Aspects of Plasminogen Activator Inhibitor 1 in Metabolic Syndrome

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2016

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"Phantasie ist wichtiger als Wissen denn das Wissen begrenzt ist"

Albert Einstein

To my family

ABSTRACT

Plasminogen activator inhibitor 1 (PAI-1) is the main inhibitor of the fibrinolytic system and binds irreversibly to tissue-type plasminogen activator (t-PA), and thereby inhibits the protective action of t-PA against thrombus formation. Elevated levels of plasma PAI-1 is observed in subjects with type 2 diabetes (T2D) and the metabolic syndrome (MetS), which is a combination of metabolic features including obesity. PAI-1 has become recognized as a central molecule linking the MetS and thrombotic vascular events. However, the origin of plasma PAI-1 is not fully established in these conditions. The aim of this thesis was to investigate the role of platelet PAI-1 and source of plasma PAI-1 in T2D, obesity and healthy subjects.

Our group has previously shown that platelets can *de novo* synthesize PAI-1 and the amount synthesized *in vitro* in 24 hours is 35-fold higher than required to maintain normal plasma levels. Therefore we wanted to investigate if platelets are the cellular origin of plasma PAI-1. Tissue-specific glycosylation patterns on PAI-1 from different tissues were determined. The results indicate that platelets are the source of plasma PAI-1, since no glycans were detected on PAI-1 isolated from plasma or platelets from healthy lean subjects. PAI-1 isolated from the other tissues expressed heterogeneous glycosylation patterns.

In obese subjects plasma PAI-1 had a glycan composition similar to that of adipose tissue suggesting that obese subjects, with elevated PAI-1 levels, may have an additional contribution from adipose tissue. Since glycosylated PAI-1, in comparison to unglycosylated forms, exhibit a lower inactivation rate and a stronger inhibitory effect, we also studied the level of glycosylated plasma PAI-1 in obesity. This was increased in obese subjects by 54% compared to lean subjects and a weak but significant correlation between the level of glycosylation and BMI was observed. These results suggest that an elevated level of glycosylated plasma PAI-1 is an important contributor to the increased risk of cardiovascular events associated with increased plasma PAI-1 levels in obese subjects.

Diabetic platelets are known to be hyper-reactive and larger in size; however, whether these features affect their contribution to the elevated levels of plasma PAI-1 in T2D is unknown. To elucidate the role of platelet PAI-1 in T2D, we characterized the PAI-1 antigen content and the mRNA expression in platelets from T2D subjects compared to both obese and lean control subjects. In order to analyze the mRNA content in platelets, reference genes to normalize for qPCR were identified and YWHAE was the most stable transcript in platelets. Furthermore, there was no significant difference in PAI-1 mRNA expression or PAI-1 antigen in platelets comparing the three groups, but an elevated level of plasma PAI-1 was seen in both T2D and obese control subject, indicating an uneffected role of platelets.

In conclusion, in balanced physiological conditions, platelets may be the major source of plasma PAI-1, however, the platelet contribution seems less important for the increased plasma levels and hence the risk of cardiovascular events in MetS and T2D.

Keywords: PAI-1, metabolic syndrome, obesity, type 2 diabetes, cardiovascular disease, fibrinolysis, thrombi, plasma, platelets, adipose tissue, mRNA, qPCR, reference gene

LIST OF ORIGINAL PAPERS

This thesis is based on the following papers, which will be referred to in the text by their roman numerals:

I   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.
Thromb Res. 2008;122:271-281.

II   Mossberg KE, Nilsson J, Sihlbom C, Larson G, Jern S, Brogren H. Increased glycosylation of plasma Plasminogen Activator Inhibitor 1 in obesity.
In manuscript.

III   Mossberg KE, Svensson PA, Gidlöf O, Erlinge D, Jern S, Brogren H. Normalization of qPCR in platelets – YWHAE a potential generic reference gene.
Platelets. 2016. DOI: 10.1080/09537104.2016.1180349. In Press

IV   Mossberg KE, Olausson J, Fryk E, Jern S, Jansson PA, Brogren H. The Role of the Platelet Pool of Plasminogen Activator Inhibitor 1 in type 2 diabetes.
In manuscript.

*Paper published under the previous family name Wallmark

LIST OF RELATED PAPERS

Additional peer-reviwed papers, not included in the thesis:

- Mossberg KE, Pournaras DJ, Welbourn R, le Roux CW, Brogren H. Differential response of plasma Plasminogen Activator Inhibitor-1 after Weight Loss Surgery in patients with or without Type 2 Diabetes.
  SOARD. 2016. dx.doi.org/10.1016/j.soard.2016.03.007 In press.

- Brogren H, Wallmark* K, Deinum J, Karlsson L, Jern S. Platelets retain high levels of active plasminogen activator inhibitor 1.
  PLoS One. 2011;6(11):e26762.

- 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. 2008;121(6):793-7.

*Papers published under the previous family name Wallmark

POPULÄRVETENSKAPLIG SAMMANFATTNING

Hjärtkärlsjukdomar är idag den vanligaste dödsorsaken i västvärlden. Detta är ett resultat av bl a ett utbrett stillasittande och ett kraftigt ökat kaloriintag. En annan effekt av denna livsstil är en lavinartad ökning av det metabola syndromet, vilket är ett tillstånd som innefattar ökad bukfetma, högt blodtryck, förhöjd insulinkänslighet och höga blodfetter. Alla dessa egenskaper ökar risken för hjärtkärlsjukdomar och kan i värsta fall leda till döden. Exempelvis är risken att drabbas av en hjärtinfarkt tre till fem gånger större hos individer med metabolt syndrom och typ 2 diabetes, jämfört med en frisk individ. Med tanke på de stora konsekvenserna, är det av stor vikt att förstå och kartlägga orsakerna till den ökade risken för hjärtkärlsjukdomar i det metabola syndromet.

Blodet i kroppen strävar efter att vara i balans. Om det är för tjockt kommer blodproppar att uppstå, men om det och andra sidan är för tunt uppkommer blödningar. Om en kärlskada sker, fäster blodplättar eller trombocyter som det också heter, i kärlväggen och till varandra. Kroppens koagulationskaskad aktiveras samtidigt och formar ett nätverk av fibrin, där trombocyterna också kan fästa. På detta sätt bildas en blodpropp. Kroppens eget proppupplösningssystem, fibrinolysen, kommer att lösa upp proppen när kärlskadan har läkt. Vid åderförkalkning i blodkärlen, vilket är vanligt vid exempelvis högt blodtryck, höga blodfetter och hög ålder, kommer inflammationsceller och fett lagras i kärlväggarna. Om det innersta lagret av kärlväggen spricker och inlagrat fett exponeras för blodet, fäster trombocyter och det bildas en blodpropp. En hjärtinfarkt uppstår om hjärtats blodkärl drabbas av en propp som är så stor att blodflödet hindras helt. Omkringliggande vävnad, i detta fall hjärtmuskeln, får syrebrist och vävnaden kan dö om syrebristen varar för länge. Plasminogen activator inhibitor 1 (PAI-1) är ett protein i blodet som hindrar fibrinolysen från att lösa upp blodproppar. Vid metabolt syndrom och typ 2 diabetes har man observerat ökade nivåer av PAI-1 i blodet eller plasma, vilket alltså förhindrar kroppen från att spontant lösa upp blodproppar som uppkommer i kärlen och därmed ökar risken för hjärtkärlsjukdomar. Man vet också att trombocyterna innehåller mycket PAI-1 och kan frisätta detta i blodet. Syftet med denna avhandling var att undersöka var PAI-1 i plasma kommer ifrån hos friska individer, samt hos individer med fetma och typ 2 diabetes.

Först undersöktes vilket som är källan till PAI-1 i blodet hos friska individer. Det är generellt svårt att ta reda på var plasmaproteiner kommer ifrån, men ett sätt är att analysera glykosylering, vilket innebär att studera sockermolekyler som finns på proteinet. Därför undersöktes om PAI-1 har olika mönster av glykosylering, som skulle kunna fungera som ett "fingeravtryck" för att avslöja källan. PAI-1 från lever, fettväv, blodkärl, vita blodkroppar och trombocyter, som alla är möjliga källor, undersöktes och jämfördes med PAI-1 från plasma. PAI-1 från trombocyter och plasma var inte glykosylerat, medan PAI-1 från alla de andra vävnaderna uppvisade ett olikartat sockermönster. Detta fynd talar för att trombocyterna skulle kunna vara källan till plasma PAI-1 hos friska, normalviktiga individer.

Eftersom nivåerna av PAI-1 i plasma är förhöjda hos individer med fetma, har det spekulerats i om PAI-1 hos dessa individer kommer från fettväven. Följaktligen observerades sockermönstret på PAI-1 från fettväv och detta jämfördes med PAI-1 i

plasma från individer med fetma. Det visade sig att de sockermolekyler som hittades på plasma PAI-1 från individer med fetma, även återfanns i stor utsträckning på PAI-1 från fettväv. Tidigare studier har visat att proteiner som har sockermolekyler på sig kan ha ändrade egenskaper och vidare har man konstaterat att glykosylerat PAI-1 har en starkare effekt i att förhindra blodproppar från att lösa upp sig i blodet. Därför undersöktes också i vilken utsträckning PAI-1 i plasma från individer med fetma var glykosylerat. Intressant nog observerades en dubbel ökning av graden av glykosylering av plasma PAI-1 hos kraftigt överviktiga individer jämfört med normalviktiga. Dessa fynd visar att fettväven kan vara källan till de ökade nivåerna av plasma PAI-1 i fetma, men också att den ökade graden av glykosylering kan bidra ytterligare till att PAI-1 motverkar kroppens förmåga att lösa upp blodproppar vid hjärtkärlhändelser.

Tidigare studier har visat att trombocyter hos individer med typ 2 diabetes är mer reaktiva och större i storlek, jämfört med friska individer. Innehållet av PAI-1 i trombocyterna undersöktes från individer med fetma och typ 2 diabetes och jämfördes med normalviktiga och kraftigt överviktiga kontrollindivider. För att kunna göra detta vidareutvecklades först en metod för att observera innehållet av PAI-1 på genetisk nivå i trombocyterna. Resultaten visade att det inte fanns någon skillnad i innehåll av PAI-1, varken på protein- eller genetisk nivå, i trombocyterna från de tre grupperna. Men som väntat var plasma PAI-1 förhöjt i typ 2 diabetes och fetma jämfört med de normalviktiga individerna. Dessa fynd talar för att det är en annan källa än trombocyterna som bidrar till de höga nivåerna av plasma PAI-1 i typ 2 diabetes.

Sammantaget visar resultaten i denna avhandling att hos friska individer är det troligt att källan till plasma PAI-1 är trombocyterna. Att ha förhöjda nivåer av PAI-1 i blodet, så som vid det metabola syndromet och typ 2 diabetes, ökar risken att drabbas av hjärtkärlsjukdomar. Hos dessa individer verkar de ökade nivåerna av PAI-1 i blodet komma från en annan vävnad, där fettväv är en trolig källa.

CONTENTS

ABBREVIATIONS

ACD	acid citrate dextrose
ADP	adenosine diphosphate
ASA	acetylsalicylic acid
ASPI	arachidonic acid
B2M	beta-2-microglobulin
BMI	body mass index
cDNA	complementary DNA
CID	collision induced dissociation
CHO	chinese hamster ovary
C_T	threshold cycle
CVD	cardiovascular disease
ELISA	enzyme-linked immunosorbent assay
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GM-CSF	granulocyte monocyte colony stimulating factor
GLP-1	glucagon-like protein-1
H3F3A	H3 histone, family 3A
HCD	high-energy collision dissociation
HUVEC	human umbilical vein endothelial cells
ICR	ion cyclotron resonance
ITM2B	integral membrane protein 2B
kDa	kilo dalton
MetS	metabolic syndrome
miRNA	micro RNA
mRNA	messenger RNA
MPV	mean platelet volume
MS	mass spectrometry
MW	molecular weight
NSAIDs	non-steroid anti-inflammatory drugs
NYHA II	New York association functional classification II
PAI-1	plasminogen activator inhibitor 1
PBMCs	peripheral blood mononuclear cells
PF4V1	platelet factor 4, variant 1
PGE_1	prostaglandin E_1
PIPES	1,4-piperazinediethanesulfonic acid
PPIA	peptidylprolyl isomerase A

PRP	platelet-rich plasma
qPCR	quantitative polymerase chain reaction
RN18S1	18S ribosomal 1
SDS	sodium dodecyl sulphate
SERPIN	serine protease inhibitor
SFLLRN	PAR receptor agonist
sFSH	serum follicle stimulating hormone
siRNA	short interfering RNA
T2D	type 2 diabetes
TGF-ß	transforming growth factor beta
t-PA	tissue-type plasminogen activator
TRAP-6	thrombin receptor activating peptide 6
YWHAE	tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein

INTRODUCTION

*T*he major cause of morbidity and mortality in the Western world is cardiovascular disease (CVD) [1]. Our modern western lifestyle has caused this harmful evolution. The metabolic syndrome (MetS), as a result of the newly adopted lifestyle, is a combination of metabolic and clinical features that includes abdominal obesity, insulin resistance and impaired glucose tolerance, hypertension, and atherogenic dyslipidemia [2]. The definition of MetS has been widely debated and revisions have been made throughout the years. Despite variations in definition, however, an alarming increase in prevalence has been observed according to international and Swedish reports [3-8]. The features of MetS are strongly associated with CVD and mortality [7, 9-11]. For instance the risk of suffering a myocardial infarction is three to five-folds higher in MetS including type 2 diabetes (T2D), compared to healthy subjects [12, 13]. Cardiovascular events in the MetS and T2D are indeed becoming a health concern worldwide [14-17]. Given this massive impact on global health, it is of major importance to elucidate the underlying mechanisms of the increased risk of thrombosis in MetS, in order to find means to reduce cardiovascular events.

