Fibrinolytic factors in relation to anthropometry and incident type 2 diabetes.

Jenny Hernestål Boman
Ett svar på en fråga kan vara rätt eller fel, båda två eller ingetdera. De flesta svar är dock lite av varje.

Okänd

The answer to a question can be right or wrong, both or neither. However, most answers are a bit of each.

Unknown
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Abstract

Fibrinolytic imbalance is associated with cardiovascular disease and its risk factors. The longitudinal changes in the fibrinolytic factors tissue plasminogen activator (tPA), plasminogen activator inhibitor-1 (PAI-1) and tPA/PAI-1 complex have been inadequately studied in the general population and in relation to incident type 2 diabetes mellitus (T2DM). The measurements, questionnaires and blood samples prospectively collected in the World Health Organisation-project MONItoring trends and determinants in CArdiovascular disease (MONICA) and in the Västerbotten Intervention Programme (VIP) enable such studies. The samples have been stored since 1985, at the Northern Sweden Medical Research Biobank. However, it is unknown how these factors are affected by long-term storage.

The aims of this thesis were to evaluate the effects of long-term storage on fibrinolytic factors, and to determine how these factors are related to incident T2DM, how these factors change over time and how these factors are related to changes in anthropometric measurements.

Storage time was shown to have a negligible impact on plasma antigen levels of fibrinolytic factors. After adjustments for traditional diabetic and cardiovascular risk markers the fibrinolytic factors tPA, PAI-1 and tPA/PAI-1 complex were associated with incident T2DM. PAI-1 was associated with incident T2DM in subjects with normal fasting and 2-hour plasma glucose levels. In MONICA-Västerbotten, tPA, PAI-1 and tPA/PAI-1 complex increased over 9 years in both men and women. PAI-1 appears to interact in a complex manner with anthropometric, inflammatory, glycaemic and lipidemic measurements, but the pattern of components correlating with the changes in PAI-1 differed markedly between the sexes.

In conclusion, PAI-1 is a potential risk marker of incident T2DM. PAI-1 increased markedly over nine years, but the pathophysiological background to these findings needs to be further investigated, separately for each sex.
Sammanfattning på svenska

För vår kropp är blodcirkulationen nödvändig för att syre, näring och restprodukter ska kunna transporteras till och från cellerna. Hemostas är kroppens sätt att balansera den process som täpper till kärlskador, koagulationen, med den process som ser till att transport kan fortgå under reparationsskedet och att kärlen resas efter att skadan har läkt, fibrinolysen. Felaktigt inställd balans mellan koagulation och fibrinolys ökar risken för blödning eller blodproppsbildning. Förändrade nivåer av fibrinolysfaktorer är kopplat till ökad risk för hjärt- och kärlsjukdom. Två av huvudbeståndsdelarna i hemostasen är tPA (tissue plasminogen activator) och dess hämmare PAI-1 (plasminogen activator inhibitor-1).


Denna avhandling baseras på fyra delarbeten. I delarbete I visades att frysförvaringen i -80°C hade försumbar påverkan på nivåerna av fibrinolysfaktorer i blodplasma. Delarbete II påvisade en koppling mellan fibrinolysfaktorer och framtidiga insjuknande i typ 2 diabetes mellitus (incident T2DM). De som senare insjuknade hade högre nivåer av tPA, PAI-1 och tPA/PAI-1 komplex vid provtagningsställfallet än deras matchade kontroller. PAI-1 och tPA/PAI-1 komplex var även kopplat till incident T2DM hos personer med normalt blodsocker vid provtagningsställfallet före insjuknandet. Delarbete III visade att tPA, PAI-1, tPA/PAI-1 komplex, VWF och inflammationsfaktorn CRP ökade i den västerbottniska befolkningen mellan 1990 och 1999. Delarbete IV fokuserade på PAI-1-ökningen och att dess koppling till andra förändringar, bl.a. i antropometri, uppvisade olika mönster hos män respektive kvinnor.

Abbreviations

2hPG  2 hour capillary plasma glucose after OGTT
CI    confidence interval
CRP   C-reactive protein
CV    coefficient of variance
ELISA enzyme-linked immunosorbent assay
FDP   fibrin degradation products
FPG   fasting plasma glucose
HDL   high density lipoprotein
IFG   impaired fasting glucose
      (P-glucose 6.1-6.9 mmol/L)
IGT   impaired glucose tolerance
      (capillary P-glucose 8.9-12.1 mmol/L)
IQR   interquartile range
LDL   low density lipoprotein
MONICA MONItoring trends and determinants in CArdiovascular
disease (global WHO study)
OGTT  oral glucose tolerance test
OR    odds ratio
PAI-1 plasminogen activator inhibitor-1
P-glucose plasma glucose
SD    standard deviation
SERPIN SERine Protease Inhibitor family
T2DM  type 2 diabetes mellitus
tPA   tissue-type plasminogen activator
TGF-β transforming growth factor-β
TNF-α tumour necrosis factor-α
uPA   urokinase-type plasminogen activator
VIP   Västerbotten Intervention Programme
VWF   von Willebrand factor
WHO   World Health Organisation
Original papers

This thesis is based on four papers, which are referred to in the text by Roman numerals.


IV. Hernestål-Boman, J., Jansson, J-H., Eliasson, M., Nilsson, T.K., Johansson, L. Individual PAI-1 increase over nine years relate to anthropometric, glycaemic, inflammatory, and lipid markers, although differently in men and women. *In manuscript*

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Preface

I grew up in Sorsele, one of the highland municipalities of northern Sweden, during the 1980s. I remember as a child reflecting upon death as the result of one of four causes, in descending order: myocardial infarction, stroke, accident, or cancer. Indeed, a message of someone’s passing away was usually followed by one of my parents asking - Was it the heart? Too often, I noticed the quick inhale of the person responding (to produce the typical up- north sound of agreeing).

In the mid 1980s the situation was alarming, and the two northernmost counties of Sweden, Norrbotten and Västerbotten, joined the world-wide MONICA study at the same time as the Västerbotten Intervention Programme (VIP) began in the Västerbotten municipality of Norsjö (eventually covering all of Västerbotten, including my home town Sorsele).

The material collected, during my time as a child and an adolescent, has yielded a large number of results in many research areas connected to, or separate from, cardiovascular disease – including diabetes, haematology, clinical chemistry, cancer, virology, mental health and occupational therapy. New insights in therapy and prevention have led to better health. In northern Sweden fewer people die from cardiovascular disease these days, and those who fall ill experience less severe symptoms and better recovery.

The studies are still on-going, and the number of collected samples has reached a large quantity administered by the Northern Sweden Medical Research Biobank.

It was with great gratitude and quite a lot of pride that I began analysing the samples and data collected in these major studies, in order to produce this thesis on fibrinolytic factors.
Introduction

Circulation is fundamentally essential to the human body. In the circulatory system, nutrients, oxygen and degradation products are constantly transported to and from the periphery, to maintain the functions of all cells and organs. Circulation depends on the pressure and flow arising from the contraction and dilatation of the heart. If the vessel walls are damaged the vascular circuit could malfunction due to the leakage of blood and/or drop in blood pressure, which could cause cell death and increase the risk of organ failure. Rapid acting vascular repair is, therefore, of greatest importance to preserve body functions but, at the same time, cannot be left uncontrolled because this process might obstruct the system. Thus, a finely tuned balance has evolved to maintain haemostasis in the human body.

Fibrinolysis and haemostasis

The term heamostasis sums up the life-preserving balance of coagulation and fibrinolysis. Coagulation is a crucial process that hinders excessive bleeding from the vessel via intrinsic or extrinsic systems that react at the site of injury, going from the rolling of platelets to the formation of polymerized fibrin that stabilizes the clot. Fibrinolysis is the well-regulated proteolytic degradation of polymerized fibrin that prevents the clot from clogging the system as well as clears the path once the damage has healed. Hyperfibrinolysis can be caused by increased enzymatic activity and might manifest as excessive bleeding. Hypofibrinolysis, on the other hand, can be caused by decreased fibrinolytic activity and could result in thrombosis. The fibrinolytic factors studied in this thesis are tissue plasminogen activator (tPA), plasminogen activator inhibitor-1 (PAI-1) and tPA/PAI-complex, together with the von Willebrand factor (VWF) for primary haemostasis.

