by RiboGreen® RNA Quantitation Kit. Fluorescence was measured using a FLUOstar Galaxy microplate reader (BMG Labtechnologies). mRNA was converted to cDNA by reverse transcription (GenAmp RNA PCR kit, Applied Biosystems Inc).

Relative quantification was performed on an ABI PRISM® 7700 Sequence Detector (Applied Biosystems Inc). The principle of the assay is that when a fluorescent probe is hybridized to its target sequence during the PCR, the Taq polymerase cleaves the reporter dye from the non-extendable probe. The reporter dye is thereby released to the solution and the increase in the dye emission is monitored in real-time. The threshold cycle ($C_T$) is defined as the cycle number at which the reporter fluorescence reaches a fixed level, and there is a linear relationship between $C_T$ and the initial target copy number [Higuchi, *et al* 1993]. Expression levels of the target gene was analyzed using the relative standard curve method (User Bulletin #2, Applied Biosystems Inc).

Oligonucleotide primers and TaqMan probes were designed using the Primer Express version 1.0 software (Applied Biosystems Inc). Each primer pair was selected so that the amplicon spanned an exon junction, to ensure that amplification of genomic DNA was avoided. GAPDH and cyclophilin were selected as endogenous controls and the sequence of primers and probes are listed in Table 1.

**Table 1.** Oligonucleotide primers and probes used for real-time quantitative PCR

| Gene | Oligonucleotid | Sequence | Position |
|---|---|---|---|
| PAI-1 | Sense primer | 5´ - ggc tga ctt cac gag tct ttc a - 3´ | 11616-11637 |
| | Antisense primer | 5´ - ttc act ttc tgc agc gcc t - 3´ | 11782-11800 |
| | Probe | 5 ´- (FAM)acc aag agc ctc tcc acg tcg cg(TAMRA) - 3´ | 11758-11780 |
| Cyclophilin | Sense primer | 5´ - gta cta tta gcc atg gtc aac ccc - 3´ | 1648-1671 |
| | Antisense primer | 5´ - cag tca aag gag acg cgg cc - 3´ | 1711-1727, 4178-4180 |
| | Probe | 5 ´- (FAM)cgt cga cgg cga gcc ctt g(TAMRA) - 3´ | 1692-1710 |
| GAPDH | Sense primer | 5´- cca cat cgc tca gac acc at - 3´ | 2171-2190 |
| | Antisense primer | 5´- cca ggc gcc caa tac g - 3´ | 3857-3872 |
| | Probe | 5´- (FAM)aag gtg aag gtc gga gtc aac gga ttt g(TAMRA) -3´ | 2195-2217, 3850-3854 |

Typically, PCR was carried out in a 25 µl reaction mixture containing cDNA from 6.25 ng total RNA, TaqMan® Universal PCR mastermix (Applied Biosystems Inc), 10 pmol of each primer, and 5 pmol probe. All samples were analyzed in triplicate.

*Methodological comment*: RNA from nucleated cells is commonly measured in comparison with certain constitutively expressed genes, for example so-called house-keeping genes [Thellin, *et al* 1999]. Expression of these control genes should be insensitive to the experimental conditions [Karge, *et al* 1998] and are used to correct for potential variation in RNA loading or efficiencies of the reverse transcription. To evaluate platelet PAI-1 mRNA levels, we used the traditional house-keeping gene approach with GAPDH and cyclophilin as endogenous controls. However, once mRNA

is packed within the platelets, the lack of transcription makes mRNA levels solely dependent on degradation. In previous studies, we have shown that degradation rates of different platelet mRNA species vary substantially [Wang, *et al* 2003], and more specifically that platelet PAI-1 mRNA has a degradation rate which is 4- and 8-fold higher than that of GAPDH and cyclophilin, respectively [Brogren, *et al* 2004]. Therefore, differences in platelet PAI-1 mRNA content depend not only on its transcription rate in the megakaryocyte but also on differences in platelet age distribution. Hence, the use of GAPDH and cyclophilin might not be the optimal reference genes and although one is to assume that platelets also contain stable transcripts, no such are currently known and future identification of stable RNA species could possibly facilitate studies of mRNA in platelets.

### *Genotyping of the 4G/5G polymorphism*

In order to identify homozygous subjects for the 4G/5G promoter polymorphism, EDTA-blood was collected and DNA was extracted using QIAamp® 96 DNA Blood Kit and QIAamp® DNA Blood mini Kit (Qiagen) according to the manufacturer's instructions. Genotyping was performed using a 5′ nuclease Taqman genotype assay and the ABI PRISM® 7700 Sequence Detection System (Applied Biosystems Inc). The principle of this assay is similar to the real-time PCR described above. The polymorphic site is amplified in the presence of two differently labeled probes complementary to the two alleles (forward primer: TCT TTC CCT CAT CCC TGC C, reverse primer: CCA ACC TCA GCC AGA CAA GG, 4G-probe: (VIC)ACA CGG TGA CT**C CCC** ACG T, 5G-probe: (FAM)ACG GCT GAC T**CC CCC** ACG T) [Tjarnlund, *et al* 2003]. Directly after PCR, the allelic content of a sample can be determined by comparing the fluorescent contribution of each dye.

### *Enzyme-linked immunosorbent assay*

PAI-1 antigen levels in plasma and platelets as well as the amount of PAI-1 synthesized *in vitro* by platelets during 24 hours was determined by enzyme-linked immunosorbent assays (ELISA). The principle of this method is that sample and standard are added to wells coated with PAI-1 specific antibodies. After binding and subsequent washing, a second PAI-1 specific enzyme-labeled antibody is added and after binding and a final wash of the wells to remove unbound antibody, a substrate is added which is converted by the enzyme to a coloured product. The product is spectrophotometrically measured, and the amount of converted substrate is directly proportional to the amount of PAI-1 in the sample. All samples were analyzed in duplicate. Four different commercially available ELISA kits were used; Coaliza® PAI-1 (Chromogenix) in Study I and III, TintElize® PAI-1 (Biopool) in Study III, Imubind® Plasma PAI-1 (American Diagnostica) in Study III, and Zymutest PAI-1 (Hyphen BioMed) in Study II and III.

*Methodological comment*: When studying absolute amounts of PAI-1 antigen the choice of assay is critical. In a multicenter study of seven different ELISA kits, considerable variation in the absolute values was observed [Declerck, *et al* 1993]. However, as concluded in that study, inter-assay variations are most likely due to the calibration of the standard and there were good correlations between the kits. Since we compared paired samples in Study I and differences between groups in Study II, the

need for an exact absolute level was limited in these studies and the choice of assay was therefore not considered critical. However, to adequately determine the level of activity in Study III the absolute antigen determination was crucial. For this reason, we used four different assays for confirmation of the results.

### *Metabolic radio-labeling and immunoprecipitation*

To study synthesis of PAI-1 in platelets, metabolic radio-labeling and immunoprecipitation was performed to distinguish newly synthesized from pre-formed proteins. Cells were incubated in medium with radio-labeled $^{35}S$-methionine. This amino acid is incorporated during synthesis and the resulting proteins will be radioactive. To isolate PAI-1 from medium and platelet lysate, immunoprecipitation was performed using two different monoclonal antibodies (MAI-12, Biopool and PAI-1 (ab1), Calbiochem) and protein G-agarose beads. The precipitates were separated by polyacrylamide gel electrophoresis and analyzed by autoradiography.

### *PAI-1 activity assay*

Activity of the PAI-1 present in platelets was evaluated by studying tPA-PAI-1 complex formation by two different methods. Western blot analysis was first used, and to verify the results a non-immunologic assay using $^{125}I$-labeled tPA was performed. Platelets were lysed in the presence of increasing concentrations of single chain tPA (Biopool), or, alternatively, a constant amount of tPA was used in the presence of increasing numbers of platelets. To determine the amount of active PAI-1, we identified the sample with the highest tPA concentration added without further increase of the complex formed. This was also the highest tPA concentration without detection of free tPA at 68 kDa in the Western blot analysis.

#### *Detection of tPA-PAI-1 complex by Western blot analysis*

After lysis, platelet proteins were loaded on 10% Tris-Glycine-polyacrylamide gels and immediately separated. Following electrophoresis, proteins were blotted onto PVDF (polyvinylidene fluoride) membranes (Hybond™-P Amersham Biosciences). The membranes were incubated in blocking buffer (Invitrogen) for 30 min and subsequently incubated with either PAI-1 monoclonal antibody (mab) MAI-12 or tPA mab PAM-3 (Biopool). After incubation with a peroxidase labeled secondary antibody, bands were visualized with Super Signal® West Pico Chemiluminescent Substrate (Pierce Perbio) and photographed.

In the comparison of lysis methods in Study III, results of the western blot analysis were further analyzed by quantitative scanning densitometry. Photographed membranes were analyzed using Kodak 1D Image Analysis software to estimate the relative differences in tPA-PAI-1 complex concentration in samples prepared with different lysis methods. The model net intensity (a mathematical approximation of the net intensity using a Gaussian model) of tPA-PAI-1 in sonicated or freeze/thawed samples were related to that of the tPA-PAI-1 from platelets lysed with Triton X-100.

#### *Detection of tPA-PAI-1 complex by $^{125}I$ labeled tPA*

Labeling was performed using the Iodogen method [Fraker and Speck 1978]. The concentration of tPA after labeling was determined using both total protein assay

(Bio-Rad) and ELISA (Tintelize® tPA, Biopool). Recovery of functional tPA (*i.e.* the ability to complex PAI-1) was evaluated by addition of a molar excess of active PAI-1, and labeling with $^{125}$I was shown to reduce the activity of tPA by 40%. Platelet lysates were prepared as described above but in the presence of labeled tPA. After electrophoresis, gels were dried and analyzed by autoradiography. tPA and tPA-PAI-1 complex were cut out and quantified by analysis on a Packard Cobra II Auto Gamma counter (Perkin-Elmer).

### *Affinity chromatography*

To analyze the glycosylation pattern of PAI-1 with mass spectrometry (MS), purification and concentration of the protein is crucial. Isolation of PAI-1 from conditioned media and cell lysates were made by affinity chromatography. The principle of this technique is to separate proteins on the basis of a reversible interaction between a protein and a specific ligand coupled to a chromatography matrix. The ligand in this case was a PAI-1 specific monoclonal antibody MAI-12 (Biopool), which previously has been shown to bind all different forms of PAI-1 with high affinity [Bjorquist, *et al* 1994]. MAI-12 was coupled over night to CNBr-activated Sepharose 4B (GE Healthcare) according to the manufacturers instructions. Samples were thawed and Triton X-100 was added, or samples were diluted to a final concentration of Triton X-100 of 0.025%. Samples were mixed end-over-end with MAI-12-Sepharose for 4 hours. The Sepharose was subsequently washed with 2 x 10 ml Pipes buffer containing 0.025% Triton X-100, the Sepharose was transferred to Poly-Prep® Chromatography columns (Bio-Rad Laboratories) and further washed with 20 x 500 µl Pipes-buffer. PAI-1 was eluted by 500 µl fractions of 0.2 M glycine-HCl pH 2.5. Identification of PAI-1 was made with Western blot analysis and PAI-1 containing fractions from each preparation were pooled and concentrated on Amicon Ultra filter (Millipore).

### *Mass spectrometry*

To study glycosylation patterns of PAI-1 from different cellular origins, high mass accuracy mass spectrometry was used. Isolated and purified PAI-1 samples from the different sources were separated by polyacrylamide gel electrophoresis and the gel was subsequently stained with SYPRO® Ruby or Coomassie blue. Gel bands corresponding to the MW of PAI-1 were cut and in-gel trypsin digestion of protein was performed as previously described [Shevchenko, *et al* 1997] and peptides were extracted.

NanoLC-MS/MS was performed on a $C_{18}$ – fused silica column, connected to a hybrid linear ion trap-Fourier Transform Ion Cyclotron mass spectrometer (FT-ICR MS) (LTQ-FT, Thermo Electron), equipped with a 7 T magnet. The principle of mass spectrometry is that by determining the specific masses it is possible to identify the molecules present in a sample. The molecules are ionized and vaporized, separated in an electric field, and detected according to their mass-to-charge ratio. Ion cyclotron resonance (ICR) offers a high mass resolving power and high mass accuracy. Accurate mass determination is necessary to characterize an exact modification whereas high mass resolving power is necessary to distinguish between related forms of the peptide or protein with different degrees of modification.

Sample injections were made (HTC-PAL auto sampler, CTC Analytics AG) and the tryptic peptides were trapped on a $C_{18}$-precolumn (4.5 cm x 100 μm *i.d.*) and separated in a 20 cm x 50 μm *i.d.* fused silica column packed with ReproSil-Pur C18-AQ 3 μm porous particles (Dr. Maisch GmbH). After 3 min linear run, loading the precolumn, the gradient was 0-50% $CH_3CN$, starting with 0.2% formic acid for 40 min and the eluent was electrosprayed into the mass spectrometer. The mass spectrometer was operated in the data-dependent mode to automatically switch between MS and MS/MS acquisition. Survey MS spectra (from m/z 400–1600) were acquired in the FT-ICR and the three most abundant doubly, triply or quadruply protonated ions in each FT-scan were selected for MS/MS in the linear ion trap. The monoisotopic precursor selection was turned off to enable selection of large, multiply protonated glycopeptides. The typical mass accuracy is <2 ppm in MS mode and 300-400 ppm in MS/MS mode.

Measured peptide masses and their CID spectra were analyzed by MASCOT database search software version 2.1.6 (Matrix Science, www.matrixscience.com), which incorporates a probability-based scoring. The Swiss-Prot database, 5 ppm precursor-ion mass tolerance window, 0.5 Da fragment-ion mass tolerance window and one allowed missed tryptic cleavage was used for protein identification. Mass values for peptides that could not be matched to the identified protein sequence by Mascot, were examined for the presence of glycosylation by use of the GlycoMod tool (http://us.expasy.org/tools/glycomod). The SwissProt accession number corresponding to the protein identity and unmatched monoisotopic masses were entered, and a mass deviation of 5 ppm was tolerated. Predicted glycopeptides were checked for the mass-to-charge (m/z) 366 and/or 657 oxonium ions of the oligosaccharides HexHexNAc and HexHexNAcNeuAc in the corresponding MS/MS spectrum, and thus confirming the presence of glycosylation.

*Methodological comment*: In this study, we used a mass spectrometry-based method without release of the glycans before analysis. The advantage of analyzing intact glycopeptides is that important information about which glycans are linked to the protein at a specific glycosylation site can be preserved. If the protein or peptide is deglycosylated prior to analysis, information can be obtained about the glycan pool, but these may not be assigned to their original glycosylation sites [Hakansson, *et al* 2003, Haslam, *et al* 2006]. To obtain information both on glycan position and composition, MS with a mass accuracy of about 2 ppm together with tandem MS fragment information and database interrogations were used. High mass accuracy is crucial in distinguishing different isoforms of glycopeptides, because the number of possible compositions with small mass differences decreases with increasing mass accuracy.

In tandem MS analysis of a glycopeptide, glycosidic bonds are more susceptible to CID fragmentation than peptide bonds, resulting in a sequential loss of glycan components from the terminal end of the attached glycan and leaving the peptide intact. The MS/MS spectra of a glycopeptide (Figure 2) is therefore easier to annotate than the complex spectra of a glycan moiety detached from its protein. Due to the low order of glycopeptides in a mixture of tryptic digests from a gel band and the typical microheterogeneity of glycosylation, very sensitive MS instruments with capability

of high molecular mass detection are needed, as for example Q-TOF or FT-ICR mass spectrometers [Wuhrer, *et al* 2007]. Glycopeptide analysis depends on the ionization efficiency of the peptide composition and the mixture of the tryptic digest. By using nanoLC separation directly coupled to the MS instrument, the low abundant glycopeptides are more frequently detected. The sensitivity of the described nanoLC-MS/MS is in the range of a few femtomoles loaded onto the column.