Thrombosis

Thrombi are formed on atherosclerotic plaques under conditions of high blood flow and are mainly composed of aggregated platelets [18]. Thrombosis over plaques occurs either when the subendothelial connective tissue is exposed or if the plaque is disrupted causing an exposed lipid core. Both events trigger an enhanced inflammatory process within the plaque. The formation of the clots activates the coagulation cascade that stabilizes the clot by creating a fibrin network [19]. If not restricted, the clot will grow to occlude the lumen of the vessel and obstruct normal circulation that eventually causes hypoxia in the supplied tissue. This may result in reduced function or even death of the tissue.

Clot dissolution

When a blood clot is formed, either as a response to traumatic vascular injury or in relation to an atherosclerotic plaque, the fibrinolytic system is immediately activated to prevent intraluminal clot growth and vessel occlusion. The fibrinolytic system is ultimately responsible for lysis of blood clots and restriction of thrombus propagation beyond the site of injury [20]. Thereby fibrinolysis acts as a counteracting system to coagulation. The efficacy to the fibrinolytic system is illustrated by the fact that spontaneous reperfusion occurs in about 30% of patients suffering from myocardial infarction [21, 22]. The key factor of the fibrinolytic process is the local release of tissue-type plasminogen activator (t-PA) from the endothelial cells that facilitates the conversion of the inactive precursor plasminogen into plasmin, which in turns degrades the blood clot by cleaving the fibrin network (Figure 1) [23-25]. The fibrinolytic system is regulated at several levels to prevent premature and/or excessive degradation, most importantly by specific serine protease inhibitors (serpins). The main inhibitor of t-PA is plasminogen activator inhibitor 1 (PAI-1) which is the subject of this thesis [26-28].
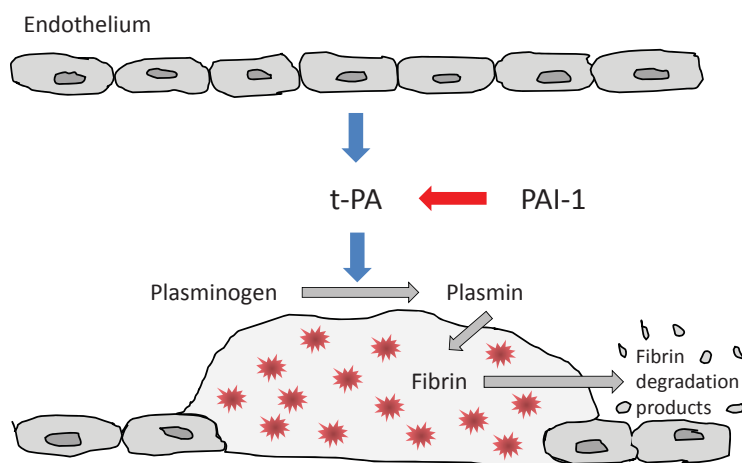
Figure 1. Schematic presentation of the intravascular fibrinolytic system. When a clot is formed, factors from the clotting process stimulate a rapid secretion of tPA from the endothelial cells. tPA cleaves the pro-enzyme plasminogen to plasmin, which in turn degrades the fibrin mesh of the clot and resolves the clot. The main inhibitor of the fibrinolytic system, PAI-1, irreversibly binds to t-PA, and thereby inhibits the protective action of tPA against thrombus formation.

PAI-1

The role of PAI-1 is to inhibit the dissolution of blood clots by inhibiting the enzymatic activity of t-PA. At an intravascular level, PAI-1 is present in blood at low concentrations, approximately 10-20 ng/ml [20, 29]. It is widely distributed in tissues and synthesized in a variety of cells *in vitro* [30, 31]. However, the source of plasma PAI-1 has been enigmatic, but endothelial cells, adipose tissue, liver, macrophages and platelets have been considered the most likely sources [32].

The PAI-1 gene consists of eight introns and nine exons and is encoded on chromosome 7, distributed over approximately 12.3 kb DNA [33]. The regulation of the translation of the PAI-1 gene is not fully understood. The promotor has been investigated and its expression is controlled by a variety of signals, cytokines, mechanical and physical stresses [34]. For instance, metabolic factors such as increased levels of plasma glucose and hypertriglyceridemia activate the PAI-1 gene [35-38]. In addition, the promotor contains a common polymorphism consisting of a single base pair insertion/deletion (4G or 5G), but it has been debated whether it is of importance for PAI-1 synthesis or not [39-42]. It appears to be of less significance, however, since the average PAI-1 mRNA levels in platelets from healthy young men were similar among the two genotypes [43].

PAI-1 is a single chain protein and has a molecular weight of ~45 kDa. The mature protein consists of 379 amino acids. PAI-1 belongs to the serpine superfamily; which all share the common feature of a reactive center that mimics the natural substrate [44]. t-PA and PAI-1 form a ~110 kDa stoichiometric 1:1 stable complex and the

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inactivation of t-PA by PAI-1 is remarkably rapid [45-51]. PAI-1 occurs both in an active and a latent form *in vivo*, but it is always synthesized in the active from that has the ability to bind and inhibit t-PA [52-54]. The active PAI-1 molecule is spontaneously inactivated with a half-life of ∼1-2 hours in plasma at physiological temperature (37°C) and pH (7.4), although in lower temperature and pH the half-life is prolonged [55-57]. In plasma, PAI-1 binds to vitronectin, which stabilizes the active form of PAI-1 and extends its half-life [58-60].

The biological properties of a protein may change upon glycosylation and glycosylated PAI-1 has a 2.3-fold stronger inhibitory effect on t-PA and a lower inactivation rate than non-glycosylated [61, 62]. PAI-1 has three potential glycosylation sites for N-linked glycosylation; N232, N288 and N353 [63]. Heterogeneous glycosylation patterns have been observed on two of these three sites (N232 and N288) on human PAI-1 expressed naturally or recombinantly by human cell lines [61].

PAI-1 and thrombosis

It has been known for decades that a decreased fibrinolytic activity is related to thrombotic events. The important function of PAI-1 in the fibrinolytic cascade was observed in the 1990s, ten years after its discovery [64-66]. The significance of PAI-1 in thrombosis has been demonstrated in several studies. Deficiency or absence of PAI-1 causes bleeding and accelerated fibrinolysis in both humans and PAI-1 knock-out mice [64, 67]. Conversely, transgenic mice that over-express active PAI-1 have an increased risk of spontaneous thrombi and the rate of progression of thrombus formation following vascular injury is prolonged [68-70]. In addition, arterial thrombi contain 2-3 fold more PAI-1 than venous thrombi [71, 72]. Several epidemiological studies have shown an association of raised plasma PAI-1 levels with an increased risk of cardiovascular events [73-75].

The exact mechanism of PAI-1 in atherosclerotic disease is not completely understood. It may play an important role by promoting vascular atherosclerosis and thrombosis [76, 77]. Increased PAI-1 expression in atherosclerotic lesions strongly suggests a primary role of PAI-1 in the atherogenic process [78-80]. However, experimental studies have shown diverging results, both promotive and preventive properties of PAI-1 on atherosclerosis [81, 82]. The complex vascular function of PAI-1 may depend on the presence of fibrin. For instance, PAI-1 may stabilize fibrin in case of a vascular injury and fibrin formation [83]. Conversely, in absence of fibrin, PAI-1 may inhibit cell migration within the vascular wall and thereby inhibit formation of intimal hyperplasia that promotes atherosclerotic plaque rupture [84].

PAI-1 and platelets

Platelets are anucleate cytoplasts primarily formed in the bone marrow by fragmentation of the cytoplasm of megakaryocytes [85]. They circulate in the blood stream in a concentration of 150-400 x 10^9/L for approximately 10 days [86]. There are different types of granules in the platelets cytoplasm of which α-granules are the most abundant. They contain a vast number of biologically active molecules crucial for platelet function; including growth factors, cytokine-like proteins, adhesion proteins

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and components of coagulation and fibrinolysis [87]. Upon activation, platelets secrete the contents of the granules. Furthermore, it is now evident that platelets are able to synthesize proteins, despite the fact that they lack nuclei. As early as in 1967, megakaryocytic-derived translationally active mRNAs were observed in platelets, and later the machinery to translate it into proteins (i.e. rough endoplasmic reticulum and polyribosomes) was discovered in platelets [88-92]. In 1998, regulated synthesis of a specific protein (Blc-3) was discovered in platelets [93]. The nature of the platelet transcriptome has been widely studied during the last decade and platelets express some 2,300 transcripts [94-97]. However, the regulation of translation of proteins in platelets is not completely understood. The levels of mRNA transcripts in platelets are mainly determined by the amount of mRNA derived from the megakaryocytes when the platelets are formed, as platelets are anucleated and the pool of mRNA transcripts degrades over time [98]. There is even some evidence of active sorting of transcripts into platelets by megakaryocytes [99]. Moreover, platelets are uniquely able to splice pre-mRNA into mature mRNA by a functional spliceosome located in the cytoplasm and thereby increase the level of mature mRNA [100]. Furthermore, human platelets contain an abundant and diverse array of microRNAs (miRNA), which are a class ~22 nucleotide RNAs that regulate protein-coding genes primarily by repressing gene expression either by mRNA degradation or translational repression [101-107]. In addition to functional miRNA transcripts, platelets also contain the machinery to process pre-miRNA into mature miRNA [108, 109].

The physiological significance of platelet PAI-1 has been widely debated. Most studies have shown that the vast majority of platelet PAI-1 is inactive and unable to inhibit fibrinolysis [20, 29, 110, 111]. However, platelets harbor approximately 90% of the circulating PAI-1 in blood and there is a strong correlation between the relative PAI-1 content in platelets and the resistance to thrombolysis [58, 110, 112-114]. Recently, is has been demonstrated that platelets contain PAI-1 mRNA and that they synthesize large amounts of active PAI-1 [115]. Preparatory procedures may have led to underestimation of platelet PAI-1 activity in most previously reported studies [116]. This information may be of importance in order to be able to elucidate the source of plasma PAI-1 in healthy subjects.

PAI-1 and MetS

Emerging evidence has suggested PAI-1 as a key link between the MetS and cardiovascular events [12, 117]. The most well-known hemostatic disturbance in individuals with MetS is fibrinolytic impairment defined by dramatically elevated plasma PAI-1 levels, which mediates the risk of vascular thrombotic events [118-120]. PAI-1 is even a strong predictor of myocardial infarction; however, the predictive ability of PAI-1 in epidemiological studies is reduced after adjusting for markers of the MetS [121-124]. Taken together, PAI-1 is a true component of the MetS and indeed an attractive target for future intervention strategies [125, 126].

Obesity is associated to elevated levels of PAI-1 in clinical studies [127], and plasma PAI-1 levels are reduced by removal of adipose tissue either by dieting, surgically resected or bariatric surgery [128-132]. Although the association of PAI-1 and obesity

is well established, the mechanisms of elevated plasma PAI-1 levels are complex and it is uncertain whether observed associations are correlative or causative [125]. PAI-1 has also been implicated in adipose tissue development but its exact role here is controversial [127]. However, it has been suggested that PAI-1 controls the recruitment of inflammatory cells in adipose tissue, influences the insulin signaling and affects adipose differentiation [121]. Adipose tissue is an active organ with capacity to synthesize and secrete proinflammatory adipokines, including PAI-1 [133, 134]. Elevated inflammatory cytokines in obesity may induce PAI-1 overexpression, subsequently associated with an impaired fibrinolysis [127, 135, 136]. Reduced fibrinolytic activity observed in thrombi formation in obese mice supports an association between obesity and a pro-thrombotic state [137].