Tissue plasminogen activator, tPA

In human blood two physiological plasminogen activators have been found: tissue plasminogen activator (tPA) and urokinase plasminogen activator (uPA) [1]. The activation of tPA is primarily involved in the dissolution of fibrin in the circulation [2], whereas uPA binds to a specific uPA receptor (uPAR), which results in activation of cell-bound plasminogen to help control proteolysis during tissue remodeling and repair, macrophage function, tumour invasion, ovulation and embryo implantation [3].
Serine proteases act by cleaving proteins that have serine as the nucleophilic amino acid in their active site. tPA is a serine protease that plays a central role in the intravascular fibrinolysis by cleaving plasminogen at its Arg561-Val562 peptide bond, which transforms it into another serine protease called plasmin. Plasmin, in turn, cleaves fibrin clots to fibrin degradation products (FDP), as shown in figure 1. Both plasminogen and tPA bind to fibrin, which protects tPA from reactions of inhibitors. As fibrin is degraded new binding sites appear and the quantity of plasmin increases.

**Figure 1.** Schematic overview of the fibrinolytic system. Activating factors are shown with black arrows and the inhibiting factor is shown with a thick red arrow.

The tPA protein is encoded by the *PLAT* gene located on chromosome 8 [4]. The production and storage of tPA takes place within the endothelium [5, 6] (the monolayer of cells that constitute the inner lining of the blood vessels) possibly in the endothelial secretory granules, called the Weibel-Palade bodies[7], but this has been debated [8]. There is a basal release of tPA, but increased release occurs in response to stimuli, such as venous occlusion, physical exercise and stress [9], or in response to agonists, such as thrombin, histamine, fibrin, and epinephrine among others[10, 11].

The levels of active tPA are affected by endothelial secretion of tPA, the rate of hepatic clearance of tPA, and the inhibition of tPA. Active tPA has a half-life of about 3 minutes [12]. Approximately 15%-20% (less than 1 ng/mL) of the amount found in plasma is of the active form. The remaining amount is in complex with the main inhibitor PAI-1 or with other inhibitors (e.g. the C1 inhibitor of the complement system [13], which is known to inhibit a broad spectrum of proteases).
Previous findings have associated the increase in tPA antigen with increasing age [14-16]. Men were shown to have higher tPA antigen levels than women [15, 17, 18]. Aging appears to increase levels of tPA antigen in men, more so in sedentary men than in physically active men [19]. Increased levels of tPA antigen precede myocardial infarction [20, 21] and are associated with increased risk of cardiovascular death [22]. Directly after smoking tPA activity appears to increase, but a decreased activity was found in chronic smoking [23, 24]. Smoking has not been significantly associated with tPA levels in the MONICA study [25].

**Plasminogen activator inhibitor, PAI-1**

Inhibition of the fibrinolytic system occurs in different ways. At the level of plasminogen activation this is mainly done by PAI-1 or by thrombin activatable fibrinolysis inhibitor (TAFI). Inhibition can also occur at the level of plasmin where α2-antiplasmin hinders the action of plasmin breaking down fibrinogen.

PAI-1 was discovered in 1981 [26] and is a member of the SERine Protease Inhibitor family (SERPIN). It is the main inhibitor of both tPA and uPA, blocking fibrinolysis in the initial stages of clot formation. Another PAI, PAI-2, is secreted by the placenta and is only present in measurable amounts during pregnancy. PAI-1 is a single-chain glycoprotein, containing 379 amino acids with a molecular weight of 50 kDa [27]. There are several inactive forms in complex with, for example, tPA or uPA, and there is a latent form that can be re-activated [28, 29].

Platelets contain a large amount of PAI-1 [30, 31]. Up to 90% of PAI-1 in peripheral blood is contained in the α-granulae of platelets and released upon activation [32]. PAI-1 activity is mainly regulated by fibrin and thrombin. By binding to fibrin PAI-1 becomes fixed within the clot, which promotes the stability and extension of the clot. PAI-1 is also found in endothelial, spleen and hepatic cells [33], adipose tissue [34, 35], macrophages, neutrophil polymorphs, vascular smooth muscle cells and mesangial cells [36, 37]. PAI-1 is bound and stabilized by proteins in the plasma and extracellular matrix (i.e. vitronectin) [29]. Aside from being an inhibitor of fibrinolysis other functions of PAI-1 appear to involve tissue development [38], tumour invasion and metastasis [39]. Regulation is also performed by hormones [40, 41], cytokines, and growth factors (e.g. TNF-a, TGF-B and IL-1) [42].

PAI-1 is fast acting and has been reported to have a half-life of 6-9 minutes in rabbits [43] but binding to vitronectin, for example, increases the stability and lifetime of active PAI-1 some 30 minutes [44] to 1 hour [28]. Lowering the temperature and decreasing the pH also increases the stability [29].
There is a well-known 4G/5G polymorphism in the promoter region of the PAI-1 gene [45]. The importance of this polymorphism is controversial. Obese women with the 5G/5G genotype have been shown to have significantly lower levels of plasma PAI-1 than the 4G/4G group [46], but in healthy men no association between the 4G/5G promoter polymorphism and platelet PAI-1 mRNA or protein expression was found [47]. The morning increase in PAI-1 antigen levels is more pronounced among individuals who are homozygous for the 4G allele, whereas a 5G-homozygotic persons might be relatively protected from diurnal variation [48, 49].

PAI-1 levels have been shown to increase with age in women [16, 51] but not in men [19], although men seem to have overall higher PAI-1 antigen levels than women [17, 18]. Physical activity and weight loss have been shown to decrease the levels of PAI-1 [19, 52, 53], possibly via the decrease in adipose tissue. Macrophages within adipose tissue produce cytokines that have been shown to up-regulate the expression of PAI-1 [54, 55]. Alcohol consumption has been shown to increase the levels of PAI-1 activity, whereas the results for coffee consumption were divergent [56]. The role of smoking on PAI-1 increase is divergent and it is unclear how it affects PAI-1 in healthy individuals [56, 57]. Smoking has been shown to have an effect on fibrinolysis [57, 58], especially in disease [59], but no significant association was found in the MONICA study [25]. Obesity might increase plasma PAI-1 levels [60-62], and high levels of PAI-1 activity has been associated with many pathological conditions, including thrombosis, inflammation, and metabolic disorders [63].

**tPA/PAI-1 complex**

When the active forms of tPA and PAI-1 meet in the circulation rapid complex formation occurs and this creates the inactive tPA/PAI-1 complex. The tPA/PAI-1 complex has been much less studied than its two fibrinolytic components. Low levels of tPA/PAI-1 complex combined with low levels of tPA activity suggest decreased secretion of active tPA. High levels of tPA/PAI-1 complex and PAI-1 activity together with low levels of tPA activity suggest inhibition of active tPA by active PAI-1 [64]. Aging appears to increase the tPA/PAI-1 complex levels, but physical activity has been shown to attenuate this increase [19]. Increased levels of tPA/PAI-1 complex have been associated with future first-ever [21], as well as with recurrent, myocardial infarction [65] and with risk of stroke [66].
**Von Willebrand Factor, VWF**

VWF is a key component of primary haemostasis. VWF is produced in the endothelium, where it is stored and secreted from the Weibel-Palade bodies [67, 68], and in platelets [69]. The VWF monomer contains specific domains with binding domain specific to, for example, to factor VIII (VWF type D domain), platelet GPlb-receptor (A1 domain), and collagen (A3 domain). VWF acts as a carrier protein of FVIII and is found in complex with FVIII in circulation. It also contributes to the aggregation and rolling of platelets, as shown in **Figure 2.** Monomers are N-glycosylated, arranged into dimers and, finally, arranged into multimers by crosslinking cysteine residues via disulfide bonds. The functional multimers of VWF can consist of more than 80 subunits and are extremely large, more than 20,000 kDa [70].

**Figure 2.** VWF multimer action contributes to the aggregation of platelets.

Decreased levels of VWF are found in patients with von Willebrand Disease (VWD) and elevated levels are often the result of inflammatory reactions. Because VWF is synthesized in endothelial cells it is a good marker of endothelial damage, and increased levels have been associated with higher risk of e.g. myocardial infarction [66]. The inter- and intra-individual variations are very high, and levels of VWF increase with age [71, 72]. VWF appears to be unrelated to the metabolic syndrome [73]. Women without metabolic syndrome have been reported to have higher VWF levels than men [74]. VWF also carries ABO blood type antigens. Individuals with type O blood group have, on average, about a 30% lower VWF level than individuals with other blood types [75].
Avoiding elements of bias prior to the data analyses

Individual and intra-individual aspects

Fibrinolytic factors are proteins whose plasma concentrations are easily affected by sampling procedures, and blood sampling should be done in a consistent manner. One must decide whether donors are to be sitting or lying down, and whether the protein of interest is an acute phase reactant (with factors such as VWF and PAI-1). One must also consider diurnal variations (tPA and PAI-1), intra-individual variations (stress, physical activity), and what effects are to be expected from blood group (VWF), smoking (VWF, tPA, PAI-1 and CRP), and age (VWF) [76–79].