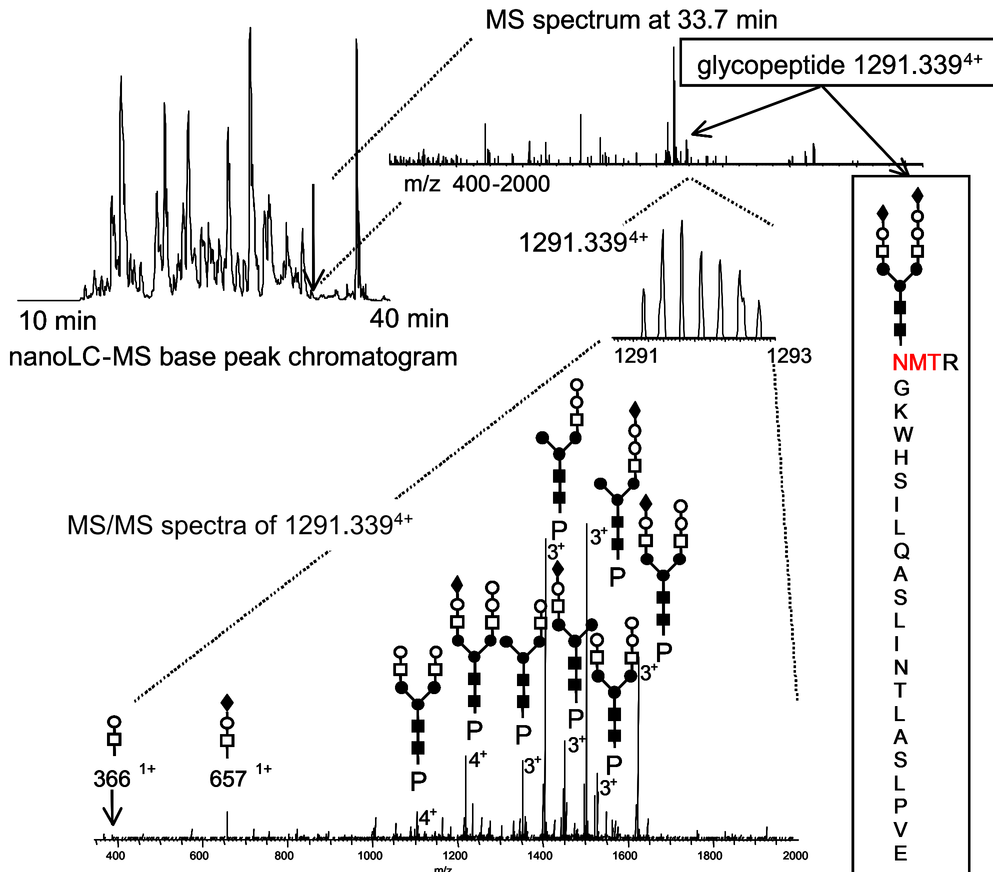


**Figure 2.** NanoLC-LTQ-FT-ICR MS analysis of plasma PAI-1 from an individual with BMI>35. The same molecular mass eluting at 33.7 min (MH+ 5162.323) corresponding to the glycopeptide with the attached glycan (Hex)3(HexNAc)2(NeuAc)2(Man)3(GlcNAc)2 was also found in adipose tissue. The quadruply protonated ion m/z 1291.339 (MH+ 5162.323) is analyzed with high resolution and a mass accuracy of about 2 ppm in the ICR cell. The bottom spectra shows the fragmentation (MS/MS) pattern of the saccharides attached to the peptide (P) and the diagnostic glycan ions at m/z 366 (Hex-HexNAc) and 657 (HexHexNAcNeuAc).

### *Glycoprotein specific staining*

To verify presence or absence of glycosylation, ProQ Emerald 300 glycoprotein gel stain kit (Molecular Probes) was used. The Pro-Q emerald stain reacts with peroxidate-oxidized carbohydrate groups, creating a bright green-fluorescent signal on glycoproteins. The detection limit of the Pro-Q emerald stain was investigated by SDS-PAGE of serial dilutions of recombinant human glycosylated PAI-1 expressed in Chinese hamster ovary cells (CHO) [Stromqvist, *et al* 1994] subsequently SYPRO® Ruby stain was performed for total protein staining. PAI-1 isolated from platelets and plasma both from lean and from obese subjects was separated by SDS-PAGE and the gels were stained with Pro-Q according to the manufacturer's instructions. As positive controls PAI-1 from adipose tissue and also 50 ng CHO PAI-1 was used. After scanning (Fluor-S MultiImager® Bio-Rad Laboratories), the gel was stained for total protein by SYPRO ruby.

## Statistical methods

Standard statistical methods were used. Data are presented as mean and standard error of the mean, unless otherwise stated. Paired Student's t-test was used after log-transformation for evaluation of the changes in platelet PAI-1 concentration in 24 hours. Comparisons of results between groups were performed with unpaired student's t-test (two-tailed), and significance tests were considered significant at $p<0.05$ (two-tailed test). Pearson correlation coefficients were calculated to determine the associations between PAI-1 mRNA and PAI-1 antigen in plasma and platelets as well as to study the associations between the three ELISA kits.

# RESULTS

## Synthesis of active PAI-1

To investigate if platelets are able to *de novo* synthesize PAI-1 we studied the mRNA content and protein synthesis in platelets. Platelet PAI-1 mRNA was quantified by real-time PCR and substantial amounts were consistently detected in all platelet samples. At baseline, the expression level of PAI-1 mRNA was approximately 6% compared to GAPDH and 7% compared to cyclophilin. The degradation rate of PAI-1 mRNA compared to GAPDH and cyclophilin was analyzed by incubation of platelets for 0, 3, 8, and 24 hours followed by analysis of mRNA by real-time PCR. As shown in Figure 3, the degradation rate was found to be 4 and 8-fold higher than that of GAPDH and cyclophilin, respectively.

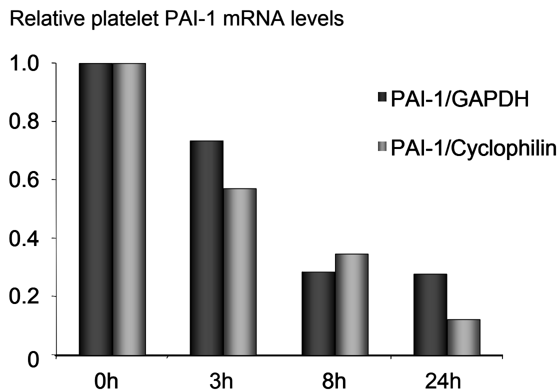


**Figure 3.** The relative degradation rate of platelet PAI-1 mRNA in comparison to GAPDH and cyclophilin. mRNA extracted from platelets incubated 0, 3, 8, and 24 hours was determined by real-time PCR and the degradation rate of PAI-1 mRNA was 4-fold higher than that of GAPDH and 8-fold higher than cyclophilin.

To study if there is an on-going synthesis and to estimate the PAI-1 protein synthesis rate, platelets were incubated and the total amount of PAI-1 antigen was analyzed by ELISA at baseline and after 24 hours. In fresh platelets, the average content of PAI-1 was 1.00 ± 0.33 (mean and SD) ng/$10^6$ platelets. After 24 hours of incubation, the concentration of PAI-1 increased in 16 out of 18 samples with individual responses ranging between 2 and 52%. On the average, the PAI-1 content increased by 25% to 1.25 ± 0.54 (mean and SD) ng per million platelets (p=0.001).

To confirm that there was an on-going *de novo* synthesis of PAI-1 in platelets, metabolic radio-labeling was performed. Following $^{35}$S-methionine incorporation for 1, 3, and 6 hours, immunoprecipitation was performed, which yielded a protein of the expected molecular mass of approximately 45 kDa. The protein was detected with two different antibodies, MAI-12 and PAI-1 (ab-1). Figure 4A shows the results of the immunoprecipitation with PAI-1 (ab-1) of $^{35}$S-labeled PAI-1. The increasing amount of radioactive PAI-1 over time confirms that there is an on-going synthesis of PAI-1 in platelets.

To inhibit protein translation, puromycin was added and as shown in Figure 4B, this resulted in a partial inhibition of protein synthesis. We also investigated if the synthesis could be stimulated by a platelet agonist. Thrombin was added and platelets were incubated for 1, 3, and 6 hours and as shown in Figure 5 thrombin activation was found to increase the rate of *de novo* synthesis of PAI-1.

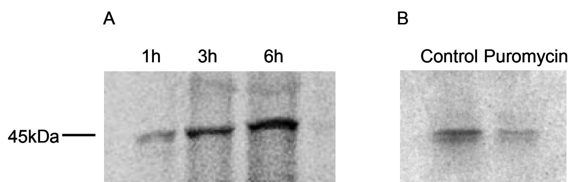


**Figure 4.** Metabolic radio-labeling and immunoprecipitation of platelet PAI-1. A. Isolated platelets incubated in the presence of $^{35}$S-methionine for 1, 3, and 6 hours. Platelet lysate and medium immunoprecipitated with PAI-1 (ab-1). The increasing amount of radioactive PAI-1 over time confirmed an on-going synthesis. B. Platelets incubated for 6 hours in the presence of 1 mM puromycin, which resulted in attenuated PAI-1 expression.

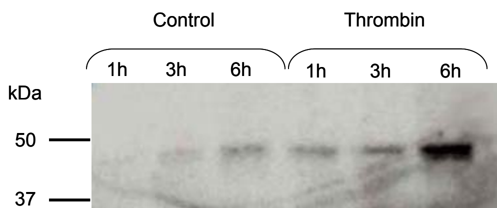


**Figure 5.** Stimulation of PAI-1 synthesis with thrombin. To investigate if the platelets could be stimulated to increase the synthesis rate, platelets were incubated for 1, 3, and 6 hours as described in Figure 4 in the absence or presence of 0.1 U/ml thrombin. Thrombin activation was found to increase the rate of PAI-1 synthesis.

Investigation of the activity of the newly synthesized PAI-1 was performed using a functional assay in which platelets were incubated with $^{35}$S-methionine in the presence of increasing tPA concentrations. PAI-1 as well as the tPA-PAI-1 complex was detected by immunoprecipitation with MAI-12. Incubations with tPA resulted in a shift of molecular weight corresponding to the expected weight of the tPA-PAI-1 complex Figure 6A. This finding indicated that the newly formed PAI-1 was in an active configuration, and, as shown in Figure 6B, addition of increasing concentrations of tPA resulted in a gradual diminution of free PAI-1 protein.

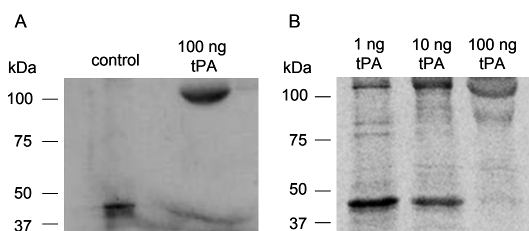


**Figure 6.** Functional analysis of the activity of newly synthesized PAI-1. Platelets were incubated in the presence of 1, 10, and 100 ng tPA for 6 hours. A. Addition of 100 ng tPA resulted in a shift in molecular weight corresponding to the expected ~110 kDa of tPA-PAI-1 complex, indicating that newly synthesized PAI-1 is active. B. Addition of increasing concentrations of tPA reduced the free PAI-1 protein and increased the tPA-PAI-1 complex.

### Influence of the 4G/5G polymorphism on platelet PAI-1 expression

To investigate if the PAI-1 promoter polymorphism influences PAI-1 expression in platelets, healthy male subjects were genotyped for the 4G/5G polymorphism. Subjects homozygous for either the 4G or 5G allele were investigated regarding PAI-1 mRNA and protein levels in platelets. Eighty-six male subjects were genotyped using the Taqman-based allelic discrimination 5´ nuclease assay and 21 (24.4%) were homozygous for the 4G allele, 48 (55.8%) were heterozygotes, and 17 (19.8%) were 5G homozygotes. Homozygous subjects (n=38) were selected for further analysis of platelet PAI-1 mRNA as well as PAI-1 antigen in plasma and platelets. Parameters analyzed and compared between the two groups are summarized in Table 2. The mean plasma PAI-1 concentration in the 4G homozygotes was on the average 25% higher than in 5G (6.53 versus 5.22 ng/ml, respectively), but the difference between the genotypes was not significant. Also, there was no significant difference in platelet PAI-1 concentration between the groups (0.23 ng/$10^6$ platelets throughout).

**Table 2.** Summary of observed parameters. Results are presented as mean and standard error of the mean.

| | **4G/4G** (n=21) | **5G/5G** (n=17) | **Significance** |
|---|---|---|---|
| Age (years) | 27.8 (1.6) | 32.5 (2.1) | ns |
| BMI (kg/m$^2$) | 23.2 (0.5) | 24.1 (0.7) | ns |
| TPK (plt/l) | 242 (12.1) | 238 (11.0) | ns |
| Triglyceride (mmol/l) | 1.25 (0.13) | 1.18 (0.16) | ns |
| Plasma PAI-1 (ng/ml) | 6.53 (1.09) | 5.22 (1.08) | ns |
| Platelet PAI-1 (ng/milj plt) | 0.229 (0.022) | 0.230 (0.027) | ns |
| Platelet PAI-1 (ng/ml) | 56.29 (6.64) | 57.28 (8.20) | ns |
| mRNA PAI-1/GAPDH | 0.0281 (0.0023) | 0.0255 (0.0024) | ns |
| mRNA PAI-1/cyclophilin | 0.0245 (0.0018) | 0.0265 (0.0030) | ns |
| mRNA PAI-1 ($C_T$) | 27.9 (0.15) | 28.0 (0.24) | ns |

The expression level of PAI-1 mRNA was 2 - 10% of GAPDH and cyclophilin in all samples. No significant differences in mRNA levels between the genotypes were found, irrespective of whether PAI-1 mRNA was normalized to GAPDH, cyclophilin, or total RNA. However, as shown in Table 3, significant correlations appeared between platelet PAI-1 antigen and mRNA both when normalizing to GAPDH and cyclophilin and also when related to total RNA.

**Table 3.** Associations between platelet PAI-1 mRNA and PAI-1 antigen in platelets and plasma.

| | PAI-1/ GAPDH | PAI-1/ Cyclophilin | PAI-1/ total RNA |
|---|---|---|---|
| Platelet PAI-1 | 0.377* | 0.445** | 0.556** |
| Plasma PAI-1 | 0.174 | 0.113 | 0.067 |

* Correlation is significant at the 0.05 level (2-tailed)
** Correlation is significant at the 0.01 level (2-tailed)

There was no significant correlation between platelet PAI-1 and plasma PAI-1 concentrations, but a significant correlation was observed between platelet count and PAI-1 in plasma ($r = 0.32$, $p < 0.05$). As expected, there was a close correlation between BMI and plasma PAI-1 ($r = 0.61$, $p < 0.001$), and also a significant correlation between plasma PAI-1 and triglycerides ($r = 0.40$, $p = 0.01$).

## Analysis of platelet PAI-1 activity

In order to evaluate the activity of platelet PAI-1, serial dilutions of platelets and tPA were performed followed by studies of tPA-PAI-1 complex formed. After lysis of platelets with Triton X-100 in the presence of tPA, tPA-PAI-1 complex was detected by Western blot analysis. To confirm the results using a non-immunologic assay, tPA was labeled with $^{125}I$ and $^{125}I$-tPA-PAI-1 was detected with autoradiography and quantified by scintigraphy.