Epidemiological studies have demonstrated that T2D is related to PAI-1 independently of established diabetes risk factors [138]. Results from a cohort of healthy non-diabetic subjects revealed that raised plasma PAI-1 levels at baseline were significantly predictive of incident diabetes over 5 years of follow-up and increasing levels over time were associated with progression of incident T2D [139, 140]. Thus, circulating levels of PAI-1 predict development of T2D in healthy individuals regardless of common diabetes risk factors [141]. Although the specific link between PAI-1 and T2D has not been completely clarified, it is known that elevated levels of PAI-1 in T2D correlate with diabetic vascular complications [76]. Clinical studies have reported that reducing blood glucose by an insulin-sensitizing strategy reduces circulation PAI-1 and lowers progression of T2D development [142].

AIMS

The overall objective of this thesis was to study the physiological significance and source of PAI-1 in the MetS and the specific aims were:

- to investigate if tissue-specific glycosylation patterns can reveal the origin of plasma PAI-1

- to investigate if the level of glycosylation differs in obese subjects compared to lean subjects

- to identify stable reference genes for normalization of qPCR in platelets

- to investigate the role of PAI-1 in T2D by characterization of platelet PAI-1 antigen and PAI-1 mRNA expression

MATERIALS AND METHODS

Subjects

Blood samples for isolation of plasma and platelets were collected from subjects with platelet counts of 150-350 x 10^9/L. Both males and females were recruited; lean or with obesity (BMI>30) and/or T2D. Due to diurnal variation of plasma PAI-1, samples were collected between 08:30 and 10:00 a.m. [143]. All participants had been advised not to take acetylsalicylic acid (ASA) or non-steroid anti-inflammatory drugs (NSAIDs) 10 days prior to blood sampling. In addition, they were asked to avoid exercise and alcoholic drinks 48h prior to the visit, eat a light meal the day before the visit and stay fasting after mid-night.

In study IV blood samples were collected only from postmenopausal women, defined as serum follicle stimulating hormone (s-FSH) >26 mU/L, as there are variations of plasma PAI-1 levels during the menstrual cycle [144, 145]. Exclusion criteria were significant cardiovascular disease (ischemic heart disease or heart failure equal to NYHA II) not including hypertension with mono therapy; smoking; significant complications from T2D; treatment with glucagon-like protein-1 (GLP-1) agonists, insulin, beta blockers, glitazones, or dipeptidyl peptidase-4 inhibitors; hematological diseases or bleeding disorders; and other concomitant disease or complications of significance as determined by medical history, physical examinations or screening laboratory evaluations. Medical treatment was withdrawn 10 days prior to the visits and the subjects were attending an extra visit three to four days prior to the visit to register fasting plasma glucose and blood pressure.

According to the Helsinki declaration, all participants signed a written informed consent prior to participation. The protocols were approved by the Ethical Committee at the University of Gothenburg.

Preparation of cells and lysates

Preparation of platelets, plasma and serum

Blood was drawn using no stasis through butterfly needles into syringes containing acid citrate dextrose (ACD) and prostaglandin E_1 (PGE$_1$), to minimize platelet activation during sampling. Platelet-rich plasma (PRP) was prepared by centrifugation at 150 x g for 20 min. After re-centrifugation at 150 x g for 10 min the PRP was pelleted at 800 x g for 15 min. Plasma was removed and the platelet pellet was re-suspended in Pipes/saline/glucose buffer containing PGE$_1$. Finally, platelets were pelleted (800 x g, 15 min), the supernatant was discarded and pelleted platelets were immediately used for subsequent preparations and analysis. In addition, for studies of reference genes in platelets, large amounts of mRNA had to be obtained. Therefore platelets were collected from blood donors at the Blood Center, Sahlgrenska University Hospital, Gothenburg, Sweden. Platelet-rich buffy coat was produced from whole blood from four healthy donors by component manufacture, where after four buffy coats were pooled in platelet medium (NaCitrate, NaCl, NaAc, pH 7.2). Platelet concentration was 515-905 x 10^9/L. Pooled platelets were either activated or inhibited. Activated

platelets were initially pelleted at 800 x g for 15 min and re-suspended in Pipes/saline/glucose buffer containing PGE$_1$. Finally, platelets were pelleted (800 x g, 15 min), the supernatant was discarded and pelleted platelets were immediately incubated in M199 without phenol red (Sigma) for 0, 24 and 48 hours in three different activation conditions; initially inhibited by 100 nM PGE$_1$ and activated after 6 hours, activated by 25 μM PAR receptor agonist SFLLRN (Bachem) or in M199 without phenol red only. Inhibited platelets were kept inhibited in medium (NaCitrate, NaCl, NaAc, pH 7.2) from the Blood Center for 0, 24, and 48 hours. After incubation, platelets were pelleted (800 x g, 15 min), the supernatant was discarded and pelleted platelets were immediately used for subsequent preparations and analysis.

Plasma, which was removed after pelleting the platelets, was re-centrifuged at 2000 x g for 20 min to remove residual platelets. For analysis of plasma PAI-1, in which no platelets were collected, blood was drawn in 0.129 M citrate and centrifuged at 2000 x g for 20 min, plasma was collected and stored at -80°C until analysis. For preparation of serum, blood was drawn in SST™-tubes, centrifuged at 2000 x g for 20 min at 4°C. Serum was collected and stored at -80°C until analysis.

Platelets were lysed to enable analysis of PAI-1 content. PRP was centrifuged at 2000 x g for 20 min and subsequently re-suspended in lysis buffer (0.1% Triton® X-100 (Roche Diagnostics GmbH), 1% bovine serum albumin (BSA) (Sigma-Aldrich) in phosphate buffered saline (PBS) (Lonza) at pH 7.4). After lysis for 30 minutes on ice the platelet lysate was centrifuged at 10 000 x g for 10 min at 4°C to remove cell debris. The supernatant of platelet lysate was diluted 1:10 in 1% BSA in PBS prior to protein analysis.

Methodological considerations: In studies analyzing platelet mRNA it is of importance to evaluate the leukocyte contamination, as the relatively high amount of RNA in leukocytes potentially could interfere with the outcomes. To ensure that the analysis of mRNA represents platelet mRNA, the number of leukocytes has to be kept at minimum. It has previously been presented that less than three-five leukocytes per 10^5 platelets may be considered to be below interfering leukocyte RNA levels [95]. The preparation methods of platelets used in these studies have carefully been evaluated and have resulted in minimal leucocyte contamination [115]. Leukocytes were eliminated by filtration in platelets collected at the blood center and the contamination rate was 1 leukocyte per 25 million platelets at the most.

Preparation and incubation of adipose tissue

For studies of adipose tissue PAI-1 glycosylation, omental adipose tissue 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. Blood vessels and connective tissue were removed and 500 mg tissue was incubated in Medium 199 with Hank´s salts (Gibco), supplemented with 30 mM NaHCO3, 1% human serum albumin (Immuno AG), 150 mM adenosine, 7175 pM insulin (Novo Nordisk), and 0.1 mg/mL cephalothin (Lilly France), during 1-3 days and the medium was changed daily [146]. 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. According to the method of Jaffe et al human umbilical vein endothelial cells (HUVEC) were isolated by collagenase digestion [147]. In brief, under sterile conditions the umbilical vein was catheterized and the blood was removed by infusion of PBS at pH 7.4, 37°C. By incubation with 0.1% collagenase, endothelial cells were explanted following gentle manipulation of the umbilical cord. Isolated cells were maintained in EGM-2 complete culture medium (Cambrex/Clonetics) at 37°C in humidified 5% CO^2 incubator and medium was collected after two days.

Preparation and culturing of monocytes/macrophages

Blood was obtained from three healthy blood donors and buffy coat was prepared from 500 ml whole blood at the Blood Center. By using Ficoll-Paque™ PLUS (GE Healthcare) peripheral blood mononuclear cells (PBMCs) were prepared. 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, after separation and washing. The cells were allowed to adhere for 1 h at 37°C in a humidified 5% CO2 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. After washing, macrophage-specific medium without GM-CSF was added every three days. Macrophages were stimulated with 10 ng/ml TGF-ß (Sigma-Aldrich), and medium was collected after 3 days for PAI-1 isolation. In one of the three preparations, macrophages were stimulated in both 10 ng/ml TGF-ß and 400 ng/ml dexamethasone (Sigma-Aldrich).

Preparation and incubation of hepatocytes

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

Preparation of megakaryocytes

The megakaryocyte cell line MEG-01 (kind gift from Prof. Anders Bengtsson, Lund University) was maintained in RPMI 1640 supplemented with 10% fetal boval serum (FBS) and 1% penicillin/streptomycin. To investigate if miRNAs affect the stability of the reference genes in platelets, the MEG-01 was transfected with 30 nM of *DICER1* short interfering RNA (siRNA) or scrambled negative control siRNA using nucleofection (Lonza) according to manufacturer's protocol. Seventy two hours after transfection, cells were harvested and immediately used for subsequent extraction of RNA.

Methodological considerations: To study the function of single miRNAs *in vitro*, miRNAs of interest are knocked down and effects on target mRNA expression is observed. However, to investigate whether possible reference genes were under miRNA influence in platelets, specifically, a novel technique was developed. Since platelets lack nuclei, observations were conducted on megakaryocytes, from which platelets

derive. By silencing *DICER1* with siRNA, normal miRNA biogenesis is inhibited and genes regulated by miRNAs will be derepressed. *DICER1* is a ribonuclease that cleaves immature pre-miRNA to mature and functional miRNA. Reference genes unaffected by dicer knock down were considered as stable mRNA transcripts.

Chemical analysis

In study IV, all chemical analysis of venous blood was conducted at the Laboratory for Clinical Chemistry at Sahlgrenska University Hospital (Gothenburg, Sweden). Mean platelet volume (MPV) was analyzed by Celldyn Sapphire (Abbott).

Preparation of RNA

For microarray hybridization, total RNA was extracted at time 0 h and 24 h using Chomszynski/Sacci method [148], prepared with MaXtract High Density Tubes 50ml (Qiagen GmbH). To improve the quality and purity of RNA, RNeasy MinElute Clean-up kit was used according to manufacturer's instruction (Qiagen). RNA quality was analyzed on 1% agarose gel and RNA quantity was analyzed using a spectrophotometer (A_{260}/A_{280}) respectively.

For qPCR in platelets, total RNA was extracted using Trizol according to manufacturer's instructions (Ambion by Life Technologies). Concentrations of total RNA were determined on Qubit 2.0 Fluorometer using Qubit RNA HS Assay Kit according to manufacturer's instructions (Life Technologies). mRNA was converted to cDNA by reverse transcription 1x RNA-to-cDNA master mix and High-capacity RNA-to-cDNA master mix (Applied Biosystems).

Methodological considerations: To extract RNA from platelets is challenging. Since platelets are anucleated, they merely contain small amounts of mRNA transcripts in their cytoplasm. A RNA extraction method, which results in sufficient amounts of RNA to perform qPCR and simultaneously minimizes contamination of mRNA, must to be conducted. Since 2008, we have carefully evaluated and optimized different methods; Chomszynski/Sacci method, Trizol and other commercial kits available. In conclusion, extraction by Trizol results in purified and sufficient mRNA to perform qPCR.

For qPCR in MEG01, RNA was prepared using miRNeasy mini kit (Qiagen). mRNA was converted to cDNA by RevertAid H- First Strand cDNA synthesis kit (Thermo Fisher Scientific).

Analyzing techniques – principle and method

Mass spectrometry

Principle in brief
Mass spectrometry (MS) identifies molecules of interest present in a sample, by determining the specific masses. The molecules are vaporized and ionized, separated in an electric field, and detected according to their mass-to-charge (*m/z*) ratio. Ion cyclotron resonance (ICR) and Orbitrap detection offers high mass accuracy and high mass

resolving power. To characterize an exact modification, accurate mass determination is required, and to distinguish between related forms of the peptide or protein with different degrees of modification, high mass resolving power is necessary.

Method

Isolated and purified PAI-1 samples 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 [149]. The digestion was stopped, peptides/glycopeptides extracted, liquid evaporated and finally the samples were reconstituted for LC-MS/MS analysis. Peptides/glycopeptides were separated by C_{18}-fused silica column using an acteonitrile gradient in low concentration formic acid and electrosprayed in positive mode into the mass spectrometer.

In study I and II, samples were analyzed using 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 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 using CID (collision induced dissociation) in the linear ion trap. The typical mass accuracy is <2 ppm in MS mode and 300-400 ppm in MS/MS mode. In addition in study II, the glycopeptides were analyzed on an Orbitrap Fusion Tribrid mass spectrometer. MS scans were performed at 120 000 resolution, *m/z* range 380-1800, MS/MS analysis was performed in a data-dependent mode, with top speed cycle of 3s for doubly or multiply charged precursor ions. Ions in each MS scan were selected for fragmentation (MS2) by high-energy collision dissociation (HCD) at 30% and detection in the Orbitrap at 30000 resolution. Precursors were isolated in the quadrupole with a 2.5 *m/z* window and dynamic exclusion within 20 ppm during 20 seconds was used for *m/z*-values already selected for fragmentation. Similarly, samples were also analyzed on an LTQ-Orbitrap XL (Thermo Fisher Scientific). The settings were: 1 microscan for MS1 scans at 60 000 resolutions (*m/z* 400), full MS mass range *m/z* 400-2000. The LTQ-Orbitrap XL was operated in a data-dependent mode with one MS1 FTMS scan of precursor ions followed by six CID (collision induced dissociation) MS2 scans at 30% energy. Dynamic exclusion of an already selected precursor was set to 10s.