Ideally participants should be calm and relaxed when donating a sample, should have arrived 15 to 20 min before sampling, without having to hurry to the examination and should have fat-fasted since midnight. Usually sampling for haemostatic factors is recommended during the morning hours, (i.e. 7 a.m. to 10 a.m.) [76].

Acute phase reactants are very sensitive to stress, whether brought upon by inflammation, infection or surgery [76]. Inflammation and infection can be estimated by the analysis of C-reactive protein, CRP [80]. It is unclear how CRP relates to haemostatic factors such as PAI-1 and VWF [73, 74]. There are conflicting results for raised levels of VWF in relation to all patients with rheumatoid arthritis [81, 82], and only those with a future thromboembolic event [83].

Seasonal effects

Many of the haemostatic factors are affected by time and vary with diurnal (during 24 hours), circaseptan (during 7 days) and circannual (over the year) rhythms [84]. Previous research showed that tPA activity rose during the day [18] until 6 p.m., and then decreased until morning and that tPA antigen peaked at 9 a.m. and then dropped until midnight when it started to increase again [77]. Another study showed that tPA activity and tPA antigen levels continued to increase from 9 a.m. to 12 a.m. [85]. PAI-1 activity and PAI-1 antigen were shown to follow each other during the day and peaked around 3 a.m., dropped until 6 p.m., and then started increasing again [77], a pattern confirmed by others as well [18, 86].

A disrupted diurnal rhythm of plasma tPA and PAI-1 was shown in shift workers on weekly shift rotations. Acute and chronic diseases might affect the diurnal variation, the amplitude, or the timing of the fluctuation [77]. The variability in VWF over 24 hours has been shown to be 10% and the 24-
hour pattern observed in healthy individuals differs from that of individuals with health issues [87].

Fibrinolysis has also been shown to have circaseptan variation, with minimum activity on Mondays and maximum levels between Tuesdays and Thursdays [88].

Fibrinogen in plasma has a circannual variation, and in healthy older adults with hypertension, the levels were at its highest during the cold months [89-91]. Another study, disregarding levels of blood pressure levels and only including male participants, found that the highest levels of fibrinogen occurred during May and June [92]. Myocardial infarction is more common during the winter season, and significantly higher levels of body mass index (BMI), glucose, total cholesterol, low density lipoprotein (LDL), triglycerides, lipoprotein (a), fibrinogen and thrombocytes and significantly lower levels of high density lipoprotein (HDL) were shown to occur in winter compared to summer. Patients who had a myocardial infarction were also shown to have a yearly variation of tPA activity and antigen [93].

Vitamin D is significantly correlated to tPA [94] and PAI-1 antigen levels [95]. Vitamin D levels vary over the year, with the lowest levels during late winter due to less sun exposure [96]. Ingestion of vitamin C also varies over the year [91], and low levels of vitamin C and vitamin D might be associated with changes in levels of fibrinolytic factors.

**Pre-analytical considerations**

When sampling for analysis of haemostatic factors care must be taken in not to over- or underestimate the levels of these proteins.

High molecular weight proteins, such as VWF, can be sensitive to posture and the levels can vary greatly between samples taken when the person is sitting or in a lying position. VWF is also affected by temperature. After thawing at 4°C overnight, and pipetting from the bottom or the upper layer of the samples, the analysis results can differ by 50%-300%. After thorough mixing of the samples the difference between the bottom and upper layers decreased. It was concluded that VWF samples must be thawed in a 37°C waterbath and mixed properly to ensure accurate measurements [97].

Up to 90% of the plasma concentration of PAI-1 is released from platelets [30-32], so pre-analytical care must be taken to minimize platelet contamination when preparing samples for PAI-1 estimation. After centrifugation platelets are found in the middle layer of the test tube, between the red blood cells in the bottom layer, and the plasma in the upper
layer, and great care must be taken to avoid the middle layer when aliquoting plasma.

**Collecting samples in long-term studies**

Inclusion of subjects in public health studies can span over months, years or decades. During this time many changes can occur that affect the analytical results. Therefore, sampling instructions must be clear and consistent, or constantly re-evaluated, to maintain sample-taking procedures, fasting instructions, and the same test tubes should be used regardless of the changes that occur in general practice. The long-term span of these studies also creates two alternatives for the performance of laboratory analysis, a problem that was focused on in study I.

The samples collected can be analysed directly after sampling at different time points, which eliminates storage as a cause of variance. However, the reagent kits used for analysis are undergoing constant modifications for better precision (repeatability) and accuracy (closeness to the true value). Consequently, samples analysed at several time points might use different coating antibodies or conjugates, or suppliers might lower the concentrations of expensive key components to maintain profit margins. All this may lead to substantial variations in laboratory results and increase the risk of bias when data from different time points are compared.

Samples can be stored in freezers and later analysed on one occasion. The storage time will then differ between samples. The stability of frozen plasma samples stored over long periods of time is a sparsely studied research area. Fresh frozen plasma stored at -70°C for 7 to 59 months showed no evidence of sample degradation for tPA and PAI-1 antigen [98]. A number of other haemostatic factors have been reported to be minimally affected in fresh frozen plasma when stored at -30°C for 12 months [99], or stable for 18 months or more when kept at -74°C [100]. However, storage time for prothrombin, and activated partial thromboplastin time were strongly influenced by freezing and storage over 4 months [101].

Ideally the same staff should be employed throughout the entire study period. It is also preferable that laboratory work for each analysis is conducted by the same staff, who are unaware of the status of participants (blinded analysis). Laboratory work should be performed with reagent kits of the same batch number [79].
**Metabolic syndrome and type 2 diabetes**

Over the years it has been difficult to reach any consensus regarding the definitions of metabolic syndrome (MetSy), and there have been debates on whether or not the syndrome actually exists [102-104]. In this thesis, the WHO definition of MetSy is used, which defines MetSy as having an impaired glucose regulation identified as type 2 diabetes mellitus (T2DM), impaired fasting glucose (IFG), impaired glucose tolerance (IGT), or lowest quartile glucose uptake under hyperinsulinemic/hyperglycaemic conditions. At least two of the following are also present:

- Blood pressure $\geq 140/90$ mmHg, and/or taking anti-hypertensive medication
- Triglycerides $\geq 1.7$ mmol/L
- HDL-cholesterol level $\leq 0.9$ mmol/l in men, or $\leq 1.0$ mmol/l in women
- BMI $\geq 30$ (or waist-hip ratio $\geq 0.9$ in men, or $\geq 0.85$ in women)
- Urinary albumin $\geq 20$ ug/min (or albumin/creatinin ratio $\geq 30$ mg/g)

The characteristics have been chosen based on their association with the development of insulin resistance. They increase with age and are also associated with an increased risk of cardiovascular events. There are different versions of the levels of impaired glucose metabolism and diagnosis of T2DM as well. In paper II, III and IV the WHO definition from 1999 were used [105], shown in **table 1**.

**Table 1.** WHO definitions of impaired glucose regulation, measured by fasting- or 2-h plasma glucose levels.

<table>
<thead>
<tr>
<th></th>
<th>Venous samples</th>
<th>Capillary samples</th>
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</thead>
<tbody>
<tr>
<td><strong>Diabetes</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fasting plasma glucose</td>
<td>$\geq 7.0$ mmol/L</td>
<td>$\geq 7.0$ mmol/L</td>
</tr>
<tr>
<td>2–h plasma glucose</td>
<td>$\geq 11.1$ mmol/L</td>
<td>$\geq 12.1$ mmol/L</td>
</tr>
<tr>
<td><strong>Impaired Glucose tolerance (IGT)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fasting plasma glucose</td>
<td>$&lt;7.0$ mmol/L</td>
<td>$&lt;7.0$ mmol/L</td>
</tr>
<tr>
<td>2–h plasma glucose</td>
<td>7.8 to 11.1 mmol/L</td>
<td>8.9 to 12.1 mmol/L</td>
</tr>
<tr>
<td><strong>Impaired Fasting Glucose (IFG)</strong></td>
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<tr>
<td>Fasting plasma glucose</td>
<td>6.1 to 6.9 mmol/L</td>
<td>6.1 to 6.9 mmol/L</td>
</tr>
<tr>
<td>2–h plasma glucose</td>
<td>$&lt;7.8$ mmol/L</td>
<td>$&lt;8.9$ mmol/L</td>
</tr>
</tbody>
</table>
**Risk factors for MetSy and T2DM**

“The common soil” theory states that the risk factors connected with development of cardiovascular disease and T2DM are of the same origin, and that cardiovascular disease and T2DM have an effect on each other [106]. Performing oral glucose tolerance tests (OGTT) on patients suffering from an acute myocardial infarction showed that approximately 40% had previously unknown IGT or T2DM [107, 108], and a majority of T2DM patients die as a result of a cardiovascular event [109]. Smoking [110, 111], low physical activity [112], obesity [113], insulin resistance and hyperglycaemia [114] have been independently associated with incident T2DM. Insulin resistance, in turn, is associated with central obesity, elevated triglyceride levels, low high-density lipoprotein levels, hyperglycaemia and hypertension [106, 115].