Western blot analysis of lysates was performed with specific antibodies directed against PAI-1 and tPA. PAI-1 mab MAI-12 detected both free PAI-1 at ~47 kDa and the ~110 kDa complex with tPA but with higher affinity for the tPA-PAI-1 complex, as previously reported [Huisman L.G.M 1992]. With increasing tPA concentrations, the amount of tPA-PAI-1 complex increased until a molar excess of tPA was reached. Using tPA mab, the same dose-dependent response of the complex was observed. When the amount of tPA added to the platelets exceeded the amount of active PAI-1, a 68 kDa band appeared representing free tPA (Figure 7). The highest concentration of tPA added without detection of free tPA was used to calculate the concentration of active PAI-1, assuming a 1:1 stoichiometry complex and a molecular weight of 47 kDa and 68 kDa for PAI-1 and tPA, respectively.

To determine the total amount of PAI-1 antigen in platelets, we used three different commercially available PAI-1 ELISA kits. The average PAI-1 antigen level was 0.48 (±0.08), 0.79 (±0.13), and 0.64 (±0.23) ng/$10^6$ platelets with TintElize, Coaliza, and Imubind respectively and mean was 0.64 ng/$10^6$ platelets.

The calculated amount of functionally active PAI-1 was related to the total PAI-1 antigen determined by each assay. This resulted in an activity of 117% (±9), 65% (±5), and 73% (±11) related to the results with TintElize, Coaliza, and Imubind, respec-

tively. This suggests an underestimation of total platelet PAI-1 antigen by TintElize. When using the average PAI-1 antigen concentration from the three ELISA kits, the amount of functionally active PAI-1 was estimated to 81%.

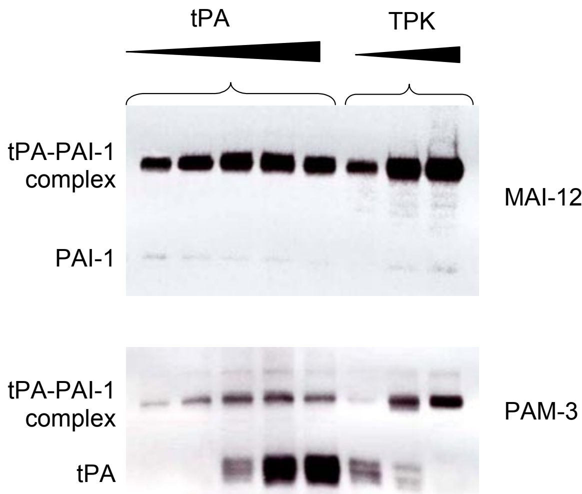


**Figure 7.** Platelets lysed in the presence of tPA. Lane 1-5; constant number of platelets lysed in the presence of increasing tPA concentration. Lane 5-8; constant tPA and increasing number of platelets. The upper membrane is incubated with PAI-1 mab MAI-12 and the lower membrane is incubated with the tPA mab PAM-3.

Using $^{125}$I-tPA and scintigraphy, it was possible to determine the amount of $^{125}$I-tPA added without any further increase of the tPA-PAI-1 complex (Figure 8). The highest concentration of $^{125}$I-tPA added without reaching maximum binding was compared to the total PAI-1 in the samples determined by the three ELISA kits as described above. The mean PAI-1 activity in the samples was 72%. Using the total PAI-1 antigen determined by TintElize, the calculated activity was 82% (±5,6). The correspondings figures for Coaliza and Imubind were 53% (±3,2) and 82% (±13.4), respectively.

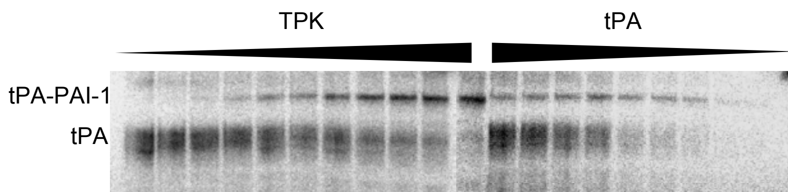


**Figure 8.** Platelets lysed in the presence of $^{125}$I labeled tPA. Lane 1-11 shows a constant amount of tPA in the presence of increasing numbers of platelets. In lane 12-20 a constant amount of platelets with decreasing concentrations of tPA.

## Analysis of the effect of different lysis methods on PAI-1 activity

To assess whether the method of platelet disruption affects the activity of PAI-1, we performed a series of experiments with sonication and freezing/thawing to compare with the results from lysis in the presence of Triton X-100. As shown in Figure 9, the results demonstrate that sonication dramatically reduced the activity of PAI-1. Furthermore, there appeared to be a dose-response relationship between sonication energy and degree of inactivation, since the activity in samples sonicated with high energy was considerably lower (Figure 9 A) compared to samples prepared with low energy (Figure 9 B). Analyzing the ratio between densitometric intensity of the tPA-PAI-1 bands, sonication with high energy reduced the activity by approximately 90% whereas with low energy there was a 50 - 60% reduction.

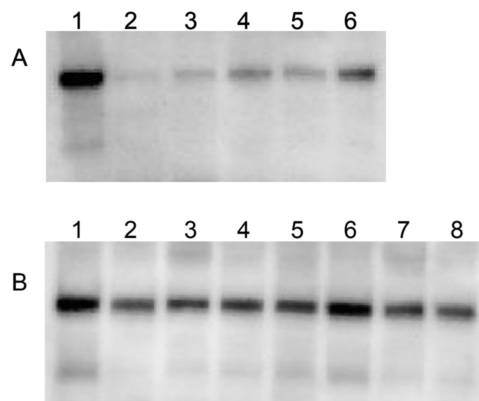


**Figure 9.** Western blot analysis showing the differences of PAI-1 activity depending on lysis method. tPA-PAI-1 complex is detected by MAI-12. Membrane A shows the samples sonicated with setting 7 and membrane B represents the samples sonicated with setting 2. Same number of platelets is lysed and the same amount of tPA is added in all samples. The results of the densitometry are presented in brackets. Lane 1: Platelets lysed with 0.1% Triton X-100 in the presence of tPA (100%). Lane 2: Platelets lysed by sonication in homogenization buffer (A 8%, B 44%). Lane 3: Platelets sonicated in Pipes buffer (A 12%, B 51%). Lane 4: Platelets lysed in Pipes buffer by freezing and thawing (A 21%, B 53%). Lane 5: Platelets lysed by sonication in Pipes buffer with tPA present (A 16%, B 54%). Lane 6 on membrane A: Platelets lysed by freezing and thawing in the presence of tPA (39%). Lane 6 (100%), 7 (58%) and 8 (54%) on membrane B represent sample 1, 3 and 4 with the addition of 0.1% Triton X-100 to sample 3 and 4 after sonication or freezing/thawing.

There was no major difference in complex formation between samples sonicated with or without tPA present, indicating that sonication alone causes the reduction in activity and it is not merely an effect of freezing and thawing of the samples. The reduction in activity was similar between samples sonicated and lysed by freezing and thawing. However, samples freeze/thawed with tPA present had a higher activity than samples to which tPA was added after lysis. Addition of 0.1% Triton X-100 to the sonicated and freeze/thawed samples did not affect the results of the Western blot analysis. The effect of sonication on control plasma from Biopool was determined by Chromolize activity assay and sonication with setting 7 resulted in a 50% reduced activity. In contrast, with sonication at setting 2 there was no significant decrease.

## Mass spectrometry analysis of glycosylation patterns of PAI-1

Next, we investigated if it would be possible to reveal the source of plasma PAI-1 by studying tissue specific glycosylation patterns. PAI-1 was purified and concentrated by affinity chromatography from plasma, platelets, adipose tissue, HUVEC, hepatocytes, and macrophages. The amount obtained from the different sources, as well as the purity of the retrieved PAI-1, varied considerably. Significant amounts of PAI-1 were purified from pooled plasma PAI-1 from lean subjects as well as from platelets, adipose tissue, and HUVEC and were all visible with Coomassie blue staining. However, the more sensitive SYPRO Ruby stain had to be used for detection of PAI-1 from macrophages and the plasma samples from each of the four obese subjects because of the limited amount of protein obtained from these sources. Figure 10 shows typical gel images of PAI-1 from the different sources. Gel bands corresponding to the MW of PAI-1 were analyzed and identified as PAI-1 using several significant MS/MS spectra of unmodified PAI-1 peptides.

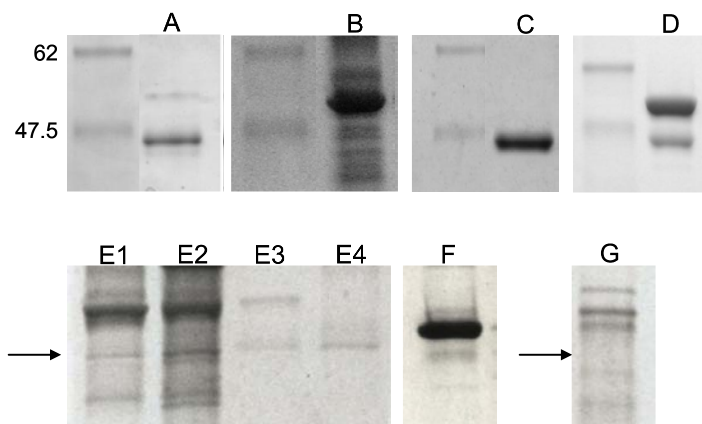


**Figure 10.** Gel images of PAI-1 isolated from the different sources. The upper gels are stained with Coomassie and a pre-stained molecular weight protein standard was used. The lower gels are stained with the more sensitive SYPRO Ruby stain without staining of the protein standard. The lanes represent the different sources as follows A: platelets, B: plasma from lean subjects, C: HUVEC, D: adipose tissue, E: plasma from subjects with BMI >35, F: macrophages and, G: hepatocytes. PAI-1 was identified by several significant MS/MS spectra of unmodified peptides in the bands of ~45 kDa. PAI-1 was not identified in any of the bands isolated from hepatocytes.

Table 4 summarizes glycopeptides and predicted glycan compositions at the two N-linked glycosylation sites found in the nanoLC-MS and MS/MS analysis of PAI-1 from the specific sources. For some of the glycopeptide masses, there are two different explanations of glycan moieties with different compositions but the same mass. For example, the mass 2654.016 of one of the glycopeptides can be explained as $(Hex)_2(HexNAc)_2(Deoxyhexose)_1(NeuAc)_1$ + $(Man)_3(GlcNAc)_2$ or as $(Hex)_3(HexNAc)_2(NeuAc)_1$+ $(Man)_3(GlcNAc)_2$.

**Table 4.** Summary of the masses and possible compostions of glycans found at the two sites on PAI-1 (N232 and N288) from the different sources. Glycans found in more than one tissue are indicated in bold type and masses with two possible glycan compositions are indicated in italic. The mass accuracy is specified in ppm, number of missed proteolytic cleavage is presented as well as presence of oxidised methionine (Y=yes).

**Glycostructures at position N288 at peptide position GNMTR**

**Adipose tissue**

| MH$^+$ | Glycan | ppm | Missed cleavage | M$_{ox}$ |
|---|---|---|---|---|
| **2929.107** | **(Hex)$_2$ (HexNAc)$_2$ (Deoxyhexose)$_1$ (NeuAc)$_2$ + (Man)$_3$(GlcNAc)$_2$** | **1.9** | - | - |
| **2784.075, *5001.303*** | **(Hex)$_2$ (HexNAc)$_2$ (Deoxyhexose)$_2$ (NeuAc)$_1$ + (Man)$_3$(GlcNAc)$_2$** | **3.8, 4.6** | **-,1** | **-,Y** |
| **5162.323** | **(Hex)$_3$ (HexNAc)$_2$ (NeuAc)$_2$ + (Man)$_3$(GlcNAc)$_2$** | **2.1** | - | **Y** |
| ***5001.303*** | **(Hex)$_3$ (HexNAc)$_2$ (Deoxyhexose)$_1$ (NeuAc)$_1$ + (Man)$_3$(GlcNAc)$_2$** | **4.6** | **1** | **Y** |
| ***2962.131*** | **(Hex)$_3$ (HexNAc)$_2$ (Deoxyhexose)$_2$ (NeuAc)$_1$ + (Man)$_3$(GlcNAc)$_2$** | **6.3** | - | **Y** |
| **5017.303, 5033.286** | **(Hex)$_4$ (HexNAc)$_2$ (NeuAc)$_1$ + (Man)$_3$(GlcNAc)$_2$** | **5.5, 3.2** | **1, 1** | **-,-** |
| ***2962.131*** | **(Hex)$_4$ (HexNAc)$_2$ (Deoxyhexose)$_1$ (NeuAc)$_1$ + (Man)$_3$(GlcNAc)$_2$** | **6.3** | - | - |
| 3365.253 | (Hex)$_2$ (HexNAc)$_2$ (NeuAc)$_4$ + (Man)$_3$(GlcNAc)$_2$ | 5.5 | - | - |
| 2638.013, *2654,016* | (Hex)$_2$ (HexNAc)$_2$ (Deoxyhexose)$_1$ (NeuAc)$_1$ + (Man)$_3$(GlcNAc)$_2$ | 2.5, 5.5 | -,- | -,Y |
| *2566.000* | (Hex)$_2$ (HexNAc)$_3$ (Deoxyhexose)$_1$ + (Man)$_3$(GlcNAc)$_2$ | 5.7 | - | Y |
| 2841.080 | (Hex)$_2$ (HexNAc)$_3$ (Deoxyhexose)$_1$ (NeuAc)$_1$ + (Man)$_3$(GlcNAc)$_2$ | 1.9, 5.5 | - | Y |
| *2654.016* | (Hex)$_3$ (HexNAc)$_2$ (NeuAc)$_1$ + (Man)$_3$(GlcNAc)$_2$ | 5.5 | - | Y |
| *2566.000* | (Hex)$_3$ (HexNAc)$_3$ + (Man)$_3$(GlcNAc)$_2$ | 5.7 | - | - |
| 5074.311 | (Hex)$_3$ (HexNAc)$_3$ (NeuAc)$_1$ + (Man)$_3$(GlcNAc)$_2$ | 2.9 | 1 | Y |

**Plasma BMI >35**

| MH$^+$ | Glycan | ppm | Missed cleavage | M$_{ox}$ |
|---|---|---|---|---|
| **2929.103, *5146.327*, 5130.318** | **(Hex)$_2$ (HexNAc)$_2$ (Deoxyhexose)$_1$ (NeuAc)$_2$ + (Man)$_3$(GlcNAc)$_2$** | **0.5, 1.9, 0.8** | **1,1,1** | **-,Y,-** |
| ***2800.062*** | **(Hex)$_2$ (HexNAc)$_2$ (Deoxyhexose)$_2$ (NeuAc)$_1$ + (Man)$_3$(GlcNAc)$_2$** | **1.0** | - | **Y** |
| ***5146.327*, 5162.334** | **(Hex)$_3$ (HexNAc)$_2$ (NeuAc)$_2$ + (Man)$_3$(GlcNAc)$_2$** | **1.9, 4.2** | **1, 1** | **-,Y** |
| ***2800.062*** | **(Hex)$_3$ (HexNAc)$_2$ (Deoxyhexose)$_1$ (NeuAc)$_1$ + (Man)$_3$(GlcNAc)$_2$** | **1.0** | - | - |
| **3312.363** | **(Hex)$_3$ (HexNAc)$_2$ (Deoxyhexose)$_2$ (NeuAc)$_1$ + (Man)$_3$(GlcNAc)$_2$** | **2.3** | **1** | - |
| ***3182.310, 5017.258*** | **(Hex)$_4$ (HexNAc)$_2$ (NeuAc)$_1$ + (Man)$_3$(GlcNAc)$_2$** | **5.5, 3.4** | **1** | **Y** |
| **5179.306** | **(Hex)$_4$ (HexNAc)$_2$ (Deoxyhexose)$_1$ (NeuAc)$_1$ + (Man)$_3$(GlcNAc)$_2$** | **4.3** | **1** | **Y** |
| *4984.262* | (Hex)$_1$ (HexNAc)$_2$ (Deoxyhexose)$_1$ (NeuAc)$_2$ + (Man)$_3$(GlcNAc)$_2$ | 0.5 | 1 | - |
| 2783.046, 3165.290, *4984.262*, 5000.262 | (Hex)$_2$ (HexNAc)$_2$ (NeuAc)$_2$ + (Man)$_3$(GlcNAc)$_2$ | 0.9, 4.2, 0.5, 0.6 | -,-,1,1 | -,-,Y,- |