Measured peptide masses and their CID or HCD spectra were submitted for database matching using probability-based scoring. The proteins were identified in the Swiss-Prot database against the mammalian sequences by 5 ppm precursor-ion mass tolerance window, 0.5 Da fragment-ion mass tolerance window and one allowed missed tryptic cleavage. Mass values for peptides that could not be matched to the identified protein sequence by the search-engine Mascot, were examined for the presence of glycosylation by use of the GlycoMod tool, (http://web.expasy.org/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 *m/z* 366 and/or *m/z* 657 oxonium ions of the oligosaccharides *HexHexNAc* and HexHexNAcNeuAc in the corresponding MS/MS

spectrum, and thus confirming the presence of glycosylation. In study II, data-base matches also included modification of Neu5AcGalGalNAc and $(Neu5Ac)_2GalGalNAc$ (656.2276 and 947.3230 amu) of Ser/Thr and Tyr residues, to more directly examine the presence of glycosylation. In addition, the neutral loss of the same masses was included in the data-matching to account for the deglycosylation of b- and y-ions including the glycosylation site. Extracted ion chromatograms of glycopeptide specific oxonium ions at m/z 138.06 and m/z 274.09 in the HCD spectra were plotted using the Xcalibur software (Thermo Scientific). Potential hits were manually evaluated and matched against possible glycan masses using Glycomod. A mass tolerance of 10 ppm was used. The proposed compositions were manually evaluated against the observed fragmentation patterns.

Methodological considerations: We used a mass spectrometry-based method without release of the glycans before analysis. Important information about which glycans are linked to the protein at a specific glycosylation site can be preserved, when analyzing intact glycopeptides. If the protein or peptide is deglycosylated prior to analysis, information can be obtained about the range of glycan structures, but specific structures may not be assigned to their original glycosylation sites [150, 151]. MS with a mass accuracy of 5-10 ppm together with tandem MS fragment information and database interrogations were used to obtain information both on glycan attachment sites and composition. Because the number of possible compositions, with similar masses, decreases with increasing mass accuracy, high mass accuracy is vital for the characterization of different glycoforms of glycopeptides. In the tandem MS analysis of a glycopeptide, the glycosidic bonds are more susceptible to CID fragmentation than the peptide bonds, resulting in a sequential loss of monosaccharide units from the terminal end of the glycan, leaving the peptide part intact. The MS/MS spectra of a glycopeptide (Figure 2) are therefore more straightforward to annotate compared to the MS/MS spectra of released glycans. Due to the low order of glycopeptides in the tryptic digests of a gel band and the typical mircoheterogeneity of glycosylation, very sensitive MS instruments with the capability of high molecular mass detection are needed, as for example Orbitrap or FT-ICR mass spectrometers. Glycopeptide analysis depends on the ionization and the nano-LC separation directly coupled to the MS instruments, the sensitivity of the described nanoLC-MS/MS is in the range of a few femtomoles loaded onto the column.

Glycoprotein specific staining

Principle in brief

Glycoprotein specific staining is used to verify absence, presence or level of glycosylation of a protein. Glycoproteins are separated by electrical charge on a gel and subsequently stained with a substance that reacts with carbohydrate groups, creating a bright green-flourescent signal. The signal is detected by a camera and visualized for analysis.

Method

PAI-1 isolated from platelets and plasma from obese and lean subjects was separated by 10% SDS-PAGE and the gels were stained by ProQ Emerald 300 glycoprotein gel stain kit (Molecular Probes) according to the manufacturer´s instruction. After
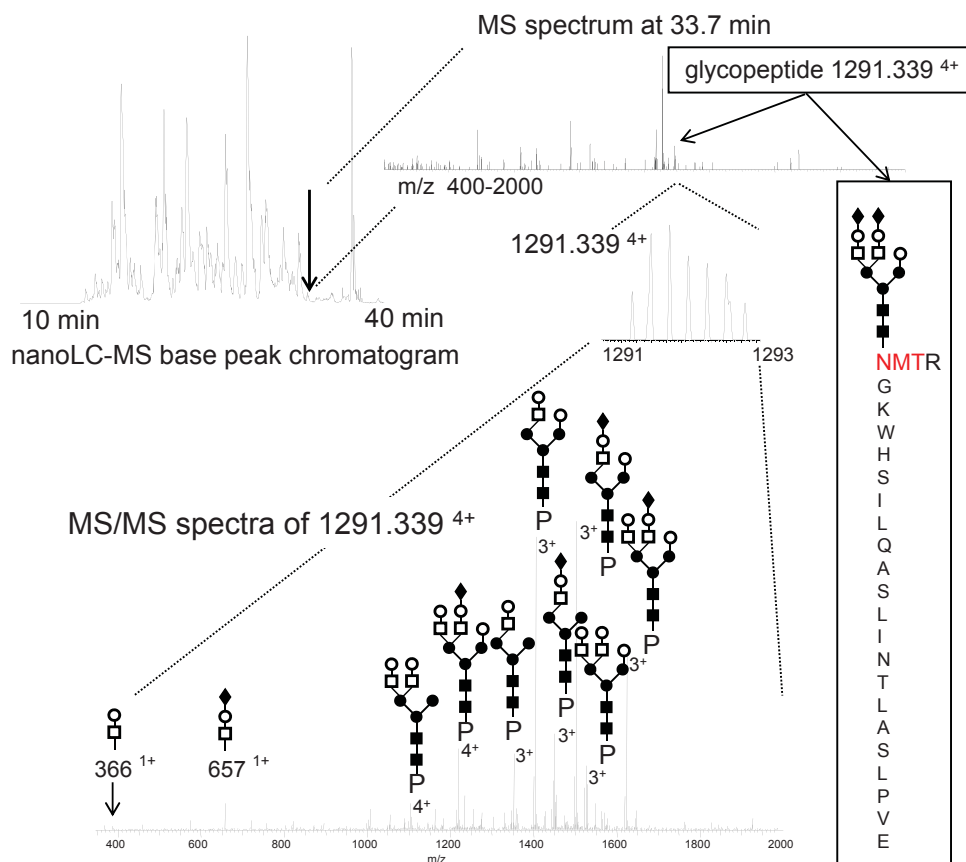
Figure 2. NanoLC-LTQ-FT-ICR MS analysis of plasma PAI-1 from an individual with BMI>35. The same molecular mass eluting at 33.7 min (MH$^+$ 5162.323) corresponding to the glycopeptide with the attached glycan (Hex)3 HexNAc)2(NeuAc)2(Man)3(GlcNAc)2 was also found in adipose tissue. The quadruply protonated 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 (HexHexNAc) and 657 (HexHexNAcNeuAc).

scanning (Fluor-S MultiImager® and Chemidoc® Bio-Rad Laboratories), the gels was stained for total protein by SYPRO® Ruby stain. The detection limit of the ProQ Emerald stain was investigated by SDS-PAGE of serial dilutions of recombinant human glycosylated PAI-1 expressed in Chinese hamster ovary cells (CHO), stained by SYPRO® Ruby for total protein. PAI-1 from adipose tissue and 50 ng CHO PAI-1 were used as positive controls. The method was used to confirm mass spectrometry data and to evaluate differences in level of glycosylation between obese and lean subjects by investigating optical density of the glyco specific staining and the total protein staining by densitometry (ImageJ, nih, Maryland, USA, http://imagej.nih.gov/ij/, 1997-2015). The ratio of glycosylated/total protein was compared between obese subjects and their matched controls.

Enzyme-linked immunosorbent assay (ELISA)

Principle in brief
ELISA is used to quantify proteins. Samples containing a specific protein and standard are added to wells coated with antibodies, which specifically bind the protein in question. After binding and subsequent washing, a second specific enzyme-labeled antibody is added. Wells are finally washed to remove unbound antibodies, subsequently a peroxidase substrate is added that converts the substrate to a spectrophotometrically measured colored product, which is directly proportional to the amount of protein present in the sample.

Method
Levels of PAI-1 antigen in platelets, plasma, and serum were determined with a commercially available ELISA (Technozyme® PAI-1, Technoclone GmbH). All samples were assayed in duplicate according to manufacturer´s protocol.

Microarray hybridization – transcription profiling

Principle in brief
Microarray hybridization aims to study the effects of global gene expression in samples. Thousands of DNA probes are attached to a solid surface in an ordered fashion. RNA from the samples is converted to cDNA that is fragmented, biotin labeled, and hybridized to the array. After washing, binding of biotinylated cDNA to the probes is detected by the addition of streptavidin coupled to a fluorescent dye. After streptavidin binding and washing, the intensity of the fluorescent signal for each probe is detected and is proportional to the relative expression level of the corresponding gene.

Method
Gene expression in platelets incubated before and after 24h incubation from eight donors was analyzed using Human Genome U133 Plus 2.0 chip (Affymetrix) in duplicates. Target preparation and hybridization of the microarray was performed according to procedures developed by the manufacturer. Raw data were visually inspected for hybridization artifacts and then analyzed using the MAS5 software (Affymetrix). Selection of stable genes was based on the MAS5 detection call algorithm.

Quantitative reverse transcriptase real-time PCR (qPCR)

Principle in brief
qPCR is used to quantify levels of specific mRNA transcripts in a sample. The mRNA transcript pool in a cell is purified and reverse transcribed to cDNA. The specific transcript of interest is amplified in a PCR reaction containing a dual-labeled probe. When this fluorescently labeled probe is hybridized to its target sequence during qPCR, the Taq polymerase cleaves the reporter dye from the non-extendable probe. The reporter dye is released into solution where the increase in dye emission is monitored in real-time and the threshold cycle analyzed. The threshold cycle (C_T) is defined as the cycle number at which the reporter fluorescence reaches a fixed threshold level. There is a linear relationship between C_T and the log of initial target copy number [121]. The relative expression levels of the target gene is analyzed using the relative standard curve

method or the comparative C_T method, where the difference in threshold cycles for a target and a reference gene in a treated sample, and comparing it to that of a control sample [153] . Endogenous reference genes work as internal controls to correct for potential variation in RNA loading, cDNA synthesis, or efficiency of PCR amplification.

Method

Relative quantification was performed on 7500 Fast Real-Time PCR System (Applied Biosystems) and StepOnePlus instrument (Thermo Fisher Scientific). Oligonucleotide primers and Taqman probes for quantification of PAI-1 and GAPDH were designed from the GenBank database using Primer Express version 1.0 software (Applied Biosystems). Each primer pair was selected so that the amplicon spanned an exon junction to preclude amplification of genomic DNA. All probes designed in-house were dual-labeled with 5'-reporter dye FAM (6-carboxy-flouorescein) and 3'-quencher dye TAMRA (6-carboxy-tetramethyl-rhodamine). The efficiency of the in-house-designed primer and probes were validated by using a dilution curve. PPIA, RN18S1, YWHAE, B2M, ITM2B, H3F3A, and PF4V1 were quantified with TaqMan® pre-designed gene expression assays™ (Applied Biosystems). All primers and probes are summarized in Table 1. For amplification of the genes, 0.24 µl cDNA was added to the PCR mixture consisting of TaqMan Universal PCR Master Mix Fast, 0.4 µM of each primer, 0.2 µM probe, in a final volume of 15 µl (Applied Biosystems). All samples were assayed in duplicates.

Table 1. Oligonucleotide primers and probes used for qPCR

Gene Symbol	Sequence	Position	Gene Expression Assay
YWHAE			Hs00356749_g1
B2M			Hs00187842_m1
ITM2B			Hs00222753_m1
H3F3A			Hs02598544_g1
PF4V1			Hs0060249_g1
RN18S1			Hs99999901_s1
PPIA			Hs04194521_s1
GAPDH			
forward primer	5'-cca cat cgc tca gac acc at -'3	2171-2190	
reverse primer	5'-cca ggc gcc caa tac g-3'	2195-2217, 3850-3854	
probe	5'-(FAM)aag gtg aag gtc gga gtc aac gga (TAMRA)-3'	3857-3872	
PAI-1			
forward primer	5'-ggc tga ctt cac gag tct ttc a-3'	11616-11637	
reverse primer	5'-ttc act ttc tgc agc gcc t-3'	11758-11780	
probe	5'-(FAM)acc aag agc ctc tcc acg tcg cg TAMRA)-3'	11782-11800	

Multiplate analysis

Principle in brief

Multiplate analyzes the platelets ability to aggregate. TRAP-test consists of TRAP-6 (thrombin receptor activating peptide -6) that stimulates the PAR-1 receptor and induces platelet aggregation. ASPI-test consists of arachidonic acid that inhibits COX-1 activation that results in a decreased platelet aggregation. ADP-test consists of adenosindiphosphate that stimulats ADP-receptors on platelets and induces aggre-

gation. The Multiplate records adhesion and aggregation by multiple electrode aggregometry, which is expressed as the area under the curve in arbitrary units.