**Fibrinolytic factors in MetSy and T2DM**

Diabetic patients have activated coagulation and hypofibrinolysis induced by both chronic and acute hyperglycaemia [116, 117]. Hyperglycaemia has been shown to stimulate coagulation [118] in healthy humans, and high glucose levels have been shown to increase the production of tPA antigen from human mesangial cells [36]. Hyperinsulinaemia has been associated with impaired fibrinolysis [118, 119]. PAI-1 antigen levels were shown to decrease with treatment of metformin in T2DM subjects [120, 121], but the effect of insulin treatment remains unclear.

PAI-1 has been shown to be predictive of T2DM, but the predictive ability disappeared after adjustment for markers of the MetSy [122, 123], suggesting that high plasma PAI-1 levels are associated with factors of the MetSy, especially obesity [124] and are reduced with weight loss [52, 53]. Obesity, in turn, is associated with chronic inflammation [125]. Macrophages present in the adipose tissue produce cytokines, such as transforming growth factor β (TGF-β) and tumour necrosis factor α (TNF-α), which have been shown to up-regulate the expression of PAI-1 [54, 55].

On the other hand, PAI-1 inhibition in mice has the potential to reduce obesity and improve insulin sensitivity [126-128]. In humans PAI-1 has been suggested to influence the accumulation of visceral fat [129], for example, by influencing adipocyte differentiation, by indirect effects on insulin signaling, and/or by regulating the recruitment of inflammatory cells within adipose tissue [130].
Landmark studies of fibrinolytic factors in relation to MetSy and T2DM.

There is a large quantity of studies of fibrinolytic factors. A review of the studies most relevant to the thesis follows.

In the Northern Sweden MONICA study, a previous prospective cohort study showed that high tPA antigen was predictive of future T2DM, independent from the MetSy. Impaired fibrinolysis was also seen in individuals with normal glucose tolerance, which later developed T2DM. Incident cases had lower baseline tPA activity and higher PAI-1 activity and tPA antigen than referents. The risk of diabetes increased linearly across quartiles of PAI-activity (p = 0.007) and tPA antigen (p < 0.001) and decreased across quartiles of tPA activity (p = 0.026). The highest quartile of tPA antigen showed an association with incident T2DM (OR: 6.5, 95% CI: 1.3-33, p = 0.024) after adjustment for diastolic blood pressure, waist circumference, insulin, triglycerides, fasting and post-prandial glucose levels[131]. Another MONICA sub study found that insulin resistance, measured indirectly from the levels of fasting plasma glucose (FPG) and fasting insulin, was closely associated with low tPA and PAI-1 activity. Serum triglyceride levels were shown to interact with insulin resistance to predict the fibrinolytic activity. Subjects in the upper tertile of insulin resistance had a PAI-1 activity that was three times higher in men and twice as high in women, than that of the lower third. High levels of insulin and triglycerides together, compared to low levels, were associated with a fivefold difference in PAI-1 activity in men and a threefold difference in women. Activity levels of tPA showed an inverse correlation to both insulin resistance and triglyceride levels [132].

A study of 1,047 non-diabetic subjects who participated in the American Insulin Resistance Atherosclerosis Study (IRAS) investigated incident T2DM within a five-year period. After adjustments for age, sex, ethnicity, clinical center, smoking, BMI, insulin sensitivity, physical activity, and family history of diabetes, PAI-1 was related to incident T2DM (OR for 1 SD increase: 1.61, 95% CI: 1.20-2.16) [133]. High baseline PAI-1 levels and the progression of PAI-1 levels over time were associated with incident T2DM. PAI-1 levels continued to increase with the rising glucose levels and the development of diabetes. Baseline and follow-up levels of PAI-1, adjusted for demography and smoking status, were higher in those who developed T2DM, than those who did not. After adjustments for demography and smoking status, the change in PAI-1 was related to incident T2DM (OR for 1 SD increase: 1.75, 95% CI: 1.37-2.22) after adjusting for baseline PAI-1 levels. After further adjustments for insulin sensitivity or waist circumference, the PAI-1 change remained significantly associated with incident T2DM [134].

In the Framingham Offspring Study, 282 participants without MetSy developed MetSy within 3 years. After adjustment for clinical risk factors,
PAI-1 remained associated with incident MetS. Evaluating longitudinal change in MetS components showed that PAI-1 was significantly associated with changes in fasting glucose, systolic blood pressure, and triglycerides (all $p<0.05$) [135].

Platelet PAI-1 release has been studied in 27 T2DM patients and 16 healthy controls. Platelet aggregation (the decrease in platelet count in whole-blood during shaking at 180 rpm at 37$^\circ$C) and plasma PAI-1 antigen levels were measured in parallel at times from 0 to 180 min of shaking. The increase in plasma PAI-1 antigen levels at baseline was higher for the group with T2DM compared to their matched controls. After 180 min of shaking there was no difference in platelet aggregation, but PAI-1 antigen increase was higher in the T2DM group. Platelets of T2DM patients released significantly more PAI-1 than platelets of healthy participants at the same level of platelet aggregation. PAI-1 levels did not correlate to HbA1c levels [136].

A randomized controlled trial on subjects with IGT and obesity (n=186), studied the effects of lifestyle intervention on cardiovascular risk factors, especially fibrinolysis. Ninety-three participants were randomized to a low-fat, high-fiber diet and regular physical exercise, which resulted in a mean PAI-1 activity decrease of 31% (-10.1 IU/mL). This was significantly more than the decrease in the usual care group (12%; -3.0 IU/mL). The corresponding reductions in tPA antigen were 14% (-1.65 µg/L) and 6% (-0.69 µg/L). The mean weight decline after one year was 5.4 kg, compared to 0.5 kg in the usual care group. This study showed that an intense lifestyle intervention had beneficial effect on fibrinolysis for at least one year [137].

Juhan-Vague and colleagues have performed several studies on PAI-1 and metabolic dysregulation. In one study three groups of patients presenting insulin resistance (14 obese subjects, 6 patients with Cushing disease and 7 with acromegaly) were compared to controls regarding levels of PAI-1 activity and antigen. Fasting insulin levels were elevated in the insulin resistant groups compared to controls. PAI-1 activity and PAI 1 antigen levels were elevated in the obese group only (34.3 ± 13.0 IU/mL for PAI 1 activity) and not in the others: 10.2 ± 10.0 IU/mL, and 7.0 ± 4.6 IU/mL for hypercortisolic and acromegalic patients respectively, and 9.7 ± 5.4 IU/mL in normal controls. The mechanism of insulin resistance was different in the three studied conditions, suggesting that PAI-1 levels might be more directly associated with insulin resistance than via an increase caused by hyperinsulinaemia [138]. In another study elevated PAI-1 levels were associated with insulin resistance, together with the increase in adipose tissue and inflammatory response, which suggested that proinflammatory cytokines might regulate PAI-1 expression in the insulin resistance syndrome [139]. PAI-1 was also suggested to be involved in adipose tissue development e.g. by influencing adipocyte differentiation, by indirect effects on insulin
signaling, and/or by affecting the recruitment of inflammatory cells within adipose tissue [130].

In addition to the previous findings, more investigations on the impact of fibrinolytic changes over time, especially PAI-1, and the development of T2DM are required.
Aims

The aims of this thesis were:

- To determine whether the analytical results of tPA, PAI-1 and tPA/PAI-1 complex are affected by long-term storage and/or reagent kit modifications (Paper I).

- To investigate whether levels of tPA, PAI-1, tPA/PAI-1 complex and VWF are associated with incident T2DM (Paper II).