**Macrophage**

| MH$^+$ | Glycan | ppm | Missed cleavage | M$_{ox}$ |
|---|---|---|---|---|
| 2872.046 | (Hex)$_2$ (HexNAc)$_1$ (Deoxyhexose)$_2$ (NeuAc)$_2$ + (Man)$_3$(GlcNAc)$_2$ | 12 | - | Y |

**Glycostructures at position N232 at peptide position FNYTE**

**HUVEC**

| MH$^+$ | Glycan | ppm | Missed cleavage | M$_{ox}$ |
|---|---|---|---|---|
| 5637.45 | (Hex)$_3$ + (Man)$_3$(GlcNAc)$_2$ | 2.0 | - | - |
| 5799.49 | (Hex)$_4$+ (Man)$_3$(GlcNAc)$_2$ | 0.2 | - | - |

After gel electrophoresis, protein bands were identified as PAI-1 in the platelet as well as in the plasma preparations but no glycopeptides were detected. The largest numbers of glycans were found on PAI-1 from adipose tissue and 14 suggested glycans were identified. However, four of the detected masses had two possible glycan compositions. There were different numbers of glycans identified at N288 in the three different preparations with the highest amount of glycans in the preparation were the highest amount of PAI-1 was obtained. Seven of the glycan compositions found in adipose PAI-1 were also found on plasma PAI-1 from obese subjects. The total number of glycans at N288 on plasma PAI-1 from the subjects with BMI >35 subjects was 9, but three of the masses had two possible compositions. PAI-1 was isolated separately from the four obese subjects and as can be seen in Figure 10 (lane E1 to E4) the

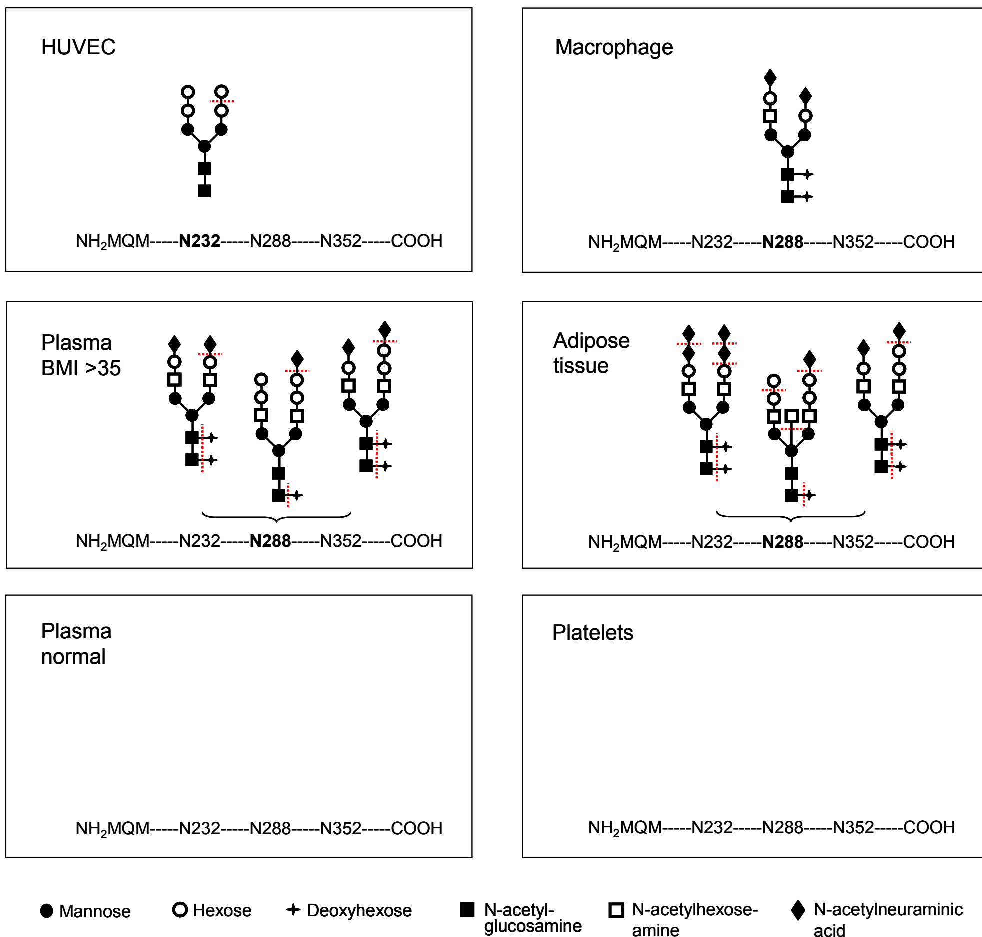


**Figure 11.** Summary of the possible glycan compositions at the two sites on PAI-1 from the analyzed cell types and plasma. Because of the heterogeneity of the glycan compositions on PAI-1 from adipose tissue and from plasma from obese subjects, three structures had to be made to summarize the possible compositions. The dashed lines indicate the different glycan compositions at that site.

amount of plasma PAI-1 from the subjects differed substantially. As in the preparations of PAI-1 from adipose tissue, the largest number of detected glycans was found on PAI-1 from the subject from which the largest amount of PAI-1 was isolated (E2). In sample E1, E3, and E4 only three glycans were identified, but they were identical and also present in E2.

The limited amount of PAI-1 obtained from macrophages made precise identification of the composition of the glycans difficult. However, diagnostic glycan ions were detected, excluding the possibility of no glycosylation.

HUVEC was the only cell type where glycans were detected on N232 at peptide position FNYTE. Two different compositions were detected at this position, but the other site was not utilized.

Figure 10G shows the preparation from hepatocytes and as indicated by the absence of bands at 45 kDa, no PAI-1 was detected with MS/MS in this sample.

In Figure 11, a summary of the glycosylation pattern of PAI-1 from the different origins is shown.

To verify the MS results of the absence or presence of glycosylated PAI-1 from different tissues, we used 1D gel electrophoresis and Pro-Q glycoprotein gel stain. The results of the glycoprotein staining verified the MS/MS results, and as shown in Figure 12A, we did not detect glycosylated PAI-1 in platelets or plasma from lean subjects. However, plasma PAI-1 from obese subjects as well as adipose tissue and CHO were all positive with the glycoprotein stain. Subsequent staining with total protein SYPRO ruby showed the presence of PAI-1 in all samples (Figure 12B).

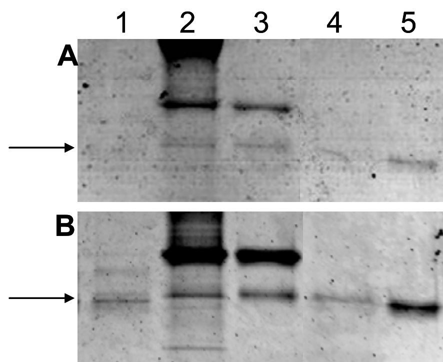


**Figure 12.** Gel image showing the results of glyco-specific staining with A: Pro-Q Emerald and B: same gel subsequently stained with SYPRO Ruby. PAI-1 from adipose tissue and from CHO cells were used as positive controls. Samples with PAI-1 from the different sources are in the following order; lane 1: plasma PAI-1 from lean subjects, lane 2: plasma PAI-1 from subject with BMI>35, lane 3: PAI-1 from adipose tissue, lane 4: platelet PAI-1 and lane 5: 50 ng of CHO PAI-1. PAI-1 from platelets and plasma from lean subjects was not positive in gel A stained with Pro-Q but visible in gel B stained with total protein stain SYPRO Ruby.

# DISCUSSION

The present studies were performed to investigate the role of platelets in inhibition of fibrinolysis by synthesis and release of PAI-1. The thesis presents three major findings.

First, our studies showed that platelets contain substantial amounts of PAI-1 mRNA, as detected by real-time PCR, and incorporation studies with radioactive methionine followed by immunoprecipitation confirmed that this mRNA was translationally active. The total amount of PAI-1 protein increased by an average of 25% in 24 hours, and the synthesis rate could be further stimulated by activation of platelets with thrombin. Importantly, the newly formed protein was active as shown by its ability to complex-bind tPA. However, the 4G/5G promoter polymorphism did not seem to influence the expression since there were no associations between genotype and levels of platelet PAI-1 mRNA or protein. These observations show that *platelets synthesize large amounts of active PAI-1*.

Second, the controversial issue of the activity of the total pool of PAI-1 in platelets was reinvestigated with a simple and direct functional approach using two assays based on reciprocating serial dilutions of tPA and platelets. Using this approach, it was found that the activity of platelet PAI-1 is considerably higher than previously reported in most studies. The average PAI-1 activity was estimated to 81% in samples analyzed by Western blot and 72% in samples analyzed with $^{125}$I-labeled tPA. Our results show that both sonication and freezing/thawing of the samples substantially reduced the detected PAI-1 activity, which may explain the low activity observed in previous studies using these lysis protocols. The results suggest that, in contrast to the traditional view, *the majority of PAI-1 in platelets is active and can inhibit fibrinolysis*.

Third, high mass accuracy mass spectrometry was used to study glycosylation of PAI-1 expressed in human plasma, platelets, adipose tissue, endothelial cells, and macrophages. Both PAI-1 from platelets and plasma from lean subjects were found to be unglycosylated. In contrast, PAI-1 from all other tissues as well as plasma PAI-1 isolated from subjects with BMI >35 was glycosylated with heterogeneous tissue-specific glycosylation patterns. This finding suggested that the *platelets may be the hitherto unknown source of plasma PAI-1*.

## *De novo* synthesis of PAI-1 in platelets

Platelets have traditionally been considered to be devoid of capacity for protein synthesis. However, even though circulating platelets are anucleate and cannot synthesize mRNA, they retain mRNA from the megakaryocyte stage [Booyse and Rafelson 1967a, Booyse and Rafelson 1967b, Newman, *et al* 1988, Gnatenko, *et al* 2003, McRedmond, *et al* 2004, Dittrich, *et al* 2006 ]. In this study we found that they also contain PAI-1 mRNA in considerable amounts. The level of PAI-1 mRNA at baseline was approximately 5 - 10% of GAPDH and cyclophilin. As shown previously, the degradation rates of different platelet mRNA species vary substantially [Wang, *et al* 2003], and this was found to be true also for PAI-1 mRNA. During the 24-hour incu-

bation period the degradation rate of PAI-1 mRNA was approximately 4-fold higher than that of GAPDH and 8-fold higher than cyclophilin. The finding suggests that the PAI-1 mRNA content of young, newly released platelets may be considerably higher.

The question that immediately arose from these studies was if this PAI-1 mRNA also could be translated into protein. Platelets have both rough endoplasmic reticulum and polyribosomes [Kieffer, *et al* 1987], and some occasional previous reports have found evidence of protein synthesis in platelets [Kieffer, *et al* 1987, Weyrich, *et al* 1998, Lindemann, *et al* 2001]. Prompted by these observations, we investigated if the platelets had the ability to *de novo* synthesize PAI-1 and found this to be the case. At baseline, the content of PAI-1 was approximately 1 ng per million platelets, which is comparable to what has been reported earlier [Booth, *et al* 1988, Declerck, *et al* 1988a]. During a 24-hour incubation period, the total amount of PAI-1 increased by 25%, showing that despite its decay rate considerable amounts of translationally active mRNA were still present. Metabolic radio-labeling with $^{35}$S-methionine and immunoprecipitation confirmed that there was an on-going *de novo* synthesis of the protein in the platelets. Also, the synthesis could be blocked by the protein synthesis blocker puromycin. However, our data also showed that the synthesis rate of PAI-1 varied considerably among individuals, possibly as an effect of the age distribution of the platelet population.

### No effect of the 4G/5G polymorphism on platelet PAI-1 expression

Besides an effect of platelet age, another interesting possibility is that the synthesis of PAI-1 could be influenced by genetic factors, such as the 4G/5G polymorphism in the PAI-1 promoter. *In vitro* promoter studies have shown that the 4G allele has a higher transcriptional activity than the 5G one, which has been suggested to be due to a differential binding of a repressor to the latter [Eriksson, *et al* 1995]. The influence on plasma PAI-1 antigen and activity levels is controversial and many studies have been performed to shed light on this issue. Some studies have found associations both in healthy subjects and in patients with hypertension, myocardial infarction, or atherosclerosis [Eriksson, *et al* 1995, Jeng 2003, Juhan-Vague, *et al* 2003, Martinez-Calatrava, *et al* 2007], whereas others have failed to demonstrate associations between PAI-1 antigen or activity levels and the 4G/5G polymorphism [Doggen, *et al* 1999, Nordt, *et al* 2003, Zietz, *et al* 2004]. Thus, it has been shown that the basal transcription might not be affected [Dawson, *et al* 1993, Zhan, *et a*l 2004], and various influences of this putative repressor might explain why some studies have found an association of the 4G/5G genotype with PAI-1 levels whereas others have not.

In our study, we did not find any significant associations between genotype and levels of PAI-1 mRNA or protein in platelets. Moreover, no correlations between the circulating plasma PAI-1 levels and the levels of platelet PAI-1 protein or mRNA was found. As expected, however, irrespective of genotype, platelet PAI-1 mRNA and PAI-1 antigen levels were found to correlate, regardless of to which of the controls the mRNA level was normalized. To validate the representativity of our results we looked for the known correlation between BMI or triglycerides and plasma PAI-1 [Cigolini, *et al* 1994, Hong, *et al* 1997] and found, as expected, highly significant cor-

relations. A potential limitation of this study is obviously that the limited number of subject has caused a Type II statistical error. However, since the average PAI-1 mRNA levels were almost identical among the two genotypes, it seems unlikely that there is a major physiological impact of the polymorphism on platelet (or megakaryocyte) PAI-1 synthesis in healthy subjects.

## Activity of platelet PAI-1

Following our initial discovery of a *de novo* synthesis of PAI-1 in the platelets, the next important issue was whether the newly formed protein was enzymatically active. Since it is difficult to directly determine the activity of PAI-1, in our first series of experiments we used a functional assay to address this question. The basis for this approach is that only active PAI-1 is capable of forming a stoichiometric 1:1 complex with tPA [Kooistra, *et al* 1986]. This analysis confirmed that both during basal conditions and when protein synthesis was stimulated by thrombin, the new PAI-1 was active. Unexpectedly, platelets lysed in the presence of tPA after 24 hours of radiolabeling showed that almost all of the PAI-1 synthesized was active. This observation indicated that the protein could be kept in an active configuration in the platelet for a much longer period of time that previously thought.