Method
Aggregation ability was measured by Multiplate® analyzer (Roche Diagnostics) in platelets from T2D subjects compared to obese and lean controls.

Statistical methods

Standard statistical methods were used. Results were analyzed using SPSS statistical software (SPSS Inc, Chicago, IL). Data are presented as mean and standard error of the mean, unless otherwise stated. The student's *t* test was used for parametric demographic data, PAI-1 antigen levels, miRNA results, and for densitometry analysis. Variance analysis ANOVA with post hoc Bonferroni was used to analyze differences of means between T2D subjects and obese and lean control groups. Non-parametric McNemar's test related samples was used when applicable. Pearson correlation coefficients were calculated to determine associations. In order to stabilize variances and to get symmetrical distributions for the residuals all statistical analyses of PAI-1 were performed on log transformed data. Results were considered significant if $P<0.05$.

RESULTS AND COMMENTS ON METHODOLOGY

Study I and II – Patterns and levels of PAI-1 glycosylation

Detection of PAI-1 by mass spectrometry

To reveal the possible source of plasma PAI-1, the specific glycosylation patterns of PAI-1 from plasma, platelets, endothelial cells, adipose tissue, macrophages and hepatocytes were studied. Gel bands corresponding to the MW of PAI-1 were analyzed and identified as the best protein hit using several significant MS/MS spectra of unmodified PAI-1 peptides in all samples from both studies. Table 2 summarizes glycopeptides and predicted glycan compositions at the two N-linked glycosylation sites found in the nanoLS-MS and MS/MS analysis of PAI-1 from the specific sources in study I. For some of the glycopeptide masses, there are two different explanations of glycan moieties with different compositions.

Examples of the glycan compositions of PAI-1 in study II are demonstrated in Figure 3. Up to 83% sequence coverage of plasma PAI-1 was accounted for in this proteomic analysis (Figure 3A). N-glycosylated peptides were subsequently searched for by tracing the presence of the glycopeptide specific oxonium ions *m/z* 138.055 and *m/z* 274.09 in the HCD-MS2 spectra (Figure 3B-3D).

Glycosylation patterns on PAI-1 from different sources

In study I, protein bands were identified as PAI-1 in the platelet as well as in plasma preparations, and despite this, no glycopeptides were detected. The majority of glycans found were from adipose tissue and 11 suggested glycans were identified. However, two of the detected masses had two alternative glycan compositions. In the three different preparations there were different numbers of glycans identified at N288, with the highest amount of glycans in the preparation where the highest amount of PAI-1 was obtained. The limited amount of PAI-1 obtained from macrophages made precise identification of the composition of the glycans difficult. The possibility of no glycosylation was excluded, since diagnostic glycan ions were detected. Glycans were detected on N232 at peptide position FNYTE in endothelial cells only. Two different compositions were detected at this position, but the other site was not utilized. We were not able to detect PAI-1 with MS/MS in hepatocytes. In Figure 4, a summary of the glycosylation pattern of PAI-1 from the different sources is shown.

Comments:
PAI-1 from endothelial cells, macrophages and adipose tissue was glycosylated with heterogeneous glycosylation patterns, in contrast to platelets and plasma from lean subjects, where no glycans were found. Previous studies have shown that the endothelium produces large amounts of PAI-1 *in vitro*, although this may be a cell-culturing artefact [154, 155]. Furthermore, PAI-1 in the endothelium is released basolaterally to the extracellular matrix and endothelial PAI-1 may therefore not be involved in the intra-vascular fibrinolysis [156]. These data support our finding that PAI-1 from the endothelium is glycosylated with two different glycans on N232 and is therefore not likely to be a major source of plasma PAI-1 in healthy individuals.

Table 2. Summary of the masses and possible compositions 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

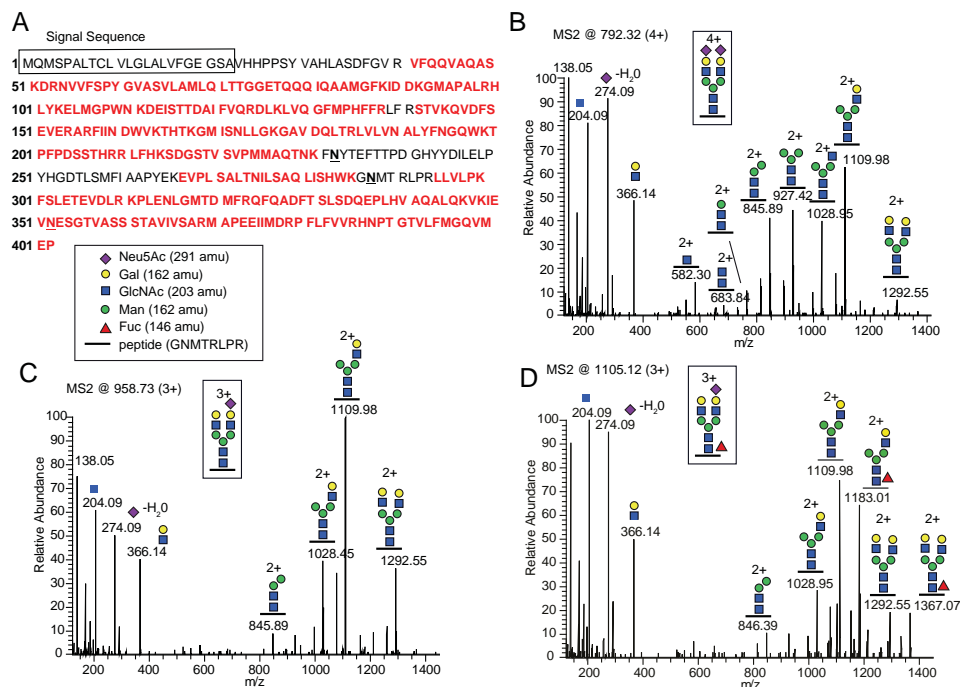| MH^+	Glycan	ppm	Missed cleavage	M_{ox}
5637.45	$(Hex)_3 + (Man)_3(GlcNAc)_2$	2.0	-	-
5799.49	$(Hex)_4 + (Man)_3(GlcNAc)_2$	0.2	-	-

Figure 3. Proteomic and glycoproteomic identification of plasma PAI-1. **A**. The sequence of PAI-1. Red residues designate identified peptides in the proteomic analysis. Underlined Asn residues are potential N-glycosylation sites. The squared sequence is the signal peptide. The N-terminal of the extracellular protein is Val-24. **B**. HCD-MS2 spectrum of disialylated (Neu5Ac*2) and biantennary N-glycan substituted GNMTRLPR (boxed structure); **C.** monosialylated biantennary N-glycan; and **D**. biantennary N-glycan. Ion charges are shown when $z > 1$. Monosaccharides are displayed according to the Consortium for Functional Glycomics. Neu5Ac=5-N-acetylneuraminic acid (sialic acid), Gal=galactose, GlcNAc=N-acetylglucosamine, Man=mannose, Fuc=fucose, amu=atomic mass units.

PAI-1 has been detected in cells of monocyte/macrophage lineage and peripheral blood monocytes as well as macrophages in the wall of atherosclerotic vessels synthesize PAI-1 [30, 157, 158]. However, it is not likely that blood monocytes contribute significantly to the plasma levels since the *in vitro* synthesized levels are close to undetectable [159, 160]. Since diagnostic ions were present and one glycan was identified on macrophage PAI-1, it is less likely that these cells are significant contributors to plasma levels in healthy individuals.

Moreover, we failed to isolate and detect PAI-1 from primary human hepatocytes. Previous studies of PAI-1 protein and mRNA levels in the liver have shown diverging results and since PAI-1 has been shown to be an acute phase protein and the expression of PAI-1 can be dramatically up-regulated in hepatocytes when stimulating with inflammatory mediators, an inflammatory stress state may be required to obtain significant amounts [31, 158, 161]. The liver might be an important source of plasma PAI-1 in inflammatory disease, sepsis, and stress. However, this results clearly suggests that unstimulated hepatocytes synthesize very low amounts of PAI-1 and further studies with larger samples will have to be performed to enable evaluation of the glycosylation pattern.
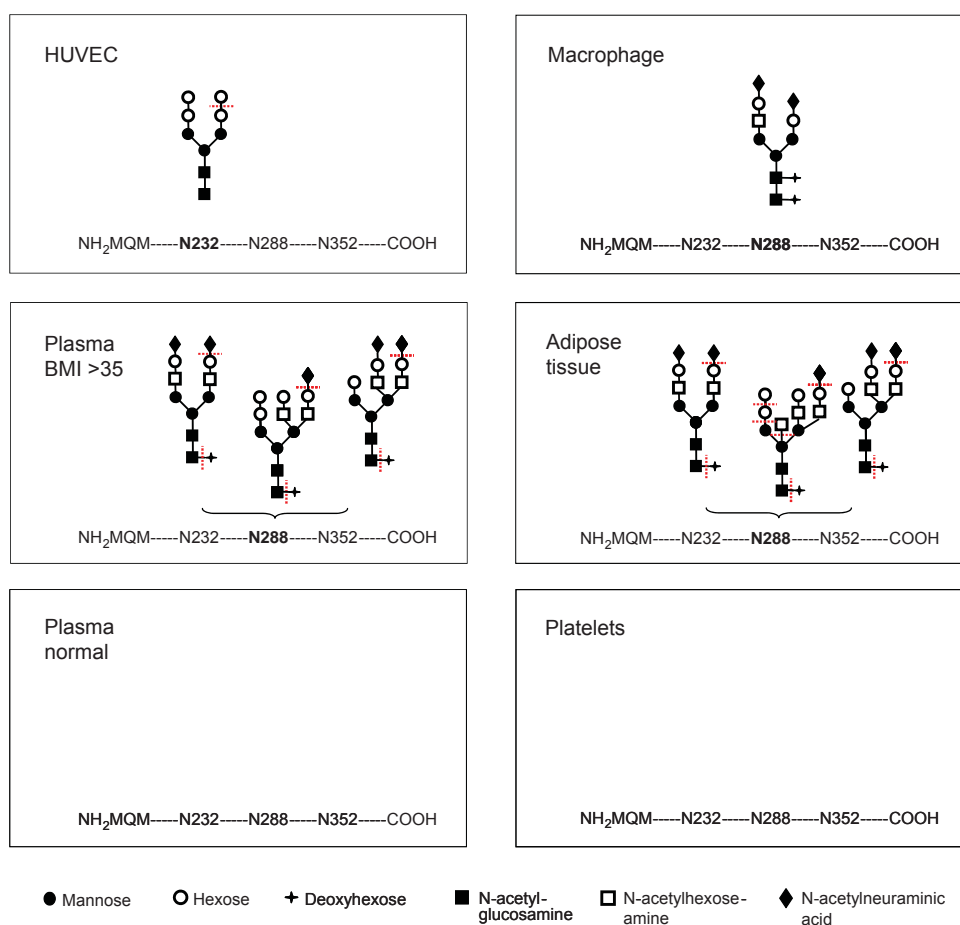
Figure 4. Schematic illustrating the suggested glycan compositions at the two sites on PAI-1 from the analyzed sources. Because of the heterogeneity of the glycan compositions on PAI-1 from adipose tissue and from plasma from obese subjects, three structures had to be made to summarize the suggested compositions. The dashed lines indicate the different glycan combinations at that site.

Glycosylation of plasma PAI-1 in obese subjects

In the second step, we investigated the glycosylation patterns on PAI-1 from plasma in obese subjects. In study I four obese subjects were included. The BMI ranged from 36.6 to 40.7, three males and one female, 47-74 years, and PAI-1 >50 ng/ml. The demographic characteristics of the 12 obese subjects and the 12 ages- and gender matched non-obese subjects in study II are listed in Table 3. Waist circumference, Waist-hip-ratio (WHR) and BMI were significantly increased in the obese subjects. Plasma PAI-1 levels were significantly elevated in obese subjects compared to non-obese subjects (44.7 ± 41.8 ng/mL vs. 11.8 ± 11.8 ng/mL; *P*=0.003). WHR, BMI and waist circumference correlated significantly to plasma PAI-1 (r=0.515, *P*=0.010; r=0.486, *P*=0.016; r=0.523, *P*=0.009 respectively).

Table 3. Characteristics of the participants. Data are means ± SD. P values are calculated by paired sample t-test or non-parametric t-test related samples when applicable.