- To describe the intra-individual changes in tPA, PAI-1, tPA/PAI-1 complex, VWF and CRP over nine years (Paper III).

- To investigate the relationship between intra-individual changes in PAI-1 antigen and changes in anthropometric measurements, glycaemic, blood pressure, lipid and inflammatory markers, separately for men and women, over nine years (Paper IV).
Materials and methods

The two northernmost counties in Sweden are Västerbotten and Norrbotten inhabited by more than 500,000 individuals. The counties cover more than a quarter of Sweden’s total area. Västerbotten has 15 municipalities, but most people live in Skellefteå and Umeå, which are located on the coast of the Gulf of Botnia [140]. Out of Norrbotten’s 14 municipalities the largest population is found in Luleå, also located on the coast [141].

Populations

VIP

The Västerbotten Intervention Programme (VIP) is an initiative of the Västerbottens County Council. It started in 1985 in the municipality of Norsjö as an attempt to decrease the high morbidity and mortality due to cardiovascular disease, which was the highest in Sweden. The project soon escalated to include all the Västerbotten County primary care centres. Inhabitants in Västerbotten were invited to participate in a health survey and individual health counselling at their local primary-care centre when they were 30 (this age group was omitted after 1995), 40, 50, or 60 years of age. At the health examination, data about age, sex, and cardiovascular risk factors were collected. Participants were also asked to donate a blood sample for research, which was stored at the Northern Sweden Medical Research Biobank. The participation rate was high; the total study population from 1989 to 2000 represented 52% of the target population [142]. The vast majority of the participants were Caucasian.

In study II we conducted a nested case-referent (case-control) study within a selection of the VIP called TRIM (Tidiga RIsk Markörer för typ 2 diabetes; Swedish for ”Early riskmarkers in type 2 diabetes”). VIP-TRIM samples were selected from the 28,736 VIP participants, from 1986 to 2000, in Umeå and surrounding municipalities, who did not have diabetes type 1 or 2 at the health examination.
MONICA

The WHO project Monitoring of Trends and Determinants in Cardiovascular Disease (MONICA) is a multinational project that has been monitoring cardiovascular risk factors and endpoints in different countries and continents since 1982. In 1986 Norrbotten and Västerbotten, joined the study. Population surveys of independent, random samples of men and women stratified into 10-year age groups (25-34, 35-44, 45-54, and 55-64 years) were performed in 1986, 1990, 1994, 1999, 2004 and 2009. For consistency, surveys always took place from January to April. 2,000 randomised participants were invited by letter, which also included questionnaires, to an examination performed by a mobile team of nurses and laboratory staff. 65% were randomised to perform an oral glucose tolerance test (OGTT) and triglyceride measurement, which requires fasting overnight. In 1999 there was a re-survey of all the previous survey populations[143]. Two attempts were made to get invited subjects to participate. If the subject did not come to the survey appointment but was able to reach by phone, a new time for the examination was scheduled. If the person did not come, and was not reached by phone, a new invitation letter was sent [143].

The northern Sweden MONICA population from 1990 consisted of 1,583 participants, out of the 2,000 invited (see Figure 3). Of these participants, 1,558 had complete data sets on fibrinolytic measurements and were re-invited in 1999 and 1,148 chose to participate. When planning for the study on changes over nine years the samples from all 566 participants from Västerbotten were selected, but due to cost-restraints only 262 individuals from Norrbotten were selected. This selection was based on changes in measurements and answers in questionnaires obtained at the examination, e.g. weight gain or changed level of physical activity. In this final group of 828 analysed samples, each individual sample from the 1990 survey had been analysed up to three times (in 1991, 2001 and/or 2005), which was the basis for study I.

Due to the diurnal variation of fibrinolytic variables papers III and IV in this thesis are based on the data collected during the morning hours (n=309) from the 566 individual from Västerbotten invited to participate in the 1990 survey, and the same individuals re-surveyed in 1999.
**Figure 3:** Schematic of the WHO-MONICA participants in Västerbotten and Norrbotten that were invited in 1990 and re-invited in 1999.

1. **2,000 individuals are invited, MONICA 1990**
   (250 men and women respectively from 4 age categories)
   Cardiovascular risk data and blood samples collected.

   - 1,583 participates
   - 1,558 complete data sets
     **Analysed 1990/1991**
     - 25 missing data

2. **1,558 individuals are re-invited, MONICA 1999**
   Cardiovascular risk data and blood samples collected.

   - 1,148 participates
   - 566 individuals from Västerbotten
   - 262 selected individuals from Norrbotten. Selection based on changes from 1990 to 1999.
   - 320 individuals from Norrbotten were not analysed

   **In total 828 individuals with data sets and plasma samples from 1990 and 1999.**
   **Analysed 2004/2005.**

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**The Northern Sweden Medical Research Bank**

A biobank contains collections of human biological specimens and related data and measurements, such as medical history, lifestyle information acquired from questionnaires, and anthropometric measurements. The Swedish Biobanks in Medical Care Act (SFS 2002:297)
says that human biological specimens that can be identified and have been
collected and/or stored within healthcare can be used for research and
clinical trials if the patients or donors have given consent for this. The
collection and use of these samples require approval by a Swedish Ethical
Board for each specific project or clinical trial. Since 1985 approximately
109,650 individuals have participated in studies and donated blood samples
that are stored at the Northern Sweden Medical Research Biobank.

**Ethical considerations**

The Declaration of Helsinki [144] states that in the search for new knowledge
the research subjects’ rights and interests must be respected, and their life,
health, dignity, integrity, right to self-determination, privacy, and
confidentiality of personal information must be protected. In reality this
means that the researcher must investigate possible risks when planning for
a study, minimize the risks found, and plan a study with methods that are
relevant to the aims of that study. To ensure that these ethical guidelines are
followed researchers apply for approval of the study at a Research Ethics
Committee. An informed consent, which emphasizes the self-determination
of the research subject, is important.

All study protocols in this thesis were approved by the Research Ethics
Committee of Umeå University: §380/97, dnr 97-331; §338/00, dnr 00-264;
§462/03, dnr 03-418 (papers I, III and IV) and §434/99, dnr 99-369 (paper
II). Data handling procedures were approved by the National Computer Data
Inspection Board.

At the time of the health examination participants in VIP and MONICA were
informed and gave written consent to future use of collected data and
donated blood samples for research purpose. Laboratory analysis was
performed on samples that were blinded for participant’s identity and case
or reference status (paper II). Participants’ identities were coded and the
code was securely kept at the MONICA administration office.

In the case of previously undetected pathological conditions, such as
hypertension or hyperglycaemia, the participants were referred to a
physician for medical advice and treatment.

In paper II the medical records of VIP participants were investigated to find
patients with T2DM in 2001, who had not been diagnosed with T2DM at the
time of their health examination. This violation of personal integrity was
justified by the possibility of finding novel early risk markers for the
development of T2DM, which could help individuals at risk to prevent or delay the development of T2DM in the future. In addition the personal identification number was removed. Data and results were analysed and presented at the group level.

Collection of data

Measurements

Anthropometry

Weight was measured in light indoor clothing without shoes and rounded to the nearest 0.2 kg. Height was measured without shoes and rounded to the nearest 0.5 cm. BMI was calculated as measured weight in kilograms divided by the square of the height in metres (kg/m^2). Waist circumference was measured with a measuring tape just above the umbilicus at the end of a normal expiration and hip circumference was measured at the broadest part of the hips. Waist and hip measurements were rounded to the nearest 0.5 centimetres.

Smoking

In study II smoking was categorized into three groups (non, ex, or daily smoking). Those who reported that they smoked irregularly were categorized as ex-smokers. In papers III and IV smoking was categorized into two groups (daily smokers or non-daily smokers). Those who reported that they smoked irregularly were categorized as non-daily smokers.

Physical activity

In paper II (VIP participants) physical activity was categorized into three levels based on the questionnaire with self-reported leisure time exercise and commuting habits during winter. The physical activity during the rest of the year was presumed to be at least as high as during winter. The groups were (1) sedentary, which included those who never exercise, those who cycle and/or walk during their leisure time less than 2-3 times per month, and those who take a bus or car to work, or cycle and/or walk to work less than 2 km each way; (2) moderate, which included those who do exercise now and then but not regularly or at most once a week, those who cycle and/or walk during their leisure time at least 2-3 times per week, and those who cycle and/or walk to work 2-5 km each way; and (3) active, which included moderately active people who exercise at least 2-3 times/week, or cycle or walk to work more than 5 km each way.
In papers III and IV (MONICA participants) physical activity was categorized into two levels based on the questionnaire with self-reported leisure time exercise. Leisure time physical activity was categorized as low (hardly any physical activity at all or mostly sitting down) or high (physically active 1-2 hours per week or more).