The activity of the total pool of PAI-1 in platelets has generally been considered to be as low as 5% [Booth, *et al* 1990, Booth, *et al* 1988, Declerck, *et al* 1988a, Lang and Schleef 1996]. It has been assumed that the low activity of platelet PAI-1 observed in most studies is explained by the rapid spontaneous inactivation of PAI-1 (half-life 1-2 hours)[Loskutoff, *et al* 1989]. However, occassional previous studies have reported unexpectedly high proportions of active PAI-1[Fay and Owen 1989, Nordenhem and Wiman 1997], and it has been suggested that platelets may possess a specific mechanism to preserve PAI-1 in an active configuration [Lang and Schleef 1996]. If so, this would provide an explanation for our previous observations of an surprisingly high activity of platelet PAI-1 [Brogren, *et al* 2004].

We therefore readdressed this issue with a similar functional approach as we used in the studies of activity of newly synthesized PAI-1. To carefully investigate the hypothesis, it was considered crucial that the method used to isolate PAI-1 from the platelet was able to capture the molecule in its active configuration (if that was the form in which it was present) and that spontaneous inactivation during the preparatory procedure was prevented. To ensure an immediate capture of active PAI-1 at the time of lysis and to circumvent the limitations of enzymatic methods [2000, Gram, *et al* 1993], we used a method in which tPA was present already when the platelets were lysed and subsequent direct detection of tPA-PAI-1 complex formation with antibodies and $^{125}$I-tPA. Interestingly, both these functional assays indicated that 70 – 80% of platelet PAI-1 was present in an active configuration that was biologically functional and could bind tPA. The findings indicate that a much larger proportion of platelet PAI-1 is active than previously assumed.

How, then, could the activity of PAI-1 be preserved for such a prolonged period of time in the platelet? A potential mechanism to explain the preserved activity has been suggested by Lang and Schleef [Lang and Schleef 1996], who have shown that plate-

lets have a unique mechanism for stabilization of PAI-1 in its active configuration by packaging together with other large α-granule proteins in a calcium-dependent manner. In plasma, active PAI-1 is stabilized by binding to vitronectin [Declerck, *et al* 1988b, Wiman, *et al* 1988] and vitronectin has also been detected in platelet α-granules. However, some studies have failed to detect a vitronectin-PAI-1 complex in platelets [Lang and Schleef 1996, Nordenhem and Wiman 1997], and it is therefore controversial whether vitronectin is the stabilizing factor of PAI-1 in platelets. This issue remains to be evaluated.

However, a potential flaw of our study is that if the procedures we used in some way could have reactivated latent PAI-1. It is known that PAI-1 can be reactivated under extreme *in vitro* conditions by denaturants such as SDS, guanidine HCl, or urea [Hekman and Loskutoff 1985]. To make sure that our results were not due to a potential reactivation and/or dissociation [Gaussem, *et al* 1993] of tPA-PAI-1 complex by the SDS used in the gel electrophoresis, we performed a series of experiments both with and without SDS in the loading buffer. However, these studies showed no detectable differences in PAI-1 activity whether SDS was present or not.

### Underestimation of platelet PAI-1 activity due to preparatory procedures

To shed light on possible mechanisms behind the discrepancy between our new results and those of previous studies, we studied the effect of commonly used preanalytic procedures to investigate if inadequate treatment of PAI-1 during the preparation might have inactivated it unintentionally. First, we studied the effect of sonication, since a recent study had demonstrated that energy levels as low as 30 W may cause protein damage [Stathopulos, *et al* 2004], and it is conceivable that a thermodynamically unstable molecule such as active PAI-1 is more susceptible to inactivation. Indeed, our results showed that even reactivated plasma PAI-1 stabilized by low pH was very sensitive to sonication and its activity was reduced by 50% with an energy load of approximately 30 W, which is 5-fold lower than the energy previously used for platelet lysis [Booth, *et al* 1988]. Taken together with the activity rates observed in the present study, one would expect sonication to reduce platelet PAI-1 activity to 5 – 10%, *i.e.* to similar levels as reported in previous studies.

The magnitude of the reduction in PAI-1 activity was similar when freezing/thawing was used for platelet lysis instead of sonication. However, whereas the reduced activity by sonication was independent of whether tPA was added before or after stimulation, the underestimation of activity by freezing/thawing could be prevented by adding tPA before lysing the platelets. Another common procedure for platelet disruption is to use detergents such as Triton X-100 [Lang, *et al* 1992, Nordenhem and Wiman 1997]. However, it has been shown that Triton X-100 decreases the half-life of PAI-1 markedly, and 0.2% Triton X-100 decreases the functional half-life of PAI-1 to less than 1 minute at 37°C [Gils and Declerck 1998, Andreasen, *et al* 1999]. Therefore, also with such protocols it is crucial to add tPA before lysis. Unfortunately, this has not been the case in the above-mentioned studies and could potentially have contributed to the low activity estimations.

## Are platelets the unknown source of plasma PAI-1?

PAI-1 is expressed in many types of cells, and both PAI-1 mRNA and protein can be retrieved from many tissues. Despite this, the source of circulating plasma PAI-1 is not known. The main pool of PAI-1 is found in the platelets [Erickson, *et al* 1985, Booth, *et al* 1988]. However, since no association between the content of PAI-1 in platelets and plasma concentration has been found, platelets have generally not been considered to contribute to the plasma levels [Booth, *et al* 1988, Simpson, *et al* 1990]. On the other hand, considering our findings that there is a large continuous synthesis of PAI-1 in the platelets, it is obvious that a very small fraction of this *de novo* synthesized protein would need to be released in order to maintain normal plasma levels [Brogren, *et al* 2004]. Indeed, a rough estimation based on these observations indicated that a release of as little as 3% of the *newly* synthesized PAI-1 would be sufficient to keep plasma levels within the normal range, even if the platelet were the only source of plasma PAI-1. In support of this view, it has been shown that the platelet count correlates with plasma PAI-1 [Cancelas, *et al* 1994, Soeki, *et al* 2000, Birdane, *et al* 2005, Brogren, *et al* 2007], a finding that may suggest that a constant low-rate release of PAI-1 from platelets could contribute to the plasma levels.

To investigate the hypothesis that platelets are the source of plasma PAI-1, we sought to find a new approach to reinvestigate this issue. In general it is difficult to determine the cellular origin of a plasma protein. However, many proteins are subjected to post-translational modifications that may alter its composition or structure. Such modifications may be tissue or cell-type specific. Of particular interest is the glycosylation pattern, which shows a considerable heterogeneity. Theoretically, such microheterogeneity may be used to reveal the origin of a plasma protein since a range of variations can be found in the precise structure of the glycan at any given glycosylation site on a given protein synthesized by a particular cell type. Even the extent of this heterogeneity can vary considerably, resulting in an almost infinite variability in glycosylation patterns [Varki 1999].

According to the contemporary view, platelets, endothelial cells, macrophages, liver, and adipocytes, are considered to be most likely contributors to plasma PAI-1 [Dellas and Loskutoff 2005]. We therefore investigated the glycosylation patterns of PAI-1 produced in these cells/tissues, and compared these patterns with that of plasma PAI-1. Interestingly, the results showed that PAI-1 isolated from platelets as well as plasma was not glycosylated at all, whereas PAI-1 from endothelial cells, macrophages, and adipose tissue was glycosylated with heterogeneous glycosylation patterns. These findings support the hypothesis that platelets could be the source of PAI-1 in plasma, at least in healthy subjects.

Endothelial cells produce large amounts of PAI-1 *in vitro* [MacGregor and Booth 1988], but this has been suggested that it may be an *in vitro* artefact [Fearns and Loskutoff 1997]. It has also been shown that endothelial PAI-1 is released basolaterally to the extracellular matrix, suggesting that it is not mainly involved in intravascular fibrinolysis [Schleef, *et al* 1990]. This conclusion is also supported by our study since we found that endothelial PAI-1 is glycosylated with two different glycans on N232, and it is therefore unlikely that the endothelium is a major source of plasma PAI-1 in

healthy individuals. Even though PAI-1 has been detected in macrophages in several tissues [Quax, *et al* 1990, Simpson, *et al* 1991, Bastelica, *et al* 2002] as well as in blood monocytes and macrophages in the wall of atherosclerotic vessels [Tipping, *et al* 1993], the amounts synthesized *in vitro* are very low [Hamilton, *et al* 1993, Tipping, *et al* 1993]. Furthermore, the presence of diagnostic ions and the suggested glycan found on macrophage PAI-1 indicate that it is unlikely that these cells contribute significantly to the plasma levels, at least not in healthy individuals.

Unfortunately, we were not able to obtain adequate amounts of PAI-1 from hepatocytes. Since PAI-1 is an acute phase protein [Kruithof, *et al* 1988] it might be necessary to add inflammatory stress to obtain sufficient amounts [Quax, *et al* 1990, Sawdey and Loskutoff 1991]. The liver might be an important source of plasma PAI-1 in inflammatory disease, sepsis, and stress. This study suggests that unstimulated hepatocytes synthesize very low amounts of PAI-1 and further studies with larger samples have to be performed to enable evaluation of its glycosylation pattern.

It has been shown that body mass index (BMI) correlates with plasma PAI-1 levels, and PAI-1 mRNA is up-regulated in adipose tissues of obese mice and humans [Sawdey and Loskutoff 1991, Loskutoff and Samad 1998, Alessi, *et al* 2000]. In addition, the elevated plasma levels of PAI-1 in obese subjects can be normalized by removal of adipose tissue both by surgery or dieting [Primrose, *et al* 1992, Mavri, *et al* 2001]. However, the notion of a release of PAI-1 from adipose tissue is controversial and diverging results have been reported [Morange, *et al* 1999, Yudkin, *et al* 1999, Lindeman, *et al* 2007]. Interestingly, the present study showed major differences in the glycosylation of plasma PAI-1 from lean and obese subjects. Ten glycans were identified on N288 in PAI-1 from plasma from obese subjects with a typical composition similar to that found on PAI-1 isolated from visceral adipose tissue, but none was found in the other tissues studied. Raised plasma PAI-1 levels are associated with an increased risk for cardiovascular events and elevated plasma PAI-1 has also been identified as a predictor of myocardial infarction [Hamsten, *et al* 1987, Collet, *et al* 2003, Hoekstra, *et al* 2004]. The results from this study suggest that the composition of the plasma PAI-1 profile is altered in obesity, leading to a highly glycosylated pool of the protein. Potentially with a prolonged activity half-life, leading to increased levels of active PAI-1 [Gils 2000, Gils, *et al* 2003].

# CONCLUDING REMARKS

The results of the series of studies presented in this thesis indicate that the platelet pool of PAI-1 may be of greater importance than previously believed. This may not only call for a redefinition of its physiological and pathophysiological function, but may also help to reconcile some of the previous seemingly contradictory findings of its role in stabilization of blood clots.

From a clinical perspective, there is compelling evidence that platelet-derived PAI-1 is pivotal for making platelet-rich blood clots resistant to both endogenous and pharmacological thrombolysis [Torr-Brown and Sobel 1993, Fay, *et al* 1994, Stringer, *et al* 1994, Konstantinides, *et al* 2001]. Despite this, most previous studies have reported activity levels of platelet PAI-1 that are probably far too low to explain its putative functional role. It was therefore of interest to find that the platelets contain considerable amounts of translationally active PAI-1 mRNA, and that there is an on-going *de novo* synthesis of PAI-1. Importantly, the majority of the newly formed protein was found to be active, and its production could be further stimulated by thrombin. It is likely that the PAI-1 synthesis may make more active PAI-1 available in a thrombin-rich environment, such as during formation of a blood clot.

However, our studies also indicate that the platelet possesses a mechanism for keeping preformed PAI-1 active for a long time. In the functional activity assays we performed, the activity of platelet PAI-1 was found to be much higher than previously suggested. This underestimation may have been due to unintentional inactivation of the protein during the preanalytic preparatory procedures. Hence, it is conceivable that the contribution of platelets for clot stabilization not only depends on their capacity for *de novo* synthesis of active PAI-1, but also on their large content of preformed PAI-1 that is in an active configuration.

Another important unresolved question has been from which source the circulating pool of PAI-1 in plasma originates. Since plasma PAI-1 is a cardiovascular risk factor and may theoretically reflect the output of several different tissues, increased knowledge of the specific source of plasma PAI-1 in different conditions with increased levels might facilitate targeted risk prevention. The new strategy to address this issue by investigating the specific glycosylation pattern of PAI-1 from different cellular sources appears to be a feasible approach to this problem. Our results show that platelets potentially could be the source of plasma PAI-1 in healthy individuals. Interestingly, however, the high levels of plasma PAI-1 in obese individuals seem to depend on a contribution from adipose tissue, which suggests that pathophysiological conditions may shift the relative contribution from different cellular sources.

In conclusion, our studies have put focus on the platelet as a potent, dynamic cytoplast with an unexpected capacity of *de novo* synthesis of the fibrinolysis inhibitor PAI-1 despite their lack of DNA and RNA synthesis. The likely consequences of this capability are well in line with the presumed role of the platelet in thrombogenesis predicted by Bizzozero more than a century ago. Moreover, despite the fact that the discoveries of the large number of enzymatic components of the hemostatic systems have shown that "blood coagulability" was by far more complex than foreseen by Virchow, the overall pathogenetic mechanisms as well as the action of the specific factors still fits remarkably well into his triad hypothesis of thrombogenesis.

# POPULÄRVETENSKAPLIG SAMMANFATTNING

När ett blodkärl skadas aktiveras blodplättarna (trombocyterna) i blodet och fäster i kärlväggen och till varandra. Samtidigt aktiveras blodets koagulationskaskad och bildar ett nätverk av fibrin i vilket trombocyterna också kan fästa. Tillsammans bildar detta den propp som ska förhindra blodförlust. När kärlskadan är läkt löses proppen upp av kroppens eget proppupplösningssystem, fibrinolysen. Hjärtinfarkt och stroke uppstår när blodproppar bildas, ofta pga kärlskador orsakade av åderförkalkning, och leder till att blodflödet förhindras. Dessa arteriella proppar är rika på trombocyter och mer resistenta mot upplösning än venösa proppar. Denna resistens har antagits bero på att trombocyterna innehåller stora mängder av proteinet plasminogen aktivator inhibitor 1 (PAI-1) som motverkar fibrinolysen. Tidigare studier har visat att trombocyternas PAI-1 bara är funktionellt till ungefär 5% varför PAI-1 och trombocyternas roll i proppstabiliteten varit svårförklarad. Syftet med denna avhandling var att undersöka de bakomliggande orsakerna till de här motsägelsefulla fynden.

I det första delarbetet undersöktes om trombocyterna har förmåga att syntetisera PAI-1. Trombocyterna bildas i benmärgen och är små fragment av en gigantisk cell (megakaryocyt), de är såtillvida inga kompletta celler eftersom de saknar kärna och därmed DNA. Man har därför länge ansett att trombocyterna inte kan syntetisera proteiner. Intressant nog har man nyligen visat att de innehåller mRNA dvs de DNA kopior som används som mall för proteinsyntes och man har funnit att trombocyterna syntetiserar några proteiner. Vi undersökte därför om så även var fallet för PAI-1. PAI-1 mRNA kunde detekteras och efter inkubering av trombocyterna i 24 timmar sågs en 25% ökning av mängden PAI-1 protein. Vi undersökte även i vilken utsträckning det nysyntetiserade PAI-1 var aktivt och fann att majoriteten var aktivt i minst 24 timmar.

I befolkningen finns en vanligt förekommande genetisk variation, så kallad polymorfi, i genen för PAI-1 och tidigare studier har visat att den kan vara av betydelse för hur mycket PAI-1 som bildas. Fyndet att trombocyter kan syntetisera PAI-1 resulterade i frågan om trombocyternas syntes var påverkad av polymorfin. För att studera detta undersöktes i delarbete II 38 friska män, 17 st med den ena genetiska varianten och 21 st med den andra, men inga signifikanta skillnader i mängden PAI-1 mRNA eller PAI-1 protein kunde ses mellan grupperna.