	Obese	**non-obese**	*P* value
n	12	12	
Sex (F/M)	6/6	6/6	NS
Age (years)	53.2 ± 12.1	53.9 ± 12.1	NS
Tobacco usage (yes/no)	3/9	1/11	NS
Hypertension (yes/no)	8/4	1/11	0.016
Waist circumference (cm)	118.8 ± 12.3	83.6 ± 7.15	<0.001
WHR (cm/cm)	0.96 ± 0.07	0.82 ± 0.06	<0.001
BMI (kg/m2)	36.3 ± 4.55	23.4 ± 1.56	<0.001
PAI-1 ag (ng/ml)	44.7 ± 41.8	11.8 ± 11.8	0.003

In study I, the total number of glycans at N288 on plasma PAI-1 from the subjects with BMI >35 subjects was seven, but two of the masses had two possible compositions (Figure 4). Five of the glycan compositions found in adipose PAI-1 were also found on plasma PAI-1 from obese subjects. The amount of isolated PAI-1 from the four obese subjects differed substantially. The largest number of detected glycans was found on PAI-1 from the subject from whom the largest amount of PAI-1 was isolated. To verify the MS/MS results of absence or presence of glycosylated PAI-1 from different tissues in study I, 1D gel electrophoresis and glycoprotein gel stain was used (data not shown). Glycosylated PAI-1 in platelets or plasma from lean subjects was not detected, however, plasma PAI-1 from obese subjects, adipose tissue and CHO were positive with the glycoprotein stain that confirmed the MS/MS results. Subsequent staining with total protein SYPRO Ruby showed the presence of PAI-1 in all samples.

In study II, glycans were found on plasma PAI-1 from both obese and lean control subjects. No significant qualitative differences in the glycosylation between the samples could be detected. Therefore, glycoprotein staining was performed in order to semi quantitatively measure the level of glycosylation between samples. To assess the level of glycosylation, 1D gel electrophoresis and Pro-Q Emerald 300 glycoprotein gel stain was performed for all subjects, as represented in Figure 5. Subsequent staining with total protein SYPRO Ruby stain showed the presence of PAI-1 in both groups (Figure 5B). There were different levels of glycosylation in obese subjects and the age- and gender matched controls (Figure 5A). Densometric analysis of the gel bands and comparisons of the ratio glycostaining/total PAI-1 between the obese subjects and the matched controls revealed that the ratio of glycosylated plasma PAI-1 compared to the total plasma PAI-1 level was 0.85 ± 0.37 in obese subjects and 0.49 ± 0.34 in lean control subjects; the difference was statistically significant (*P*=0.002). The difference in level of glycolysation between obese and control subjects was 54%. The gel analy-

sis was repeated two times with separate gel runs and the densometric measurements generated the same result. Additionally, there was a weak but significant correlation between the level of glycosylation and BMI (r=0.406, P=0.049).
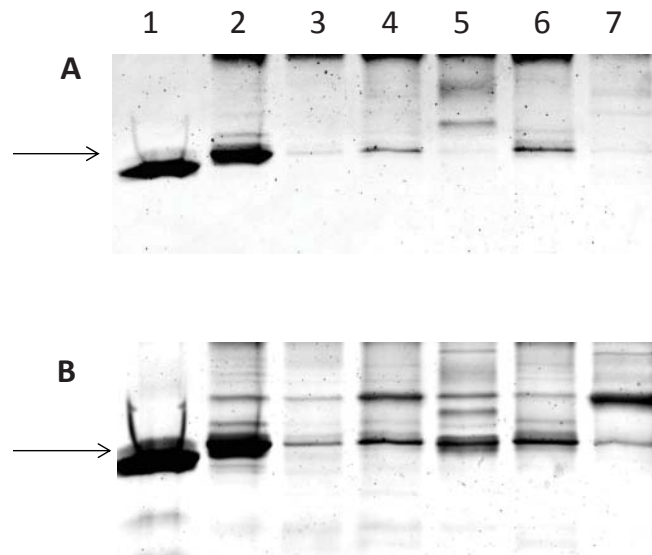

Figure 5. Glycosylation on plasma PAI-1. Gel image showing the results of glyco-specific staining with A: Pro-Q Emerald and B: same gel subsequently stained with SYPRO Ruby. PAI-1 from CHO cells were used as positive controls. Samples with PAI-1 are in the following order; lane 1: 50 ng CHO PAI-1, lane 2, 4 and 7: plasma PAI-1 from subjects with BMI>30, lane 3, 5 and 7: plasma PAI-1 from matched control subjects with BMI<25.

Comments:

No glycans were found on plasma PAI-1 from lean subjects in study I, but a variety of glycans were found on plasma PAI-1 from lean control subjects in study II. This discrepancy is unlikely to be caused by methodological differences. Although the more sensitive and much faster LC-MS/MS experiments of high resolution Orbitrap spectrometers used in study II increase the possibility to detect lower amounts of glycans, the vast amounts of PAI-1 analyzed in study I should have revealed glycans even with FT-ICR (LTQ-FT, Thermo Electron) masspectrometer.

The high performance mass spectrometry method used in study II, without isotopically labeled standards, cannot be regarded as quantitative. Therefore, mass spectrometry was only used to verify the strong presence of immunopurified PAI-1 in the gel bands, and to verify the presence of PAI-1 glycosylation at the glycopeptide level. Glycospecific and total protein staining followed by densometric measurements are indeed a semi-quantitative method, but by repeating the gel runs and staining protocols similar results were observed. The known correlation between PAI-1 and BMI was highly significant in the cohort in study II, verifying the representativeness of the study subjects. Plasma PAI-1 antigen levels were significantly increased in obese subjects, results that confirm previous findings [141].

Study III and IV – The role of platelet PAI-1 in T2D

To investigate the role of platelets on the raised plasma PAI-1 levels in T2D, mRNA expression of PAI-1 in platelets was characterized in T2D subjects. In order to study mRNA expression in platelets, a normalization strategy for qPCR was conducted. qPCR is a robust method to quantify gene expression [162], however, potential errors can occur at various stages [163]. The use of reference genes is the most effective method to correct for these errors, but the reference genes have to be validated [164-166]. It has been shown that commonly used reference genes are not necessarily the most stable ones [167] and more importantly, using the wrong reference gene may produce erroneous results [168].

Identification of stable genes in platelets

Microarray analysis was used to identify transcripts with stable expression. There is evidence that most of the mRNAs in platelets degrade over time [98], and an unusual strategy for selection of stable genes had to be employed. The MAS5 algorithm normalizes the data based on total signal intensity of the microarray. This means that the signal of transcript in the samples after 24 incubation are lower due to the transcript degradation but the signals are artificially increased by the normalization procedure to make the overall signal comparable between microarrays. The transcripts that by the MAS5 algorithm are classified as *increased,* are in fact the transcripts that have been degraded the least (hence, the most stable). A total of 83 genes were classified as *increased* in both microarray replicates. Final candidates for further evaluation was selected among these 83 transcripts, classified as increased based on the MAS5 increase call, and had a more than 2-fold increase in signals (hence a low degradation) and a high signal intensity in both replicates. The five genes, YWHAE, B2M, ITM2B, H3F3A and PF4V1 that expressed the highest signal intensity, were chosen as candidates from the microarray experiment. Additionally, GAPDH, RN18S1 and PPIA were chosen as candidates for analysis, since they have previously been used as reference genes in studies of platelets [43, 115, 169-172]. These eight genes were selected as candidate genes for further characterization, summarized in Table 4.

Table 4. Selected candidate genes

Gene Symbol	Assession Number	Gene Title	Genomic Localization
YWHAE	NM_006761	Tyrosine 3-monooxygenase/ tryptophan 5-monooxygenase activation protein	chr17p13.3
B2M	NM_004048	Beta-2-microglobulin	chr15q21-q22.2
ITM2B	NM_021999	Integral membrane protein 2B	chr13q14.3
H3F3A	NM_002107	H3 histone, family 3A /// H3 histone, family 3A	chr1q41
PF4V1	NM_002620	Platelet factor 4 variant 1	chr4q12-q21
GAPDH	NM_002046	Glyceraldehyde-3-phosphate dehydrogenase	chr12p13
RN18S1	NR_003286	18S ribosomal 1	chr22p12
PPIA	NM_021130	peptidylprolyl isomerase A (cyclophilin A)	chr7p13

Comments:

Traditional reference genes were reported as early as the 1970s to be differently expressed in tissues and despite these observations they have been used extensively, without proper validation, in many studies [173, 174]. In order to perform an unbiased search for genes that are stable over time, a microarray experiment was performed. In this experiment, YWHAE, B2M, ITM2B, H3F3A, PF4V1 were the five most stable genes in platelets over time, even though none of them has previously been suggested as a suitable reference gene in platelets. However, there are potential pitfalls in using microarray in this experimental setting. Firstly, platelets used for the microarray were inhibited until RNA isolation, and hence does not include the whole experimental conditions used for the qPCR evaluation of the reference genes. Secondly, one could also argue that no gene from the microarray experiment is an excellent reference gene, since no gene was completely stable over time as the signal from all genes decreased over time. The most likely explanation is that the mRNA content in platelets, derived from the megakaryocytes, is degraded over time despite inhibition of the platelets. Even with these potential pitfalls, the unbiased approach using microarray for selection of candidates for validation is preferable to choosing candidate reference genes from the literature since it opens up for discovery of genes not previously considered as reference genes. The validity of this approach is indicated in the results showing that the stability of the genes chosen from the microarray experiment in general was higher that the genes selected from literature.

Confirmation of the candidate genes by qPCR

All candidate genes had raw C_T values ranging from 15 to 33. Stability, identified as geometric means by RefFinder, of the candidate reference genes in platelets are shown in Table 5. At least three genes should be used as reference genes for a correct normalization according to previous findings [166]. YWHAE, PF4V1 and B2M were most stably expressed in the total material, combining all experimental data. In activated platelets YWHAE, PF4V1 and B2M were the most stably expressed genes, as seen in the total material. There was no difference in stability between the three various conditions, in which the platelets were activated (data not shown). Inhibited platelets showed a different result, where ITM2B was most stably expressed followed by PF4V1, B2M and YWHAE.

Platelets were incubated for 0, 24 and 48 hours. Initially, ITM2B was most stably expressed, followed by YWHAE, B2M and PF4V1. However, after 24 and 48 hours PF4V1, YWHAE and B2M were the most stably expressed genes. YWHAE, PF4V1 and B2M are most stably expressed genes in platelets from healthy donors. In inhibited non-incubated platelets ITM2B was most stably expressed. However, this candidate gene was not stable over time and across different types of incubations. GAPDH was consistently never ranked as the most stable gene, and never the least stable gene. PPIA and RN18S1 were the least stable genes.

In order to elucidate if there are inter-individual differences; five subjects with type 2 diabetes (T2D) and five gender- and age matched controls were studied (Table 6). In both groups YWHAE was the most stable gene followed by H3F3A and ITM2B.

Table 5. Comprehensive gene stability by RefFinder in platelets from healthy donors. Platelets are pooled from four healthy donors in three different experiments. Isolated platelets are incubated under different in vitro conditions. The three most stable genes are marked in bold for each in vitro experiment.

	YWHAE	PF4V1	B2M	ITM2B	H3F3A	GAPDH	PPIA	RN18S1
All data	**1,565**	**1,861**	**2,943**	6,236	3,722	3,162	7,238	7,445
Activated	**1,565**	**1,861**	**2,943**	6	3,722	3,162	7,238	7,737
Inhibited	2,828	**2**	**2,28**	**1,778**	4,821	5,477	7	8
Non-incubated	**1,861**	3,364	**2,449**	**1,316**	5,477	5,477	7	8
Incubated 24h	**1,861**	**1,565**	**2,913**	5,433	4,472	2,943	7	8
Incubated 48h	**1,861**	**2,060**	**2,236**	6,481	4	2,943	8	6,481

Table 6. Comprehensive gene stability by RefFinder in platelets from T2D and controls. Platelets are isolated from five subjects with type 2 diabetes and five age- and gender matched healthy control subjects. The three most stable genes are marked in bold for each experiment.

	YWHAE	PF4V1	B2M	ITM2B	H3F3A	GAPDH	PPIA	RN18S1
T2D	**1,32**	7,44	4,47	**3,13**	**1,41**	3,87	7	6,45
Non-T2D	**1,41**	7	5,23	**2,06**	**1,73**	4	8	5,73

Comments:

There are several web-based programs that can be used to identify the most stable genes among a set of candidate genes in an experiment of interest. GeNorm calculates the geometric mean of the gene expression of data converted to copy number to select the least variable gene [166]. A second program, BestKeeper, uses the same method as GeNorm, but using raw data [175]. Norm-Finder measures the variation and ranks the reference genes based on the impact of the experimental conditions between observations [176]. In this study the software RefFinder was used, since it integrates four different approaches; GeNorm, NormFinder, BestKeeper and ΔΔCT method. RefFinder uses raw CT data, and to adjust for the efficiencies of the qPCR assays used, GeNorm and NormFinder were evaluated separately to take the efficiencies into account [177]. The result was the same, independently of the method used.