**Blood pressure**

In paper II blood pressure was measured with a mercury sphygmomanometer with subjects in a supine position after a 5-min rest. Hypertension was defined as systolic blood pressure of 140 mmHg or more and/or diastolic blood pressure of 90 mmHg or more and/or reported use of antihypertensive medication during the period of 14 days prior to the health examination. In papers III and IV blood pressure was measured with a mercury sphygmomanometer with the participant in a sitting position after a 15-min rest.

**Family history of T2DM**

Participants in paper II were considered to have a family history of T2DM if they reported to have a parent or sibling with T2DM.

**Blood sampling**

All participants who donated blood samples during the morning hours were instructed to fast from midnight until the time of blood sampling. Blood samples were drawn with no or a minimum of stasis in sitting position.

In study II venous blood samples were drawn with a minimum of stasis into evacuated glass tubes containing 1/100 volume of 0.5% EDTA. The sample tube was centrifuged at 1500 x g. The plasma was immediately frozen at -20 °C and stored at -80 °C in the Northern Sweden Medical Research Biobank until analysis.

In papers I, III and IV venous blood samples were drawn into 5 ml vacuum tubes containing 0.5 mL of 0.45 mol/L citrate buffer pH 4.3 (Stabilyte, Biopool, Sweden), which is considered most suitable for fibrinolytic assessment \[51, 145\]. After blood was drawn the sample tube was centrifuged at 2000 x g. The plasma was aliquoted and frozen in liquid nitrogen in 1990 and at -20°C in 1999 and, finally, stored at -80°C at the Northern Sweden Medical Research Bank until analysis. Thus, there was a change between the 1990 and 1999 sampling procedure for the fibrinolytic samples, where Stabilyte plasma samples were being snap-frozen in liquid nitrogen during 1990 and at were frozen at -20°C in 1999. The evaluation of possible bias due to this is presented in the additional results of paper III.
**Glycaemia**

OGTTs were performed with a 75g glucose load according to WHO standards. In paper II glucose concentrations were measured in venous plasma in a fasting state (FPG) and in capillary plasma 2 hours (2-hcPG) after glucose intake on a Reflotron bench top analyser (Boeringer Mannheim GmbH, Mannheim, Germany). In papers III and IV FPG and 2-hcPG were measured with the HemoCue B-glucose analyser (HemoCue AB, Ängelholm, Sweden).

**Lipid markers**

In paper II the analysis of serum lipids was performed via routine methods at the Department of Clinical Chemistry at Umeå University Hospital. In papers III and IV total cholesterol and triglyceride level were measured on a multianalyser (Vitros 950 IRC, Johnson and Johnson, Rochester, NY, USA).

**Laboratory analysis of frozen samples**

**The ELISA-technique**

Enzyme-linked immunosorbert assays (ELISAs) combine the specificity of antibodies with the sensitivity of simple enzyme assays, by using antibodies or antigens bound to an easily measured enzyme. The ELISA can test for antigens that are recognized by an antibody or it can test for antibodies that recognize an antigen. ELISA is a procedure of several steps: (1) coat the microtiter plate wells with antigen or antibody; (2) block all unbound sites to prevent false positive results; (3) add sample to the wells; (4) add antigen or antibody conjugated to an enzyme; (5) perform the reaction of a substrate with the enzyme to produce a coloured product, thus indicating a positive reaction; and (6) stop the reaction by degradation of reactivity by addition of an acid such as H$_2$SO$_4$.

**Fibrinolytic and inflammatory analysis**

The commercial kits used to produce the results in this thesis include tPA antigen, which measures all forms of tPA, and tPA activity which react to a binding site of the free, active form of tPA. PAI-1 commonly occurs in its active form, as in the PAI-1 activity reagent kit, and a minor portion in the tPA/PAI-1 complex, which can be measured together with the active form, as in the PAI-1 antigen reagent kit (see Figure 4). The tPA/PAI-1 complex is one of the specific forms of inactivated tPA in complex with PAI-1.
Consequently, the complex level can never be higher than the antigen form of either tPA or PAI-1 antigen.

**Figure 4.** Illustration of the different assessment methods for tPA, PAI-1 and tPA/PAI-1 complex plasma levels, inspired by Nilsson et al. 1991 [56].

Laboratory analysis for papers I, II, III and IV were performed in 2005. All samples were thawed in a 37°C water bath and analysed directly afterwards. In study II samples from cases and their matched referents were analysed together in random order. Measurements were made by laboratory staff unaware of each subject’s disease status. Reagent kits with identical batch numbers, purchased from Biopool (Umeå, Sweden), were used for tPA (Chromolize tPA activity®, Imulyse tPA antigen®), PAI-1 (TintElize PAI-1 antigen®) and tPA/PAI-1 complex (TintElize tPA/PAI-1 ®). VWF levels were analysed with reagents purchased from DAKO (Copenhagen, Denmark).

**Statistical methods**

Data are reported as proportions, as means with standard deviation (SD) or 95% confidence interval (CI), or as medians with interquartile range (IQR). Significance testing was carried out using the independent sample’s t-test for normally distributed continuous variables. The Mann-Whitney test was used for continuous variables that were not normally distributed and chi-squared tests were used for categorical variables. The McNemar test, a chi-square alternative used for individuals participating twice in the same study, was also used. In papers III and IV individual changes between 1999 and 1990
were calculated as the value at 1999 minus the value at 1990 (Δ\text{1999-1990}). Changes over time (Δ\text{1999-1990}) were tested with p for trends across age groups and were calculated using analysis of variance (ANOVA). Significance testing was carried out using the Wilcoxon matched-pair signed-rank test for comparison of continuous variables.

Natural logarithm transformation was used for non-normally distributed variables in order to perform regression analysis. In paper II conditional logistic regression was used to calculate age- and sex-matched odds ratios (ORs) comparing the risk of incident T2DM in cases and referents. ORs with 95% CIs were calculated per increment of 1 SD for continuous variables. Backwards stepwise elimination was also used with variables with p<0.1 remaining in the final model. ORs with 95% CIs were calculated per increment of 1 SD for continuous variables.

Regression plots that compare method A values against method B values, as well as Bland-Altman plots, which compare increasing mean values of either method, were used for comparison of two or more analytical methods [146, 147].

Measurements of additive biological interaction were performed using the common assumption that ORs can be used instead of relative risks (RR). Rothmans relative excess risk (RERI) and attributable proportion (AP) due to biological interaction, as well as the synergy index (S), were calculated as described by Andersson et al [148]:

\[ \text{RERI} = \text{RR}_{\text{high BMI, high fibrinolytic factor}} - \text{RR}_{\text{low BMI, high fibrinolytic factor}} + 1 \]

\[ \text{AP} = \text{RERI} / \text{RR}_{\text{high BMI, high haemostatic factor}} \]

\[ S = \frac{\text{RR}_{\text{high BMI, high haemostatic factor}} - 1}{((\text{RR}_{\text{low BMI, high haemostatic factor}} - 1) + \text{RR}_{\text{high BMI, low haemostatic factor}} - 1))} \]

There is no interaction if RERI = 0, AP = 0, and S = 1. The OR was then calculated per increment of 1 SD for continuous variables.

The variability of laboratory ELISAs was estimated by the coefficient of variance (CV %), which is calculated as the SD divided by the mean. The advantage of the CV is that the SDs of the assays increase or decrease in proportion to the increase or decrease of the mean. Division by the mean removes the mean as a variability factor. Thus, the CV is a standardization of the SD that allows comparison of variability estimates regardless of the
controls concentrations. CVs for the laboratory measurements used in this thesis are found in table 3.

The Statistical Package for Social Science (SPSS®, a.k.a. Predictive Analytics Software, PASW®) versions 15.0-22.0 were used for statistical analysis. A p-value <0.05 (two-sided) was considered statistically significant. The linear regression in study I was calculated with 95% confidence intervals of slopes, intercepts and squared correlations coefficients using Method Validator version 1.19. Regression plots and Bland-Altman plots in paper I were constructed using the Analyse It® tool for Microsoft Office Excel, version 1.72. This thesis was produced using Microsoft Office Word 2003.
Paper I

To study the long term storage effect samples from 1,598 individuals stored at the Medical Research Biobank for 5-20 years, were used. The samples were collected between 1985 and 2000 and analysed in 2005, as shown in figure 5. Antigen levels of tPA, PAI-1 and tPA/PAI-1 complex were natural log transformed to achieve normal distribution. Univariate linear regression was used to examine the relationships between each fibrinolytic variable and storage time. The $R^2$ value estimates the proportion of variation in a fibrinolytic variable that is explained by storage time. A $p$-value <0.05 (two-sided) was considered statistically significant.