Aktivt PAI-1 är en instabil spänd molekyl, ungefär som en råttfälla och den inaktiveras av sig själv genom att övergå till ett mer avslappnat tillstånd och halveringstiden är 1-2 timmar. Bara aktivt PAI-1 kan binda till och hämma sitt substrat tPA (vävnads plasminogen aktivator) och tidigare studier har visat att aktiviteten på PAI-1 i trombocyter är ca 5%. Fyndet i delarbete I, att nysyntetiserat PAI-1 var aktivt i 24 timmar, tyder på att proteinet kan hållas aktivt längre tid i trombocyterna. I de tidigare studierna av PAI-1 aktivitet har man använt metoder för att spränga (lysera) trombocyterna som potentiellt kan inaktivera PAI-1. I delarbete III ville vi därför studera aktiviteten på PAI-1 med alternativa metoder och undersöka effekten av de tidigare använda lyserings-metoderna. Genom att undvika inaktivering fann vi att PAI-1 var aktivt till ca 80%. Studien visade även att lysering av trombocyter med ultraljud eller genom frysning och tining, sänker aktiviteten.

PAI-1 finns inte bara i trombocyterna utan även cirkulerande fritt i blodet. Höga nivåer av PAI-1 i blodet ses ofta hos individer med ökad risk att drabbas av hjärt-kärlsjukdom. Man vet dock inte vilka celler i kroppen som är ursprung till plasma-PAI-1. Fyndet i den första studien, att trombocyterna själva kan syntetisera aktivt PAI-1, gav upphov till frågan om trombocyterna kan vara den hittills okända källan till plasma-PAI-1. Det är generellt ganska svårt att ta reda på var plasmaproteiner kommer ifrån, men ett sätt är att titta på modifieringar av proteinerna. En proteinmodifiering är så kallad glykosylering som innebär att proteinet har sockermolekyler på sig. Genom att analysera hur olika celltyper glykosylerar PAI-1 kunde vi skilja ut olika mönster och fick på så sätt ett ”fingeravtryck” för att kunna avslöja källan till plasma-PAI-1. I delarbete IV undersöktes PAI-1 från lever, fettväv, blodkärl, vita blodkroppar, och trombocyter som alla är potentiella källor, och dessa jämfördes med plasma-PAI-1. Plasma-PAI-1 och trombocyt-PAI-1 var inte glykosylerat, medan PAI-1 från alla de andra undersökta vävnaderna uppvisade ett heterogent sockermönster. Detta fynd talar för att trombocytena skulle kunna vara källan till plasma-PAI-1.

Eftersom plasma-PAI-1 nivåerna är höga hos överviktiga individer har det spekulerats i om PAI-1 hos dessa individer kommer från fettväven. För att undersöka detta jämförde vi glykosyleringsmönstret på PAI-1 från fettväv med plasma-PAI-1 från fyra individer med body mass index (BMI) över 35. Intressant nog skilde sig glykosylleringsmönstret för PAI-1 i plasma mellan normalviktiga och överviktiga individer. De glykosyleringar som detekterades på plasma-PAI-1 från överviktiga detekterades även i stor utsträckning på PAI-1 från fettväv. Dessa fynd talar för att PAI-1 i blodet huvudsakligen kommer från trombocyter medan de höga nivåer som ses hos överviktiga beror på ett tillskott av PAI-1 från fettväv.

Sammantaget visar resultaten i denna avhandling en ny roll för trombocyterna i hämningen av fibrinolysen. Dels genom att det PAI-1 som finns i trombocyterna till största delen är aktivt och dels genom att trombocyterna dessutom kan nysyntetisera aktivt PAI-1. Vi visar även att trombocyterna kan vara källan till plasma PAI-1 medan de höga nivåer som ses hos överviktiga individer troligen beror på ett tillskott av PAI-1 från fettväv.

# ACKNOWLEDGEMENTS

A lot of people have contributed to this thesis in one way or another, I would like to express my sincere gratitude to all of you and especially…

*Sverker Jern* head of the Clinical Experimental Research Laboratory and my supervisor for accepting me as a PhD student, for all your support and encouragement during these years, for sharing some of your vast knowledge of many things, your enthusiasm and great sense of humour has been truly inspiring.

*Lena Karlsson* my co-supervisor for your brilliant support, curiosity and infectious enthusiasm. You are an incredible motivator and source of inspiration!

*Johanna Deinum* my co-supervisor for your enthusiasm, for all the stimulating discussions, and also for giving me the opportunity to work with your glycosylation hypothesis.

*Karl Swedberg*, chairman of Department of Emergency and Cardiovascular Medicine, Sahlgrenska University Hospital/Östra for support and for providing resources.

My co-authors *Maria Carlström* for being a good friend and my personal dissertation coach, *Karin Wallmark*, for being a good friend, room mate and for sharing my interest in PAI-1 and platelets, *Carina Sihlbom* for brilliant collaboration and for sharing some of your great knowledge of glycoproteomics. I have really enjoyed working with you all.

*Lingwei Wang, David Erlinge, Malin Lönn, Birgitta Oden, Richard Gustavsson, Fredrik Viklund,* and *Per Larsson* for collaboration and contribution.

*Eva Thydén* for excellent secretary skills and all the practical issues regarding PhD studies and especially for all the help with the composing of this thesis!!

*Cecilia Lundholm* and *Hannele Korhonen*, former and present room mates for excellent laboratory assistance and for all the good laughs.

My present and former colleagues at the Clinical Experimental Research Laboratory *Anna Wolf* for friendship both in and outside the lab, *Erik Ulfhammer* and *Pia Larsson* for being amusing congress company and for all the nice discussions about whatnot, *Claes Ladenvall, Niklas Bergh, Wilhelm Ridderstråle, Thórdís Hrafnkelsdottir, Per Ladenvall, Katarina Jood, Christina Jern, Karin Hultman, Jan-Arne Björkman, Thorarinn Gudnasson, Lisa Brandin, Sandra Huskanovic, Smita DuttaRoy, Ann-Britt Johansson, Ott Saluveer, Karin Wåhlander, Mikael Ekman*, *Ulrika Nimblad,* and all other people working in the group for longer or shorter periods of time.

Present and former research nurses at the Clinical Experimental Research Laboratory especially *Annika Odenstedt, Sven-Erik Hägelind, Kim Fahlén, Lill Alnäs, Ingrid Eriksson, Ann-Christine Lindahl,* and *Gunnel Hedelin* for creating a nice atmosphere.

I would also like to especially thank all blood donors, in particular my perforated colleagues at the Clinical Experimental Research Laboratory for being persistent phlebotomy volunteers!

All friends and relations supporting and encouraging me during these years, especially *Sofia* and *Maria*.

My dear family, *Lena* and *Carl*, for your endless support and encouragement. *Sara, Petter, Isak,* and *Erik* for support and for reminding me of the important things in life.

And last but not least… *Michael* for distraction, love, and support.

---

These studies were supported by grants from the Swedish Reaserch Council, the Swedish Heart-Lung Foundation, Sahlgrenska University Hospital Foundations, King Gustav V:s and Queen Victoria Foundation, Lars Hiertas Memorial Foundation, and Emelle Foundation.

# REFERENCES

Alessi, M.C., Bastelica, D., Morange, P., Berthet, B., Leduc, I., Verdier, M., Geel, O. & Juhan-Vague, I. Plasminogen activator inhibitor 1, transforming growth factor-beta1, and BMI are closely associated in human adipose tissue during morbid obesity. *Diabetes* 2000;49:1374-1380.

Alessi, M.C., Poggi, M. & Juhan-Vague, I. Plasminogen activator inhibitor-1, adipose tissue and insulin resistance. *Curr Opin Lipidol* 2007;18:240-245.

Andreasen, P.A., Egelund, R., Jensen, S. & Rodenburg, K.W. Solvent effects on activity and conformation of plasminogen activator inhibitor-1. *Thromb Haemost* 1999;81:407-414.

Aso, Y. Plasminogen activator inhibitor (PAI)-1 in vascular inflammation and thrombosis. *Front Biosci* 2007;12:2957-2966.

Bastelica, D., Morange, P., Berthet, B., Borghi, H., Lacroix, O., Grino, M., Juhan-Vague, I. & Alessi, M.C. Stromal cells are the main plasminogen activator inhibitor-1-producing cells in human fat: evidence of differences between visceral and subcutaneous deposits. *Arterioscler Thromb Vasc Biol* 2002;22:173-178.

Belloc, F., Heilmann, E., Combrie, R., Boisseau, M.R. & Nurden, A.T. Protein synthesis and storage in human platelets: a defective storage of fibrinogen in platelets in Glanzmann's thrombasthenia. *Biochim Biophys Acta* 1987;925:218-225.

Birdane, A., Haznedaroglu, I.C., Bavbek, N., Kosar, A., Buyukasik, Y., Ozcebe, O., Dundar, S.V. & Kirazli, S. The plasma levels of prostanoids and plasminogen activator inhibitor-1 in primary and secondary thrombocytosis. *Clin Appl Thromb Hemost* 2005;11:197-201.

Bizzozero, G. Über einer neuen Formbestandteil des Blutes. *Archives of Pathological Anatomy and Physiology and of Clinical Medicine* 1882;90.

Bjorquist, P., Brohlin, M., Ehnebom, J., Ericsson, M., Kristiansen, C., Pohl, G. & Deinum, J. Plasminogen activator inhibitor type-1 interacts exclusively with the proteinase domain of tissue plasminogen activator. *Biochim Biophys Acta* 1994;1209:191-202.

Booth, N.A. Fibrinolysis and thrombosis. *Baillieres Best Pract Res Clin Haematol* 1999;12:423-433.

Booth, N.A., Croll, A. & Bennett, B. The activity of plasminogen activator inhibitor-1 (PAI-1) of human platelet. *Fibrinolysis* 1990;4:138-140.

Booth, N.A., Robbie, L.A., Croll, A.M. & Bennett, B. Lysis of platelet-rich thrombi: the role of PAI-1. *Ann N Y Acad Sci* 1992;667:70-80.

Booth, N.A., Simpson, A.J., Croll, A., Bennett, B. & MacGregor, I.R. Plasminogen activator inhibitor (PAI-1) in plasma and platelets. *Br J Haematol* 1988;70:327-333.

Booyse, F. & Rafelson, M.E., Jr. In vitro incorporation of amino-acids into the contractile protein of human blood platelets. *Nature* 1967a;215:283-284.

Booyse, F.M. & Rafelson, M.E., Jr. Stable messenger RNA in the synthesis of contractile protein in human platelets. *Biochim Biophys Acta* 1967b;145:188-190.

Brogren, H., Karlsson, L., Andersson, M., Wang, L., Erlinge, D. & Jern, S. Platelets synthesize large amounts of active plasminogen activator inhibitor 1. *Blood* 2004;104:3943-3948.

Brogren, H., Wallmark, K., Jern, S. & Karlsson, L. Plasminogen activator inhibitor 1 expression in platelets is not influenced by the 4G/5G promoter polymorphism. *Thromb Res* 2007;Sep 18 [Epub ahead of print].

Brommer, E.J. The level of extrinsic plasminogen activator (t-PA) during clotting as a determinant of the rate of fibrinolysis; inefficiency of activators added afterwards. *Thromb Res* 1984;34:109-115.

Bugert, P., Dugrillon, A., Gunaydin, A., Eichler, H. & Kluter, H. Messenger RNA profiling of human platelets by microarray hybridization. *Thromb Haemost* 2003;90:738-748.

Cancelas, J.A., Garcia-Avello, A. & Garcia-Frade, L.J. High plasma levels of plasminogen activator inhibitor 1 (PAI-1) in polycythemia vera and essential thrombocythemia are associated with thrombosis. *Thromb Res* 1994;75:513-520.

Carmeliet, P., Stassen, J.M., Schoonjans, L., Ream, B., van den Oord, J.J., De Mol, M., Mulligan, R.C. & Collen, D. Plasminogen activator inhibitor-1 gene-deficient mice. II. Effects on hemostasis, thrombosis, and thrombolysis. *J Clin Invest* 1993;92:2756-2760.

Chang, Y., Bluteau, D., Debili, N. & Vainchenker, W. From hematopoietic stem cells to platelets. *J Thromb Haemost* 2007;5 Suppl 1:318-327.

Chen, Y.Q., Su, M., Walia, R.R., Hao, Q., Covington, J.W. & Vaughan, D.E. Sp1 sites mediate activation of the plasminogen activator inhibitor-1 promoter by glucose in vascular smooth muscle cells. *J Biol Chem* 1998;273:8225-8231.

Chmielewska, J., Ranby, M. & Wiman, B. Evidence for a rapid inhibitor to tissue plasminogen activator in plasma. *Thrombosis Reseach* 1983;31:427-436.

Chmielewska, J., Ranby, M. & Wiman, B. Kinetics of the inhibition of plasminogen activators by the plasminogen-activator inhibitor. Evidence for 'second-site' interactions. *Biochem J* 1988;251:327-332.

Chung, H.C., Rha, S.Y., Park, J.O., Yoo, N.C., Kim, J.H., Roh, J.K., Min, J.S., Lee, K.S., Kim, B.S. & Kim, J.J. Physiological and pathological changes of plasma urokinase-type plasminogen activator, plasminogen activator inhibitor-1, and urokinase-type plasminogen activator receptor levels in healthy females and breast cancer patients. *Breast Cancer Res Treat* 1998;49:41-50.

Chung, I. & Lip, G.Y. Virchow's triad revisited: blood constituents. *Pathophysiol Haemost Thromb* 2003;33:449-454.

Cigolini, M., Targher, G., Seidell, J.C., Schiavon, R., Manara, F., Zenti, M.G., Mattioli, C. & De Sandre, G. Relationships of plasminogen activator inhibitor-1 to anthropometry, serum insulin, triglycerides and adipose tissue fatty acids in healthy men. *Atherosclerosis* 1994;106:139-147.

Collen, D. On the regulation and control of fibrinolysis. Edward Kowalski Memorial Lecture. *Thromb Haemost* 1980;43:77-89.

Collet, J.P., Montalescot, G., Vicaut, E., Ankri, A., Walylo, F., Lesty, C., Choussat, R., Beygui, F., Borentain, M., Vignolles, N. & Thomas, D. Acute release of plasminogen activator inhibitor-1 in ST-segment elevation myocardial infarction predicts mortality. *Circulation* 2003;108:391-394.

Criteria and search for specific assays for active plasminogen activator inhibitor 1 (PAI-1) in plasma. *eJIFCC* 2000;12:http://www.ifcc.org/ejifcc/vol12no12/PAI-11assays.htm.

Dale, G.L. Platelet kinetics. *Curr Opin Hematol* 1997;4:330-334.

Dano, K., Andreasen, P.A., Grondahl-Hansen, J., Kristensen, P., Nielsen, L.S. & Skriver, L. Plasminogen activators, tissue degradation, and cancer. *Adv Cancer Res* 1985;44:139-266.

Dano, K., Behrendt, N., Hoyer-Hansen, G., Johnsen, M., Lund, L.R., Ploug, M. & Romer, J. Plasminogen activation and cancer. *Thromb Haemost* 2005;93:676-681.

Dawson, S.J., Wiman, B., Hamsten, A., Green, F., Humphries, S. & Henney, A.M. The two allele sequences of a common polymorphism in the promoter of the plasminogen activator inhibitor-1 (PAI-1) gene respond differently to interleukin-1 in HepG2 cells. *J Biol Chem* 1993;268:10739-10745.

de Lange, M., Snieder, H., Ariens, R.A., Spector, T.D. & Grant, P.J. The genetics of haemostasis: a twin study. *Lancet* 2001;357:101-105.