Confirmation of the candidate genes by miRNA experiments

Furthermore, to confirm the stability and the precision of the reference genes further observations were conducted. To study if miRNAs affect the stability of the candidate genes, the megakaryocyte cell line MEG-01 was transfected with Dicer1 siRNA. After 72 h transfection, Dicer1 expression was decreased by 61.7% (*P*=0.000008) compared to cells transfected with scrambled control siRNA. Moreover, a number of highly abundant platelet miRNAs were depleted in cells transfected with Dicer1 siRNA, confirming that Dicer1 knock-down led to disrupted miRNA biogenesis (data not shown). Global mean normalization was used to normalize for each of the candidate genes. A significant increase compared to control cells was seen for ITM2B and

PPIA, by 26.0% (*P*=0.009) and 25.3% (*P*=0.019), respectively (Figure 6). RN18S1 was increased by 24.5%, but this was non-significant (*P*=0.064). YWHAE, H3F3A, B2M, and GAPDH remained unchanged whereas PF4V1 was not detectable at all.
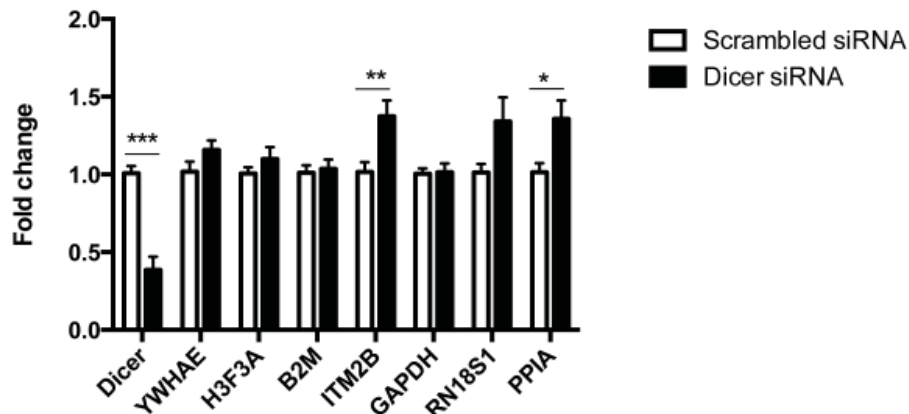

Figure 6. Effect of miRNA on candidate genes. Megakaryocytic cell line MEG01 transfected in three experiments with three replicates in each group. **P*=0.019, ** *P*=0.009, ****P*=0.000008.

Comments:
In order to evaluate the possible effect of miRNA on the stability of the selected reference genes, we performed a thorough review of the literature but could not find any evidence that any of the candidate genes are targeted by miRNAs. Dicer1 is required for miRNA biogenesis by cleaving double stranded pre-miRNA [178]. In this study, YWHAE, B2M, H3F3A and GAPDH were not influenced by Dicer1 knock down and are therefore not expected to be regulated by miRNAs, a result in agreement with the result from RefFinder analysis. ITM2B, and PPIA increased in MEG01 cells after Dicer1 knock-down, which may indicate depression of gene expression as a result of decreased miRNA levels.

There was some variation in the stability of the candidate genes, but YWHAE was the most stable ones throughout all experimental conditions and it was not influenced by miRNA. B2M, GAPDH, and H3F3A varied in stability; however, they were not under miRNA regulation and may well be used as candidates when validating reference genes in platelets. On the other hand, ITM2B, PPIA and RN18S1 were unstable and potentially regulated by miRNA and are therefore not robust candidates. GAPDH was not among the most stable genes, nevertheless it appeared not to be influenced by miRNA and since it is widely used as a reference gene in platelets it may be considered for further research if it is desired to compare results with previous studies. To our knowledge, there is only one previous study investing reference genes in platelets, and in this study platelets from healthy donors and subjects with a history of myocardial infarction were used [179]. GAPDH was the most stable gene in healthy donors

(geometric mean 2.34), although, except for GAPDH, a comparison of results is not applicable, since different candidate genes were used. In conclusion, YWHAE is a stable reference gene across different experimental conditions and intra-individual variations in platelets and is a potiential generic reference gene for qPCR in platelets.

Characterization of the platelet pool of PAI-1 in T2D

In order to investigate the role of platelet PAI-1 in T2D, the levels of PAI-1 mRNA expression and antigen were characterized. Eight subjects with T2D were included, as well as seven obese and eight lean control subjects. The characteristics of the three subject groups are shown in Table 7. The three groups were well defined regarding obesity, and T2D subjects were distinctly differentiated from the two control groups concerning factors associated with T2D.

Table 7. Characteristics of the participants. Data presented as mean±SD. One-way ANOVA was used for statistical analyses, with Post Hoc test Bonferroni; *significant difference between control and T2D, #significant difference between control and obese, and Ψsignificant difference between obese and T2D.

	T2D	**Obese**	**Lean**	***p*-value**
Sex (M/F)	4/4	2/5	3/5	NS
Diabetes duration	3.2±1.2			
Age (years)	59.5±5.6	60.4±5.6	60.1±5.8	NS
BMI (kg/m^2)	34.2±2.7*	32.4±1.9#	23.2±2.1	<0.001
Waist (cm)	112.8±7.0*	110.4±5.5#	83.7±10.7	<0.001
WHR	0.97±0.08*	0.94±0.07	0.84±0.09	0.008
SBP (mmHg)	154.5±14.2*	135.3±18.1	117.1±12.4	<0.001
DBP (mmHg)	94.1±6.8*	86.4±10.6#	74.1±9.30	0.001
fP-Glucose (mmol/L)	9.3±2.50*,Ψ	5.6±0.55	5.0±0.77	<0.001
B-HbA1c (mmol/mol)	54.3±8.6*,Ψ	35.6±3.4	32.6±1.8	<0.001
S-Cholesterol (mmol/L)	5.2±1.0	5.2±1.2	5.5±1.2	NS
S-Triglycerides (mmol/L)	1.6±0.51*	0.38±0.14#	0.18±0.06	0.001
S-HDL-c (mmol/L)	1.3±0.28*	1.2±0.27#	1.9±0.42	0.001
S-LDL-c (mmol/L)	3.6±1.00	3.7±0.99	3.6±0.97	NS
S-Creatinine (μmol/L)	70.5±7.1Ψ	88.3±7.9	78.8±13.1	0.008
S-ALAT (μkat/l)	0.77±0.33*,Ψ	0.47±0.12	0.33±0.06	0.001

Table 8 summarizes the characteristics of the diabetic platelets compared to obese and lean control subjects. There was no difference in platelet count between the groups and no significant correlations between MPV and BMI, plasma glucose or HbA1c. Platelet aggregation ability, measured by multiplate analyzer, was within reference values in all groups. There was no difference between the groups, with the exception for ASPI-test where obese subjects had increased aggregation ability in response to arachidonic acid compared to lean subjects.

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Table 8. Platelets in T2D subjects compared to obese and lean controls. Data presented as mean±SD. One-way ANOVA was used for statistical analyses, with Post Hoc test Bonferroni; *significance between control and T2D, #significance between control and obese, and Ψsignificance between obese and T2D. For TPK and MPV; T2D n=8, obese n=7, lean n=8. For multiplate results; T2D n=3, obese n=6, lean n=5.

	T2D	Obese	Lean	*p*-value
B-TPK (x10^9/L)	256.8±60.6	238.4±51.2	232.4±39.6	NS
B-MPV (fL)	7.3±0.84Ψ	9.0±0.58	8.5±1.20	0.020
P-TRAP	118.3±13.5	134.8±22.9	105.4±15.7	NS
P-ADP	78.3±26.6	86.8±9.7	67.0±23.9	NS
P-ASPI	91.7±9.5	107.0±5.7#	84.0±12.2	0.005

Differences in antigen levels of PAI-1 in platelets, plasma and serum were investigated by ELISA, illustrated in Figure 7. There was approximately a 6-fold increase in plasma PAI-1 in T2D and obese subjects compared to lean control subjects. A non-significant trend towards an increase in serum PAI-1 levels in T2D and obese subjects compared to lean control subjects was observed. Although the ratio plasma PAI-1 and serum PAI-1 between lean control subjects and T2D or obese subjects differed significantly, there was no difference in the platelet pool of PAI-1 antigen between the three groups. There was a significant difference in the ratio between plasma PAI-1 and serum PAI-1 comparing T2D subjects or obese control subjects to lean control subjects shown in Figure 8. Furthermore, plasma PAI-1 correlated significantly to BMI and waist circumference. In addition, there were significant correlations between plasma PAI-1 and factors involved in the MetS; P-glucose, HbA1c and triglycerides, however, there was no significant correlation to platelet count or MPV.

The mRNA levels of PAI-1 in platelets are shown in Figure 9. There was no difference in gene expression levels of PAI-1 between T2D, obese and lean control subjects normalized to YWHAE or B2M (*P*=0.558 and *P*=0.332 respectively).

Comments:
As expected, plasma PAI-1 was significantly increased in T2D and obese subjects and there were highly significant correlations between BMI or triglycerides and plasma PAI-1, which are well-known correlations and indicate that the subjects from this cohort are representative [43, 180, 181].

A potential limitation is the relatively low number of subjects included in this study and an increased number of subjects could possibly alter the results. Larger studies may be conducted in order to reach improved statistical power. Furthermore, the T2D subjects in this cohort had mild T2D. It cannot be ruled out that analysis of the pool of platelet PAI-1 from subjects suffering from severe T2D may have resulted in a different outcome. However, an alternative design cannot be conducted as it would be unethical to withdraw medical treatment for ten days in subjects suffering a more severe T2D.
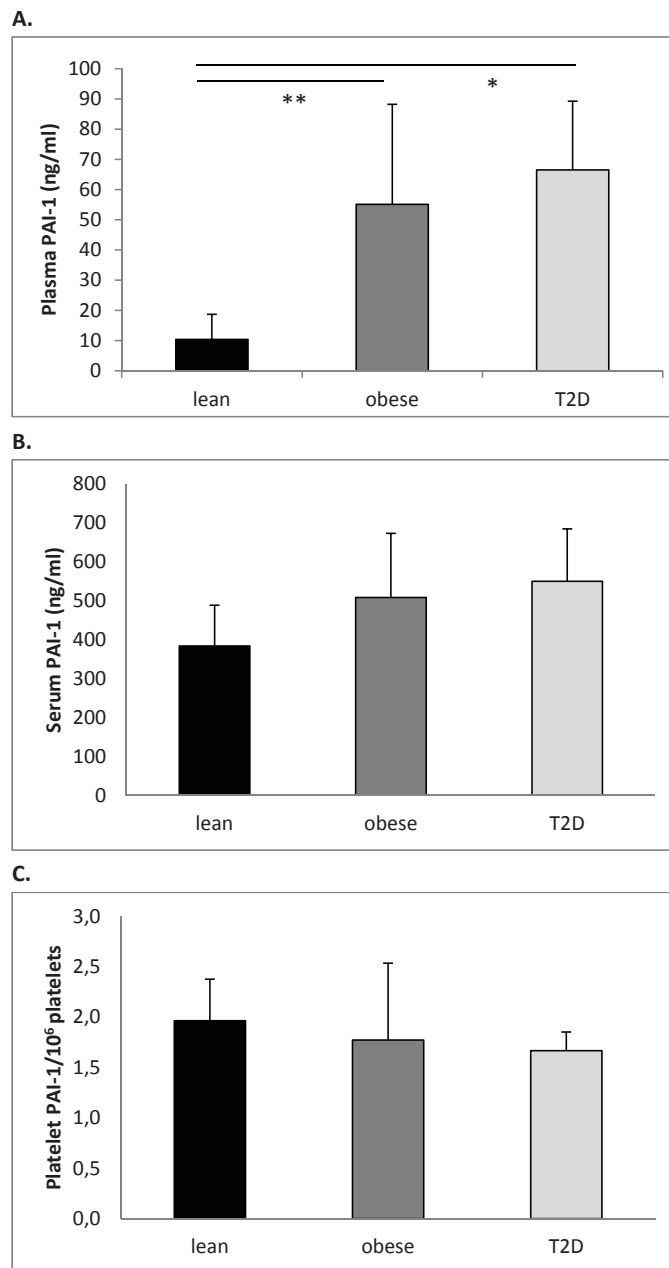
Figure 7. Pool of PAI-1 in plasma, serum and platelets in T2D subjects. **A**. There was a significant increase of plasma PAI-1 in subjects with T2D and obese subjects compared to lean control subjects (T2D n=6, obese n=7 and lean controls n=8). *P<0.001, **P<0.001. **B**. No significant difference in serum PAI-1 between the three groups (T2D n=6, obese n=7 and lean controls n=8). **C**. There was no significant difference in platelet PAI-1 between the three groups (T2D n=4, obese n=5 and lean controls n=8).
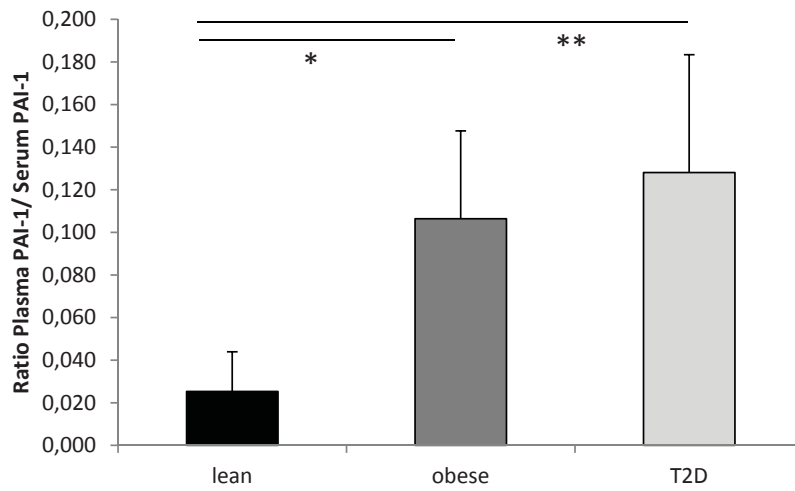
Figure 8. Ratio of plasma PAI1/serum PAI1 in T2D. There was a significant difference in ratio between lean control subjects and T2D or obese subjects (T2D n=6, obese n=7 and lean controls n=8). *P<0.001, **P=0.003.
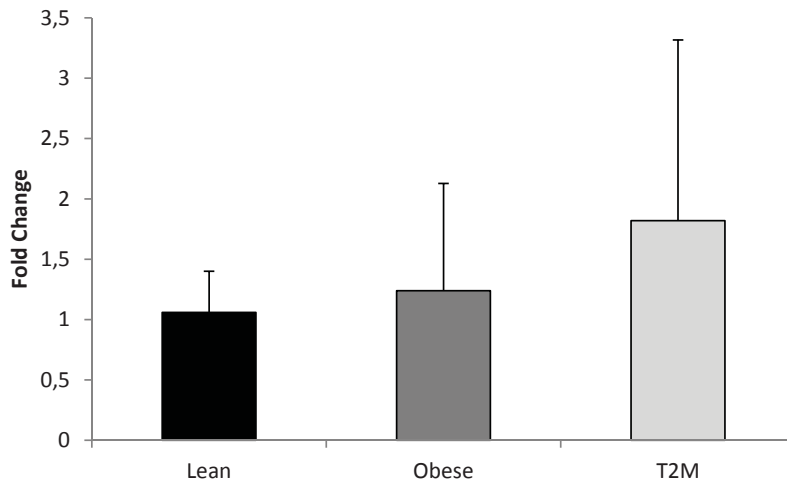
Figure 9. Gene expression levels of PAI-1 compared to reference genes. There were no significant differences in gene expression levels of PAI-1 in platelets between subjects with T2D, obese and lean controls. T2D n=6, obese n=7 and lean controls n=7.