Figure 5. Number of samples collected per year in the long-term storage study.

To study the effect of reagent kit modifications over time 1,558 samples, collected from individuals in the MONICA survey 1990, were used. Activity and antigen levels of tPA and PAI-1, as well as tPA/PAI-1 complex were analysed in total or in part on three separate occasions (1990, 2001 and 2005) using different analysis kits, or kits with different batch numbers, see Figure 6 and Table 2 for details.
Figure 6. Plasma samples from MONICA taken in 1990 were analysed on three different occasions. The reagent kit used for analysis is indicated within parenthesis.

MONICA survey 1990

2000 individuals from Västerbotten and Norrbotten were invited. Cardiovascular risk data and blood samples were collected.

| Analyzed in 1990; 1558 individuals | tPA activity (Spectrolyse) |
| Re-analyzed in 2001; 78 individuals | PAI-1 activity (Spectrolyse) |
|                                      | tPA antigen (TintElize ver 1) |

| Re-analyzed in 2005; 828 individuals | tPA activity (Chromolyse) |
|                                      | PAI-1 activity (Chromolyse) |
|                                      | tPA antigen (Imulyse or TintElize ver 2) |
|                                      | PAI-1 antigen (Imulyse) |
|                                      | tPA-PAI-1 complex (TintElize) |

Table 2. Number of comparable samples for each fibrinolytic analysis.

<table>
<thead>
<tr>
<th></th>
<th>Year of analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>tPA activity</td>
<td>46</td>
</tr>
<tr>
<td>tPA antigen</td>
<td>43</td>
</tr>
<tr>
<td>PAI-1 activity</td>
<td>n.a.</td>
</tr>
<tr>
<td>PAI-1 antigen</td>
<td>n.a.</td>
</tr>
<tr>
<td>tPA/PAI-1 complex</td>
<td>n.a.</td>
</tr>
</tbody>
</table>

Abbreviations: tPA: tissue plasminogen activator; PAI-1: plasmingen activator inhibitor-1; n.a; not available.

The kits used and their modifications over time are described in paper I.
Paper II

In paper II we conducted a nested case-referent (case-control) study within a selection of the VIP called TRIM (Tidiga RIskMarkörer för typ 2 diabetes; Awedish for "Early riskmarkers in type 2 diabetes"). In the TRIM study, VIP participants from Umeå and the surrounding municipalities, who developed T2DM from 1989 to 2000 according to patient records, were included if they had been screened prior to diagnosis (n=237), as shown in Figure 7. T2DM was diagnosed according to 1999 WHO criteria [124, 149]. Two referents with stored plasma samples, but without T2DM according to the registry, were randomly selected for each case and were matched for sex, age, and year of health examination (n=474). Cases were excluded if the subjects had chosen not to donate blood (n=34), if the samples were prioritized to other studies (n=25), if data were missing (n=20), or if there were no matching referents (n=1). Referents were excluded due to case exclusion or because blood samples were not available. In total, 157 cases with diagnosed T2DM during follow-up and available baseline blood samples were included in this study, along with 277 referents.

Figure 7. Schematic of study population for paper II.
Cases were stratified into two groups: cases with normal glucose levels (FPG <6.1 mmol/L and 2-hcPG <8.9 mmol/L at baseline) and their matched referents, and cases with elevated glucose levels (FPG 6.1-6.9 mmol/L and/or 2-hcPG 8.9-12.1 mmol/L at baseline) and their matched referents. Multivariate conditional logistic regression was performed for each fibrinolytic factor, BMI, family history of T2DM, physical activity, smoking, systolic blood pressure and total cholesterol.

Logistic regression was used to calculate age- and sex-matched ORs. Natural logarithm transformation was used for tPA, PAI-1, tPA/PAI-1 and VWF measurements. Adjustment was performed for each haemostatic risk factor and potential confounders in three models: (1) T2DM risk factors: BMI, smoking (non/ex/daily smoking), family history of T2DM and physical activity (sedentary/moderate/active); (2) cardiovascular risk factors: BMI, smoking (non/ex/daily smoking), family history of T2DM, physical activity (sedentary/moderate/active), CRP, systolic blood pressure and triglycerides; and (3) model 2 plus FPG and 2hPG levels.

**Additional sub-group analysis (paper II)**

Considering the associations of BMI with incident T2DM [150] and PAI-1 with BMI (adipose tissue) [55, 130] we investigated whether there was any interaction between PAI-1 antigen and BMI in paper II.

The primary aim in this additional statistical analysis was to investigate the interaction between obesity and elevated levels of tPA, PAI-1 and tPA/PAI-1 complex respectively, in relation to incident T2DM.

Biological interaction between BMI and fibrinolytic factors was tested. Participants were stratified into two groups by BMI level, (above or below 27) to yield approximately equal sized groups, and two groups above or below median values for tPA, PAI-1, tPA/PAI-1 and VWF. Cases with normal glucose levels (FPG <6.1 mmol/L and 2hPG <8.9 mmol/L at baseline) at the time of their health examination were chosen for multivariate logistic regression analysis. Multivariate analysis included two models: (1) each fibrinolytic factor, BMI and smoking, and (2) each fibrinolytic factor, smoking, systolic blood pressure and total cholesterol.
Papers III and IV

In Västerbotten 1,000 randomly selected persons stratified into sex and age groups (25-34, 35-44, 45-54 and 55-64 years) were invited to participate in the MONICA survey 1990 and 780 individuals participated. All of these participants were re-invited in 1999 and 566 individuals were re-examined. Because haemostatic variables are known to have diurnal variation [77], only those who participated during the morning hours (7:00 to 11:30 a.m.), at both surveys were included. Blood samples were available for 309 participants, shown in figure 8.

Figure 8. Schematic overview for selection of participants in papers III and IV.

MONICA survey 1990
1000 men and women were invited.
780 participated and were re-invited
1999.

Re-examination 1999
566 participated.

309 suitable samples were donated between 7.00-11.30 in both 1990 and 1999.

241 participants without diabetes and with results of PAI-1 antigen and 2hPG levels in both 1990 and 1999 were selected

Paper III

Paper IV
**Changes in PAI-1 activity over ten years**

To evaluate the marked increase in PAI-1 antigen observed in paper III we used another population in Northern Sweden as a validation study. In the validation study, Stabilyte samples were collected from 58 (53% female) control subjects over ten years who participated both at baseline (1997-2000) and at the ten-year follow-up (2007-2010). Plasma samples were collected in Stabilyte tubes, between 7 a.m. and 9 a.m. after an overnight fast. The samples were centrifuged at 2500 x g, for 15 min, and frozen at −80°C. PAI-1 activity analysis was performed in 2011 using Chromolize™ PAI-1 activity ELISA.

**Additional analysis (paper III)**

**Study of diurnal variation**

To test for the presence of diurnal variation in fibrinolytic factors, the effect of time of sampling was test. Time of sampling for each of the 566 participants was registered to the nearest hour. Median values of the fibrinolytic factors were then plotted against time of sampling. Because only a few individuals (≤10) participated after 14:30, sampling hours 15 and 16 were excluded.

**Freezing in liquid nitrogen (-196°C) compared to freezing at -20°C**

In MONICA 1990 the plasma samples for fibrinolytic measurements were collected in Stabilyte test tubes, snap-frozen in liquid nitrogen and stored at -80°C at the Northern Sweden Medical Research Biobank in Umeå. Plasma samples from the same individuals in 1999 were collected in Stabilyte test tubes, but were frozen at -20°C, and then stored at -80°C at the Medical Research Bank. Liquid nitrogen has a temperature of -196°C, which might prevent an over-estimation of PAI-1 plasma content by slowing down the thrombocyte release of PAI-1. To test the null-hypothesis that the PAI-1 result was comparable between samples frozen at -20°C and samples snap frozen in liquid nitrogen ten samples of Stabilyte-plasma were compared. The samples were collected from 8 a.m. to 10 a.m. and centrifuged for 15 min at 2000 x g and 4°C. The plasma fraction was divided into two aliquots: one was frozen directly in liquid nitrogen and the other was frozen at -20°C. In the afternoon the samples were transferred to -80°C. The plasma samples were analysed in pairs, using Trinilize PAI-1 antigen® purchased from Tcoag Ireland Ltd. (Bray, Wicklow, Ireland).
Results

Laboratory measurements

The inter-assay coefficients of variance (CV) for the laboratory analysis of the TRIM (paper II) and MONICA (paper III and IV) plasma samples are found in table 3.