Declerck, P.J., Alessi, M.C., Verstreken, M., Kruithof, E.K., Juhan-Vague, I. & Collen, D. Measurement of plasminogen activator inhibitor 1 in biologic fluids with a murine monoclonal antibody-based enzyme-linked immunosorbent assay. *Blood* 1988a;71:220-225.

Declerck, P.J., De Mol, M., Alessi, M.C., Baudner, S., Paques, E.P., Preissner, K.T., Muller-Berghaus, G. & Collen, D. Purification and characterization of a plasminogen activator inhibitor 1 binding protein from human plasma. Identification as a multimeric form of S protein (vitronectin). *J Biol Chem* 1988b;263:15454-15461.

Declerck, P.J., Moreau, H., Jespersen, J., Gram, J. & Kluft, C. Multicenter evaluation of commercially available methods for the immunological determination of plasminogen activator inhibitor-1 (PAI-1). *Thromb Haemost* 1993;70:858-863.

Dellas, C. & Loskutoff, D.J. Historical analysis of PAI-1 from its discovery to its potential role in cell motility and disease. *Thromb Haemost* 2005;93:631-640.

Denis, M.M., Tolley, N.D., Bunting, M., Schwertz, H., Jiang, H., Lindemann, S., Yost, C.C., Rubner, F.J., Albertine, K.H., Swoboda, K.J., Fratto, C.M., Tolley, E., Kraiss, L.W., McIntyre, T.M., Zimmerman, G.A. & Weyrich, A.S. Escaping the nuclear confines: signal-dependent pre-mRNA splicing in anucleate platelets. *Cell* 2005;122:379-391.

DeWood, M.A., Spores, J., Hensley, G.R., Simpson, C.S., Eugster, G.S., Sutherland, K.I., Grunwald, R.P. & Shields, J.P. Coronary arteriographic findings in acute transmural myocardial infarction. *Circulation* 1983;68:I39-49.

DeWood, M.A., Spores, J., Notske, R., Mouser, L.T., Burroughs, R., Golden, M.S. & Lang, H.T. Prevalence of total coronary occlusion during the early hours of transmural myocardial infarction. *N Engl J Med* 1980;303:897-902.

Dieval, J., Nguyen, G., Gross, S., Delobel, J. & Kruithof, E.K. A lifelong bleeding disorder associated with a deficiency of plasminogen activator inhibitor type 1. *Blood* 1991;77:528-532.

Dittrich, M., Birschmann, I., Pfrang, J., Herterich, S., Smolenski, A., Walter, U. & Dandekar, T. Analysis of SAGE data in human platelets: features of the transcriptome in an anucleate cell. *Thromb Haemost* 2006;95:643-651.

Doggen, C.J., Bertina, R.M., Cats, V.M., Reitsma, P.H. & Rosendaal, F.R. The 4G/5G polymorphism in the plasminogen activator inhibitor-1 gene is not associated with myocardial infarction. *Thromb Haemost* 1999;82:115-120.

Du, X.L., Edelstein, D., Rossetti, L., Fantus, I.G., Goldberg, H., Ziyadeh, F., Wu, J. & Brownlee, M. Hyperglycemia-induced mitochondrial superoxide overproduction activates the hexosamine pathway and induces plasminogen activator inhibitor-1 expression by increasing Sp1 glycosylation. *Proc Natl Acad Sci U S A* 2000;97:12222-12226.

Eren, M., Painter, C.A., Atkinson, J.B., Declerck, P.J. & Vaughan, D.E. Age-dependent spontaneous coronary arterial thrombosis in transgenic mice that express a stable form of human plasminogen activator inhibitor-1. *Circulation* 2002;106:491-496.

Erickson, L.A., Fici, G.J., Lund, J.E., Boyle, T.P., Polites, H.G. & Marotti, K.R. Development of venous occlusions in mice transgenic for the plasminogen activator inhibitor-1 gene. *Nature* 1990;346:74-76.

Erickson, L.A., Ginsberg, M.H. & Loskutoff, D.J. Detection and partial characterization of an inhibitor of plasminogen activator in human platelets. *J Clin Invest* 1984;74:1465-1472.

Erickson, L.A., Hekman, C.M. & Loskutoff, D.J. The primary plasminogen-activator inhibitors in endothelial cells, platelets, serum, and plasma are immunologically related. *Proc Natl Acad Sci U S A* 1985;82:8710-8714.

Eriksson, P., Kallin, B., van 't Hooft, F.M., Bavenholm, P. & Hamsten, A. Allele-specific increase in basal transcription of the plasminogen-activator inhibitor 1 gene is associated with myocardial infarction. *Proc Natl Acad Sci U S A* 1995;92:1851-1855.

Eriksson, P., Nilsson, L., Karpe, F. & Hamsten, A. Very-low-density lipoprotein response element in the promoter region of the human plasminogen activator inhibitor-1 gene implicated in the impaired fibrinolysis of hypertriglyceridemia. *Arterioscler Thromb Vasc Biol* 1998;18:20-26.

Evangelista, V., Manarini, S., Di Santo, A., Capone, M.L., Ricciotti, E., Di Francesco, L., Tacconelli, S., Sacchetti, A., D'Angelo, S., Scilimati, A., Sciulli, M.G. & Patrignani, P. De novo synthesis of cyclooxygenase-1 counteracts the suppression of platelet thromboxane biosynthesis by aspirin. *Circ Res* 2006;98:593-595.

Fay, W.P., Eitzman, D.T., Shapiro, A.D., Madison, E.L. & Ginsburg, D. Platelets inhibit fibrinolysis in vitro by both plasminogen activator inhibitor-1-dependent and -independent mechanisms. *Blood* 1994;83:351-356.

Fay, W.P. & Owen, W.G. Platelet plasminogen activator inhibitor: purification and characterization of interaction with plasminogen activators and activated protein C. *Biochemistry* 1989;28:5773-5778.

Fay, W.P., Parker, A.C., Condrey, L.R. & Shapiro, A.D. Human plasminogen activator inhibitor-1 (PAI-1) deficiency: characterization of a large kindred with a null mutation in the PAI-1 gene. *Blood* 1997;90:204-208.

Fay, W.P., Shapiro, A.D., Shih, J.L., Schleef, R.R. & Ginsburg, D. Brief report: complete deficiency of plasminogen-activator inhibitor type 1 due to a frame-shift mutation. *N Engl J Med* 1992;327:1729-1733.

Fearns, C. & Loskutoff, D.J. Induction of plasminogen activator inhibitor 1 gene expression in murine liver by lipopolysaccharide. Cellular localization and role of endogenous tumor necrosis factor-alpha. *Am J Pathol* 1997;150:579-590.

Fox, K.A., Robison, A.K., Knabb, R.M., Rosamond, T.L., Sobel, B.E. & Bergmann, S.R. Prevention of coronary thrombosis with subthrombolytic doses of tissue-type plasminogen activator. *Circulation* 1985;72:1346-1354.

Fraker, P.J. & Speck, J.C., Jr. Protein and cell membrane iodinations with a sparingly soluble chloroamide, 1,3,4,6-tetrachloro-3a,6a-diphrenylglycoluril. *Biochem Biophys Res Commun* 1978;80:849-857.

Fuster, V. Lewis A. Conner Memorial Lecture. Mechanisms leading to myocardial infarction: insights from studies of vascular biology. *Circulation* 1994;90:2126-2146.

Gaussem, P., Grailhe, P. & Angles-Cano, E. Sodium dodecyl sulfate-induced dissociation of complexes between human tissue plasminogen activator and its specific inhibitor. *J Biol Chem* 1993;268:12150-12155.

Giardina, E.G., Chen, H.J., Sciacca, R.R. & Rabbani, L.E. Dynamic variability of hemostatic and fibrinolytic factors in young women. *J Clin Endocrinol Metab* 2004;89:6179-6184.

Gils, A. & Declerck, P.J. Modulation of plasminogen activator inhibitor 1 by Triton X-100--identification of two consecutive conformational transitions. *Thromb Haemost* 1998;80:286-291.

Gils, A., Pedersen, K.E., Skottrup, P., Christensen, A., Naessens, D., Deinum, J., Enghild, J.J., Declerck, P.J. & Andreasen, P.A. Biochemical importance of glycosylation of plasminogen activator inhibitor-1. *Thromb Haemost* 2003;90:206-217.

Gils, A.K., I. Brouwers, E. Declerck, P, J. Glycosylation dependent conformational transitions in plasminogen activator inhibitor-1: evidence for the presence of two active conformations. *Fibrinolysis and Proteolysis* 2000;14:58-64.

Gnatenko, D.V., Dunn, J.J., McCorkle, S.R., Weissmann, D., Perrotta, P.L. & Bahou, W.F. Transcript profiling of human platelets using microarray and serial analysis of gene expression. *Blood* 2003;101:2285-2293.

Gram, J., Declerck, P.J., Sidelmann, J., Jespersen, J. & Kluft, C. Multicentre evaluation of commercial kit methods: plasminogen activator inhibitor activity. *Thromb Haemost* 1993;70:852-857.

Hakansson, K., Emmett, M.R., Marshall, A.G., Davidsson, P. & Nilsson, C.L. Structural analysis of 2D-gel-separated glycoproteins from human cerebrospinal fluid by tandem high-resolution mass spectrometry. *J Proteome Res* 2003;2:581-588.

Hamilton, J.A., Whitty, G.A., Stanton, H., Wojta, J., Gallichio, M., McGrath, K. & Ianches, G. Macrophage colony-stimulating factor and granulocyte-macrophage colony-stimulating factor stimulate the synthesis of plasminogen-activator inhibitors by human monocytes. *Blood* 1993;82:3616-3621.

Hamsten, A., de Faire, U., Walldius, G., Dahlen, G., Szamosi, A., Landou, C., Blomback, M. & Wiman, B. Plasminogen activator inhibitor in plasma: risk factor for recurrent myocardial infarction. *Lancet* 1987;2:3-9.

Haslam, S.M., North, S.J. & Dell, A. Mass spectrometric analysis of N- and O-glycosylation of tissues and cells. *Curr Opin Struct Biol* 2006;16:584-591.

Hekman, C.M. & Loskutoff, D.J. Endothelial cells produce a latent inhibitor of plasminogen activators that can be activated by denaturants. *J Biol Chem* 1985;260:11581-11587.

Higuchi, R., Fockler, C., Dollinger, G. & Watson, R. Kinetic PCR analysis: real-time monitoring of DNA amplification reactions. *Biotechnology (N Y)* 1993;11:1026-1030.

Hoekstra, T., Geleijnse, J.M., Schouten, E.G. & Kluft, C. Plasminogen activator inhibitor-type 1: its plasma determinants and relation with cardiovascular risk. *Thromb Haemost* 2004;91:861-872.

Hong, Y., Pedersen, N.L., Egberg, N. & de Faire, U. Moderate genetic influences on plasma levels of plasminogen activator inhibitor-1 and evidence of genetic and environmental influences shared by plasminogen activator inhibitor-1, triglycerides, and body mass index. *Arterioscler Thromb Vasc Biol* 1997;17:2776-2782.

Hou, B., Eren, M., Painter, C.A., Covington, J.W., Dixon, J.D., Schoenhard, J.A. & Vaughan, D.E. Tumor necrosis factor alpha activates the human plasminogen activator inhibitor-1 gene through a distal nuclear factor kappaB site. *J Biol Chem* 2004;279:18127-18136.

Hrafnkelsdottir, T., Gudnason, T., Wall, U., Jern, C. & Jern, S. Regulation of local availability of active tissue-type plasminogen activator in vivo in man. *J Thromb Haemost* 2004;2:1960-1968.

Hrafnkelsdottir, T., Wall, U., Jern, C. & Jern, S. Impaired capacity for endogenous fibrinolysis in essential hypertension. *Lancet* 1998;352:1597-1598.

Huisman, L.G.M, M.P., van Griensven J, Kluft C. Evaluation of the specificity of antigen assays for plasminogen activator inhiitor 1: Comparison of two new commercial kits. *Fibrinolysis* 1992;6:87-88.

Hunter, J. A treatise on the blood, inflammation and gun-shot wounds. London: Nicol 1794 p 87.

Ishibashi, T., Nagata, K., Ohkawara, H., Sakamoto, T., Yokoyama, K., Shindo, J., Sugimoto, K., Sakurada, S., Takuwa, Y., Teramoto, T. & Maruyama, Y. Inhibition of Rho/Rho-kinase signaling downregulates plasminogen activator inhibitor-1 synthesis in cultured human monocytes. *Biochim Biophys Acta* 2002;1590:123-130.

Jaffe, E.A., Nachman, R.L., Becker, C.G. & Minick, C.R. Culture of human endothelial cells derived from umbilical veins. Identification by morphologic and immunologic criteria. *J Clin Invest* 1973;52:2745-2756.

Jeng, J.R. Association of PAI-1 gene promoter 4g/5g polymorphism with plasma PAI-1 activity in Chinese patients with and without hypertension. *Am J Hypertens* 2003;16:290-296.

Juhan-Vague, I., Morange, P.E., Frere, C., Aillaud, M.F., Alessi, M.C., Hawe, E., Boquist, S., Tornvall, P., Yudkin, J.S., Tremoli, E., Margaglione, M., Di Minno, G., Hamsten, A. & Humphries, S.E. The plasminogen activator inhibitor-1 -675 4G/5G genotype influences the risk of myocardial infarction associated with elevated plasma proinsulin and insulin concentrations in men from Europe: the HIFMECH study. *J Thromb Haemost* 2003;1:2322-2329.

Karge, W.H., 3rd, Schaefer, E.J. & Ordovas, J.M. Quantification of mRNA by polymerase chain reaction (PCR) using an internal standard and a nonradioactive detection method. *Methods Mol Biol* 1998;110:43-61.

Kieffer, N., Guichard, J., Farcet, J.P., Vainchenker, W. & Breton-Gorius, J. Biosynthesis of major platelet proteins in human blood platelets. *Eur J Biochem* 1987;164:189-195.

Komissarov, A.A., Zhou, A. & Declerck, P.J. Modulation of serpin reaction through stabilization of transient intermediate by ligands bound to alpha-helix F. *J Biol Chem* 2007;282:26306-26315.

Konstantinides, S., Schafer, K., Thinnes, T. & Loskutoff, D.J. Plasminogen activator inhibitor-1 and its cofactor vitronectin stabilize arterial thrombi after vascular injury in mice. *Circulation* 2001;103:576-583.

Kooistra, T., Sprengers, E.D. & van Hinsbergh, V.W. Rapid inactivation of the plasminogen-activator inhibitor upon secretion from cultured human endothelial cells. *Biochem J* 1986;239:497-503.

Kruithof, E.K., Gudinchet, A. & Bachmann, F. Plasminogen activator inhibitor 1 and plasminogen activator inhibitor 2 in various disease states. *Thromb Haemost* 1988;59:7-12.

Kruithof, E.K., Nicolosa, G. & Bachmann, F. Plasminogen activator inhibitor 1: development of a radioimmunoassay and observations on its plasma concentration during venous occlusion and after platelet aggregation. *Blood* 1987;70:1645-1653.

Kruithof, E.K., Tran-Thang, C., Ransijn, A. & Bachmann, F. Demonstration of a fast-acting inhibitor of plasminogen activators in human plasma. *Blood* 1984;64:907-913.

Kucia, A.M. & Zeitz, C.J. Failed reperfusion after thrombolytic therapy: recognition and management. *Heart Lung* 2002;31:113-121.

Lambers, J.W., Cammenga, M., Konig, B.W., Mertens, K., Pannekoek, H. & van Mourik, J.A. Activation of human endothelial cell-type plasminogen activator inhibitor (PAI-1) by negatively charged phospholipids. *J Biol Chem* 1987;262:17492-17496.