PATHOPHYSIOLOGICAL DISCUSSION AND CONCLUSION

The source of plasma PAI-1 has previously been considered an unsolved question. 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 MetS with increased levels might facilitate targeted risk prevention and potential future therapies. The results of the four studies included in this thesis indicate that the platelet pool of PAI-1 is the important contributor to normal plasma PAI-1 in lean individuals. However, the increased levels of plasma PAI-1 in MetS including T2D may derive from another source where adipose tissue is a likely candidate.

The source of plasma PAI-1 in healthy individuals

Several studies of both humans and rodents have been performed to identify the source of plasma PAI-1, and both PAI-1 mRNA and protein are found in many tissues. The main pool of PAI-1 in the body is found in platelets [110, 182]. However, because of the lack of an association between the platelet PAI-1 content and the plasma concentration, platelets have not been considered to contribute to plasma PAI-1 [110, 183]. On the other hand, it has been shown that platelet count correlates with plasma PAI-1, a finding which is compatible with the view that a constant release of PAI-1 from platelets could contribute to the plasma level [43, 184-186]. There is a considerable constitutive *de novo* synthesis of PAI-1 in platelets and a release of as little as 3% of newly synthesized PAI-1 would be sufficient to maintain normal plasma levels [115]. Taken together with the results that plasma and platelet PAI-1 share the characteristic of not being glycosylated, our findings suggest platelets to be the main source of PAI-1 in plasma in healthy lean subjects.

The source of plasma PAI-1 in obesity

Previous studies have revealed that plasma PAI-1 levels correlate with BMI, and in obese mice and humans PAI-1 mRNA is up-regulated in adipose tissues [31, 187, 188]. In addition, elevated plasma levels of PAI-1 in obese subjects can be normalized by removal of adipose tissue both by surgery, dieting, or gastric bypass [128, 129, 132], suggesting that adipose tissue could be a source of plasma PAI-1 in obese subjects. Other studies have reported that visceral and subcutaneous adipose tissue explants produce PAI-1 in amounts correlated to its levels in blood [189]. Conversely, studies of arteriovenous differences across subcutaneous tissue from obese and visceral fat from non-obese subjects have not been able to demonstrate a significant release of PAI-1 [190, 191].

Interestingly, we were able to show differences in glycosylation patterns of plasma PAI-1 from lean and obese subjects. Moreover, seven 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 of those glycans were found in the other tissues studied. The higher levels of plasma PAI-1 observed in obesity seem to depend on a contribution from adipose tissue, which suggests that pathophysiological conditions may shift the relative contribution from different cel-

lular sources. Furthermore, not only the increased levels of plasma PAI-1 in obesity seem to increase the risk of thrombosis, but also its level of glycosylation. We found a higher level of glycosylation of plasma PAI-1 in obese subjects and there was a correlation between BMI and level of glycosylation indicating a relative relationship between fat deposits and level of glycosylated plasma PAI-1. Since the biochemical properties of a protein may change upon glycosylation and glycosylated PAI-1 has a 2.3-fold stronger inhibitory effect on t-PA than non-glycosylated and a lower inactivation rate [61, 62], our results suggest that it may not only be the increased absolute level of plasma PAI-1 that increases the risk for thrombotic events in obese subjects but also the increased proportion of glycosylated plasma PAI-1.

In study II, glycans were found on plasma PAI-1 from both obese and lean control subjects, which initially may appear inconsistent with study I, in which no glycans were found on plasma PAI-1 from lean subjects. However, the discrepancy between the two studies is likely to be caused by differences in metabolic phenotypes of the control groups. In study II, the lean control subjects were physically inactive compared to the physically active and fit subjects in study I. There were differences in plasma PAI-1 levels of the healthy subjects with an average of 12 ng/ml in study II compared to study I, in which none of the subjects had a plasma level above 10 ng/ml. Furthermore, there was a difference in age by 25 years between the lean subjects in the two studies and as described by Serrano et al, the contribution of plasma PAI-1 from adipose tissue may increase during aging [62]. Taken together, these differences indicate that there was a less advantageous metabolic phenotype of the lean control subjects in the second study, and this may explain the finding of some glycans on plasma PAI-1 from individuals in the latter study.

The source of plasma PAI-1 in T2D

Platelet hyper-reactivity plays a critical role in the pathogenesis of atherothrombosis in patients with obesity and T2D and these conditions are also associated with an increased MPV [192-195]. This would suggest that the platelet mRNA expression and the synthesis of PAI-1 could be altered in obesity and T2D. However, no significant difference in PAI-1 mRNA expressions in platelets between the groups was observed. Moreover, no difference was seen in platelet PAI-1 antigen between the groups, that is in contrast to previous results, in which a reduction in platelet PAI-1 was observed in T2D subjects [183]. Comparable PAI-1 mRNA expression levels and PAI-1 antigen levels in platelets between the groups may indicate an unaffected role of platelets or megakaryocytes for the plasma levels, thus indicating that a different source is contributing to the elevated levels in T2D and obesity. This is in agreement with the glycosylation results; the increase in plasma PAI-1 observed in obese subjects may be derived from adipose tissue. The significant difference in the ratio between plasma PAI-1 and serum PAI-1 between lean control subjects and T2D or obese subjects is also in line with this hypothesis.

Our group has shown that plasma PAI-1 levels are decreased in the immediate postoperative period after gastric bypass, prior to any weight-loss [132]. This may indicate that an additional mechanism, not directly related to the fat mass, regulates the PAI-1 levels. Bariatric surgery has been shown not only to be an effective treatment for obe-

sity, but is also associated with improved type 2 diabetes outcomes. This is observed before any significant weight loss has occurred and the main hypothesis is that this is related to an increased release of gut hormones and bile acids [196]. It may be speculated that hormonal factors such as insulin influence the decrease in plasma PAI-1. A strong correlation between PAI-1 and insulin has been reported [197].

Even though the similar platelet PAI-1 content in T2D, obese and lean subjects indicate an unaffected role of platelets, a higher synthesis rate and turn-over of PAI-1 in platelets from T2D compared to obese and lean control subjects could not completely be ruled out. Platelets in T2D could initially contain more PAI-1 mRNA transcripts that are translated into protein at an increased rate and the PAI-1 is directly released into the blood stream.

Several studies have previously shown that platelets from T2D exhibit an abnormal functional profile, although most of these studies revealed a high glycemic burden or significant diabetic complications among the subjects included [194, 198, 199]. Conversely, Schlomai et al have demonstrated normal platelet size and function in subjects with well-controlled T2D without prior ischemic events [200]. In addition, there is evidence that MPV values decrease in patients who achieve improved diabetic control [201]. Our results demonstrated no substantial difference in platelet count, size or aggregation ability between T2D subjects compared to obese and lean control subjects. This may be explained by the features of the T2D subjects in our cohort; they had no ischemic events or diabetic complications such as microvasculopathy, no multi-pharmacy and the duration of T2D was less than five years. In contrast to abovementioned studies, in our study population all medical treatment was withdrawn ten days prior to venous sampling, to assure that drug treatment was not influencing the platelets. For instance, oral hypoglycemic agents and statins are known to affect platelet function [202, 203].

From a pathophysiological perspective, this thesis presents three main findings:

1. Glycosylation patterns on PAI-1 indicate that platelets are the source of plasma PAI-1 in healthy subjects

2. Adipose tissue may be an additional source of plasma PAI-1 and there is an increased ratio of glycosylated PAI-1 in obesity

3. The unaffected pool of platelet PAI-1 in T2D indicates that the elevated plasma levels derive from another source

In conclusion, these studies have put focus on the origin of PAI-1 in plasma. In physiologically balanced conditions, platelets may be the major source of plasma PAI-1. However, the platelet contribution seems less important for the increased plasma levels and hence the risk of cardiovascular events in obesity and T2D, in which adipose tissue is a more likely source. The western lifestyle, mainly responsible for the features of the MetS, is causing a shift to a procoagulant and antifibrinolytic phenotype with an increased risk of CVD, in which a pathological increase in plasma PAI-1 from adipose tissue may be a key event.

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My clinical supervisor *Kerstin Holmberg*, and all wonderful colleagues at Närhälsan Herrestad Vårdcentral for supporting me in my work, encouraging me to keep going and showing vast interest in my research field. *Ann-Marie Svensson* for valuable administration skills. All former clinical colleagues, especially *Jenny Larsson-Fridén* and *Åke Johansson*, for your support.

To all of my wonderful friends, far and near, for making sure there is more to life than science. Especially thanks to *Sara* for being my dearest friend for as long as I can remember. Thank you for always being there. *Ingrid*, *Erik*, *Mimi*, and *Estelle* for good times. And to *Minna*, *Jenny* and *Karin* for listening to my research dilemmas (over a glass of wine).

To my *mother Inger* for support and giving me endless love. To my *father Björn* for support, being a role model and sharing my interest in medical research. My *brother Erik* and his family *Sofia*, *Ida* and *Elsa* for being who you are and being there. To *morfar Curt* for your great interest in understanding what my research is all about. *Mormor*, *farmor* and *farfar* for devotion. My family-in-law, *Susanne* and her family, *Monica* and *Kjell* for support and hours of baby-sitting. To *Elise* my "older-sister" and all my cousins, aunts, uncles and friends of the family.

To my wonderful children, I love you indefinitely. *Anton*, for showing me that love has nothing to do with ties of blood and I am so thankful that you, curious and thoughtful, stormed into my life. *Klara* for being my sunshine and reminding me about the most important things in life.

And to *Christian*, my husband, love of my life and best-friend. For your wise thoughts, support, and encouragement. I could not have done this without you. I will be your babe forever, even when we are old and grumpy!

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