Lang, I.M., Marsh, J.J., Moser, K.M. & Schleef, R.R. Presence of active and latent type 1 plasminogen activator inhibitor associated with porcine platelets. *Blood* 1992;80:2269-2274.

Lang, I.M. & Schleef, R.R. Calcium-dependent stabilization of type I plasminogen activator inhibitor within platelet alpha-granules. *J Biol Chem* 1996;271:2754-2761.

Lawrence, D., Strandberg, L., Grundstrom, T. & Ny, T. Purification of active human plasminogen activator inhibitor 1 from Escherichia coli. Comparison with natural and recombinant forms purified from eucaryotic cells. *Eur J Biochem* 1989;186:523-533.

Levin, E.G. Quantitation and properties of the active and latent plasminogen activator inhibitors in cultures of human endothelial cells. *Blood* 1986;67:1309-1313.

Levin, E.G. & Santell, L. Conversion of the active to latent plasminogen activator inhibitor from human endothelial cells. *Blood* 1987;70:1090-1098.

Lijnen, H.R. & Collen, D. Mechanisms of physiological fibrinolysis. *Baillieres Clin Haematol* 1995;8:277-290.

Lijnen, H.R. & Collen, D. Endothelium in hemostasis and thrombosis. *Prog Cardiovasc Dis* 1997;39:343-350.

Lijnen, H.R., Van Hoef, B. & Collen, D. On the reversible interaction of plasminogen activator inhibitor-1 with tissue-type plasminogen activator and with urokinase-type plasminogen activator. *J Biol Chem* 1991;266:4041-4044.

Lindahl, T.L., Ohlsson, P.I. & Wiman, B. The mechanism of the reaction between human plasminogen-activator inhibitor 1 and tissue plasminogen activator. *Biochem J* 1990;265:109-113.

Lindahl, T.L., Sigurdardottir, O. & Wiman, B. Stability of plasminogen activator inhibitor 1 (PAI-1). *Thromb Haemost* 1989;62:748-751.

Lindeman, J.H., Pijl, H., Toet, K., Eilers, P.H., van Ramshorst, B., Buijs, M.M., van Bockel, J.H. & Kooistra, T. Human visceral adipose tissue and the plasminogen activator inhibitor type 1. *Int J Obes (Lond)* 2007.

Lindemann, S., Tolley, N.D., Dixon, D.A., McIntyre, T.M., Prescott, S.M., Zimmerman, G.A. & Weyrich, A.S. Activated platelets mediate inflammatory signaling by regulated interleukin 1beta synthesis. *J Cell Biol* 2001;154:485-490.

Loskutoff, D.J. & Samad, F. The adipocyte and hemostatic balance in obesity: studies of PAI-1. *Arterioscler Thromb Vasc Biol* 1998;18:1-6.

Loskutoff, D.J., Sawdey, M. & Mimuro, J. Type 1 plasminogen activator inhibitor. *Prog Hemost Thromb* 1989;9:87-115.

Loskutoff, D.J., van Mourik, J.A., Erickson, L.A. & Lawrence, D. Detection of an unusually stable fibrinolytic inhibitor produced by bovine endothelial cells. *Proc Natl Acad Sci U S A* 1983;80:2956-2960.

Lowe, G.D. Virchow's triad revisited: abnormal flow. *Pathophysiol Haemost Thromb* 2003;33:455-457.

Lucore, C.L., Fujii, S., Wun, T.C., Sobel, B.E. & Billadello, J.J. Regulation of the expression of type 1 plasminogen activator inhibitor in Hep G2 cells by epidermal growth factor. *J Biol Chem* 1988;263:15845-15848.

MacGregor, I.R. & Booth, N.A. An enzyme-linked immunosorbent assay (ELISA) used to study the cellular secretion of endothelial plasminogen activator inhibitor (PAI-1). *Thromb Haemost* 1988;59:68-72.

Martinez-Calatrava, M.J., Martinez-Larrad, M.T., Zabena, C., Gonzalez-Sanchez, J.L., Fernandez-Perez, C. & Serrano-Rios, M. The 4G/4G PAI-1 genotype is associated with elevated plasma PAI-1 levels regardless of variables of the metabolic syndrome and smoking status. A population-based study in Spanish population. *Diabetes Obes Metab* 2007;9:134-135.

Mavri, A., Alessi, M.C., Bastelica, D., Geel-Georgelin, O., Fina, F., Sentocnik, J.T., Stegnar, M. & Juhan-Vague, I. Subcutaneous abdominal, but not femoral fat expression of plasminogen activator inhibitor-1 (PAI-1) is related to plasma PAI-1 levels and insulin resistance and decreases after weight loss. *Diabetologia* 2001;44:2025-2031.

McRedmond, J.P., Park, S.D., Reilly, D.F., Coppinger, J.A., Maguire, P.B., Shields, D.C. & Fitzgerald, D.J. Integration of proteomics and genomics in platelets: a profile of platelet proteins and platelet-specific genes. *Mol Cell Proteomics* 2004;3:133-144.

Mimuro, J. & Loskutoff, D.J. Purification of a protein from bovine plasma that binds to type 1 plasminogen activator inhibitor and prevents its interaction with extracellular matrix. Evidence that the protein is vitronectin. *J Biol Chem* 1989;264:936-939.

Morange, P.E., Alessi, M.C., Verdier, M., Casanova, D., Magalon, G. & Juhan-Vague, I. PAI-1 produced ex vivo by human adipose tissue is relevant to PAI-1 blood level. *Arterioscler Thromb Vasc Biol* 1999;19:1361-1365.

Myohanen, H. & Vaheri, A. Regulation and interactions in the activation of cell-associated plasminogen. *Cell Mol Life Sci* 2004;61:2840-2858.

Newman, P.J., Gorski, J., White, G.C., 2nd, Gidwitz, S., Cretney, C.J. & Aster, R.H. Enzymatic amplification of platelet-specific messenger RNA using the polymerase chain reaction. *J Clin Invest* 1988;82:739-743.

Nordenhem, A. & Wiman, B. Plasminogen activator inhibitor-1 (PAI-1) content in platelets from healthy individuals genotyped for the 4G/5G polymorphism in the PAI-1 gene. *Scand J Clin Lab Invest* 1997;57:453-461.

Nordt, T.K., Eschenfelder, E., Peter, K., Sobel, B.E. & Bode, C. Independence of growth factor induced plasminogen activator inhibitor type-1 (PAI-1) expression from the 4G/5G polymorphism of the PAI-1 gene. *Thromb Haemost* 2003;90:36-42.

Ottosson, M., Vikman-Adolfsson, K., Enerback, S., Olivecrona, G. & Bjorntorp, P. The effects of cortisol on the regulation of lipoprotein lipase activity in human adipose tissue. *J Clin Endocrinol Metab* 1994;79:820-825.

Owen, C.A. *A History of Blood Coagulation.* Mayo Foundation for Medical Education and Reserch, Rochester 2001.

Panes, O., Matus, V., Saez, C.G., Quiroga, T., Pereira, J. & Mezzano, D. Human platelets synthesize and express functional tissue factor. *Blood* 2007;109:5242-5250.

Potter van Loon, B.J., Rijken, D.C., Brommer, E.J. & van der Maas, A.P. The amount of plasminogen, tissue-type plasminogen activator and plasminogen activator inhibitor type 1 in human thrombi and the relation to ex-vivo lysibility. *Thromb Haemost* 1992;67:101-105.

Primrose, J.N., Davies, J.A., Prentice, C.R., Hughes, R. & Johnston, D. Reduction in factor VII, fibrinogen and plasminogen activator inhibitor-1 activity after surgical treatment of morbid obesity. *Thromb Haemost* 1992;68:396-399.

Quax, P.H., van den Hoogen, C.M., Verheijen, J.H., Padro, T., Zeheb, R., Gelehrter, T.D., van Berkel, T.J., Kuiper, J. & Emeis, J.J. Endotoxin induction of plasminogen activator and plasminogen activator inhibitor type 1 mRNA in rat tissues in vivo. *J Biol Chem* 1990;265:15560-15563.

Quick, A.J. Salicylates and bleeding: the aspirin tolerance test. *Am J Med Sci* 1966;252:265-269.

Rau, J.C., Beaulieu, L.M., Huntington, J.A. & Church, F.C. Serpins in thrombosis, hemostasis and fibrinolysis. *J Thromb Haemost* 2007;5 Suppl 1:102-115.

Rendu, F. & Brohard-Bohn, B. The platelet release reaction: granules' constituents, secretion and functions. *Platelets* 2001;12:261-273.

Rentrop, K.P., Feit, F., Sherman, W. & Thornton, J.C. Serial angiographic assessment of coronary artery obstruction and collateral flow in acute myocardial infarction. Report from the second Mount Sinai-New York University Reperfusion Trial. *Circulation* 1989;80:1166-1175.

Robbie, L.A., Bennett, B., Croll, A.M., Brown, P.A. & Booth, N.A. Proteins of the fibrinolytic system in human thrombi. *Thromb Haemost* 1996;75:127-133.

Sancho, E., Declerck, P.J., Price, N.C., Kelly, S.M. & Booth, N.A. Conformational studies on plasminogen activator inhibitor (PAI-1) in active, latent, substrate, and cleaved forms. *Biochemistry* 1995;34:1064-1069.

Sawdey, M.S. & Loskutoff, D.J. Regulation of murine type 1 plasminogen activator inhibitor gene expression in v5

tion of active and inactive forms of recombinant human plasminogen activator inhibitor type 1 (PAI-1) expressed in Chinese hamster ovary cells: comparison with native human PAI-1. *Protein Expr Purif* 1994;5:309-316.

Stromqvist, M., Karlsson, K.E., Bjorquist, P., Andersson, J.O., Bystrom, M., Hansson, L., Johansson, T. & Deinum, J. Characterisation of the complex of plasminogen activator inhibitor type 1 with tissue-type plasminogen activator by mass spectrometry and size-exclusion chromatography. *Biochim Biophys Acta* 1996;1295:103-109.

Thellin, O., Zorzi, W., Lakaye, B., De Borman, B., Coumans, B., Hennen, G., Grisar, T., Igout, A. & Heinen, E. Housekeeping genes as internal standards: use and limits. *J Biotechnol* 1999;75:291-295.

Thon, J.N. & Devine, D.V. Translation of glycoprotein IIIa in stored blood platelets. *Transfusion* 2007;47:2260-2270.

Thorsen, S., Philips, M., Selmer, J., Lecander, I. & Astedt, B. Kinetics of inhibition of tissue-type and urokinase-type plasminogen activator by plasminogen-activator inhibitor type 1 and type 2. *Eur J Biochem* 1988;175:33-39.

Tipping, P.G., Davenport, P., Gallicchio, M., Filonzi, E.L., Apostolopoulos, J. & Wojta, J. Atheromatous plaque macrophages produce plasminogen activator inhibitor type-1 and stimulate its production by endothelial cells and vascular smooth muscle cells. *Am J Pathol* 1993;143:875-885.

Tjarnlund, A., Andersson, M., Jood, K., Ladenvall, P. & Jern, C. Rapid genotyping of haemostatic gene polymorphisms using the 5' nuclease assay. *Thromb Haemost* 2003;89:936-942.

Torr-Brown, S.R. & Sobel, B.E. Attenuation of thrombolysis by release of plasminogen activator inhibitor type-1 from platelets. *Thromb Res* 1993;72:413-421.

Urden, G., Chmielewska, J., Carlsson, T. & Wiman, B. Immunological relationship between plasminogen activator inhibitors from different sources. *Thromb Haemost* 1987;57:29-34.

Wagner, O.F., Vetterlein, M. & Binder, B.R. Purification of an active plasminogen activator inhibitor immunologically related to the endothelial type plasminogen activator inhibitor from the conditioned media of a human melanoma cell line. *J Biol Chem* 1986;261:14474-14481.

van der Bom, J.G., Bots, M.L., Haverkate, F., Kluft, C. & Grobbee, D.E. The 4G5G polymorphism in the gene for PAI-1 and the circadian oscillation of plasma PAI-1. *Blood* 2003;101:1841-1844.

Van Guilder, G.P., Hoetzer, G.L., Smith, D.T., Irmiger, H.M., Greiner, J.J., Stauffer, B.L. & DeSouza, C.A. Endothelial t-PA release is impaired in overweight and obese adults but can be improved with regular aerobic exercise. *Am J Physiol Endocrinol Metab* 2005;289: E807-813.

Wang, L., Ostberg, O., Wihlborg, A.K., Brogren, H., Jern, S. & Erlinge, D. Quantification of ADP and ATP receptor expression in human platelets. *J Thromb Haemost* 2003;1:330-336.

Varki, A.C., R. Esko, J. Freeze, H. Hart, G. Marth, J. (1999) *Essentials of Glycobiology.* Cold Spring Harbor Laboratory Press, New York.

Vaughan, D.E., Declerck, P.J., Van Houtte, E., De Mol, M. & Collen, D. Studies of recombinant plasminogen activator inhibitor-1 in rabbits. Pharmacokinetics and evidence for reactivation of latent plasminogen activator inhibitor-1 in vivo. *Circ Res* 1990;67:1281-1286.

Weiss, H.J. & Aledort, L.M. Impaired platelet-connective-tissue reaction in man after aspirin ingestion. *Lancet* 1967;2:495-497.

Verheijen, J.H., Chang, G.T. & Kluft, C. Evidence for the occurrence of a fast-acting inhibitor for tissue-type plasminogen activator in human plasma. *Thromb Haemost* 1984;51:392-395.

Weyrich, A.S., Dixon, D.A., Pabla, R., Elstad, M.R., McIntyre, T.M., Prescott, S.M. & Zimmerman, G.A. Signal-dependent translation of a regulatory protein, Bcl-3, in activated human platelets. *Proc Natl Acad Sci U S A* 1998;95:5556-5561.

Wiman, B., Almquist, A., Sigurdardottir, O. & Lindahl, T. Plasminogen activator inhibitor 1 (PAI) is bound to vitronectin in plasma. *FEBS Lett* 1988;242:125-128.

Virchow, R. *Gesammelte Abhandlungen zur Wissenschaftlichen Medicin.* Meindinger Sohn & Co., Frankfurt 1856.

Wuhrer, M., Catalina, M.I., Deelder, A.M. & Hokke, C.H. Glycoproteomics based on tandem mass spectrometry of glycopeptides. *J Chromatogr B Analyt Technol Biomed Life Sci* 2007;849:115-128.

Xue, Y., Bjorquist, P., Inghardt, T., Linschoten, M., Musil, D., Sjolin, L. & Deinum, J. Interfering with the inhibitory mechanism of serpins: crystal structure of a complex formed between cleaved plasminogen activator inhibitor type 1 and a reactive-centre loop peptide. *Structure* 1998;6:627-636.

Yudkin, J.S., Coppack, S.W., Bulmer, K., Rawesh, A. & Mohamed-Ali, V. Lack of evidence for secretion of plasminogen activator inhibitor-1 by human subcutaneous adipose tissue in vivo. *Thromb Res* 1999;96:1-9.

Zhan, M., Zhou, Y. & Han, Z.C. No association of the plasminogen activator inhibitor-1 promoter 4G/5G polymorphism with inhibitor level during basal transcription in vitro. *Int J Hematol* 2004;79:400-404.

Zietz, B., Buechler, C., Drobnik, W., Herfarth, H., Scholmerich, J. & Schaffler, A. Allelic frequency of the PAI-1 4G/5G promoter polymorphism in patients with type 2 diabetes mellitus and lack of association with PAI-1 plasma levels. *Endocr Res* 2004;30:443-453.