Table 3. Inter-assay coefficients of variance and number of control measurements.

<table>
<thead>
<tr>
<th></th>
<th>TRIM</th>
<th>MONICA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
<td>Inter-assay CV</td>
</tr>
<tr>
<td>tPA activity</td>
<td>n.a.</td>
<td>n.a.</td>
</tr>
<tr>
<td>tPA antigen</td>
<td>12</td>
<td>10.8</td>
</tr>
<tr>
<td>PAI-1 antigen</td>
<td>24</td>
<td>7.1</td>
</tr>
<tr>
<td>tPA/PAI-1 complex</td>
<td>12</td>
<td>8.3</td>
</tr>
<tr>
<td>VWF</td>
<td>12</td>
<td>6.5</td>
</tr>
<tr>
<td>CRP</td>
<td>8</td>
<td>3.8</td>
</tr>
</tbody>
</table>

Abbreviations: tPA: tissue plasminogen activator; PAI-1: plasmingen activator inhibitor-1; CRP: C-reactive protein, n.a; not applicable.

Long-term stability of fibrinolytic factors stored at -80 °C. (Paper I)

Basic characteristics of both study populations are given in Table 2 in paper I. In the long term storage study the participants were older and there were more men, the prevalence of diabetes and hypertension was more common, and BMI and blood pressure were higher.

Long-term storage study

The samples had been stored for a median time of 11.6 years (10th percentile 8.2 years to 90th percentile 15.0 years). The squared correlations coefficient, $R^2$, indicating effect of storage time on the measured variables in these samples, was calculated for each factor: tPA antigen, $R^2=0.01$; PAI-1 antigen,
$R^2=0.01$; and tPA-PAI-1 complex, $R^2=0.02$ (p<0.001 for all variables). The regression plots are shown in Figure 9.

**Figure 9.** Relation between ln tPA, ln PAI-1 and ln tPA-PAI-1 complex and storage time in the long term storage study. Regression plots and squared correlations coefficients, $R^2$, are given for all 1,598 studied subjects.

Reagent kit modifications study

Samples were drawn in 1990 and analysed in 1990, 2001 and 2005. Regression plots of tPA activity, tPA antigen and PAI-1 antigen are shown in the left-hand panels of Figures 10-12. Bland-Altman plots are shown in the right-hand panels of these figures. The linear regression equations and $R^2$ values for tPA activity, tPA antigen, PAI-1 activity, PAI-1 antigen and tPA-PAI-1 complex are given in paper I. The tPA activity slope coefficients were between 1.06 and 1.09, with $R^2$ between 0.67 and 0.93, as shown in Figure 10. For the tPA antigen the slope coefficients were between 0.72 and 0.95 with $R^2$ between 0.47 and 0.75, as shown in Figure 11. The slope coefficient for PAI-1 antigen was 0.87 ($R^2 = 0.85$), as shown in Figure 12.

**Figure 10.** Regression plot and Bland-Altman plot for the reagent kit study showing tPA activity measured with Spectrolyse in 1990 and Chromolize in 2001 (x) in 78 subjects, and with Spectrolyse in 1990 and Chromolize in 2005 (o) in 786 subjects.
**Figure 11.** Regression plot and Bland-Altman plot for the reagent kit study showing tPA antigen concentrations measured with TintElize in 1990 and Imulyse 2001 (x) in 75 subjects, and with TintElize in 1990 and Imulyse 2005 (o) in 702 subjects, in the reagent kit study.

**Figure 12.** Regression plot and Bland-Altman plot for the reagent kit study showing PAI-1 antigen concentrations measured with Imulyse in 2001 and TintElize in 2005 (Δ) in 44 subjects in the reagent kit study.
Signs of dysregulated fibrinolysis precede incident type 2 diabetes mellitus in a population-based study. (Paper II)

Cases with T2DM were diagnosed a median 5.5 years (range 0.1-10.6 years) after the baseline health examination in the VIP. These cases reported having a parent or sibling with diabetes more often than referents. No difference was found in smoking status or physical activity at baseline. Hypertension was more common among cases. Cases with incident T2DM had higher baseline systolic and diastolic blood pressure, FPG, 2hPG, BMI, and triglyceride, CRP, tPA, PAI-1, tPA/PAI-1, and VWF levels compared with referents.

The univariate conditional logistic regression analysis showed a significantly increased risk of incident T2DM for family history of diabetes, hypertension, systolic and diastolic blood pressure, FPG, 2hPG, BMI, triglycerides, CRP, tPA, PAI-1, tPA/PAI-1 complex, and VWF. The exclusion of patients taking antihypertensive medication did not affect the association of haemostatic variables with incident T2DM.

Multivariate regression analysis was performed for each haemostatic factor. The results are shown in Figure 13. In model 1 tPA, PAI-1, tPA/PAI-1 complex and VWF were independently associated with incident T2DM. In model 2, tPA, PAI-1, and tPA/PAI-1 complex were related to incident T2DM, whereas VWF was not. In model 3, only PAI-1 and tPA/PAI-1 remained independently associated with incident T2DM.
Figure 13. Risk of incident T2DM in relation to tPA antigen, PAI-1 antigen, tPA/PAI-1 complex and VWF. ORs with 95% CIs, per 1 SD increment.

Conditional logistic regression analysis performed with complete data sets.

*Model 1: Adjusted for body mass index, smoking (non/ex/daily smoking), family history of T2DM and physical activity (sedentary/moderate/active).

**Model 2: Adjusted for model 1 factors plus C-reactive protein, systolic blood pressure and triglycerides.

***Model 3: Adjusted for model 2 factors plus fasting glucose level and 2-hour capillary glucose level.

OR: odds ratio; CI: confidence interval; SD: standard deviation; T2DM: type 2 diabetes mellitus; tPA: tissue plasminogen activator; PAI-1: plasminogen activator inhibitor-1; VWF: von Willebrand factor.
# Subgroup analysis

Conditional univariate and multivariate analysis were performed separately for the subgroups of participants with normal glucose levels and elevated glucose levels at baseline. The results are shown in **Figure 14**. The multivariate analysis was carried out with adjustments for BMI, smoking, family history of diabetes, physical activity, CRP, systolic blood pressure, triglycerides, and each haemostatic variable. Because the subgroups were based on normal or elevated glucose levels, no adjustments were made for glucose levels.

**Figure 14.** Forest plots illustrating risk for incident type 2 diabetes in A) cases with normal glucose levels, and B) cases with elevated glucose levels. ORs with 95% CIs per increment of 1 SD.

* FPG <6.1 and 2hPG<8.9 mmol/L.
** FPG 6.1-6.9 and/or 2hPG 8.9-12.1 mmol/L.

Conditional logistic regression analysis performed with complete data sets, multivariate analysis included BMI, smoking (non/ ex/ daily smoking), family history of T2DM, physical activity (sedentary/ moderate/ active), CRP, systolic blood pressure and triglycerides and one of the haemostatic variables (tPA, PAI-1, tPA/PAI-1 complex or VWF).
Additional results for paper II

A multivariate regression analysis was performed separately for men and women for each fibrinolytic factor. The results are shown in Figure 15. In model 1 tPA, PAI-1, and tPA/PAI-1 complex were independently associated with incident T2DM. In model 2, tPA antigen levels in men were no longer significantly associated with incident T2DM. In model 3, no significant association with incident T2DM was found.

Figure 15. Risk of incident type 2 diabetes in relation to tPA antigen, PAI-1 antigen and tPA/PAI-1 complex in men and women, respectively. OR and 95% CI per 1 SD increment.

Conditional logistic regression analysis performed with complete data sets.
*Model 1: Adjusted for body mass index, smoking (non/ex/daily smoking), family history of type 2 diabetes and physical activity (sedentary/moderate/active).
**Model 2: Adjusted for model 1 factors plus C-reactive protein, systolic blood pressure and triglycerides.
***Model 3: Adjusted for model 2 factors plus fasting glucose level and 2 hour capillary glucose level.
Interaction analysis

All fibrinolytic factors interacted with BMI in the association with incident T2DM, as shown in Appendix I and Figure 16 A-C.

Figure 16. Age-adjusted binary logistic regression showing the risk of incident type 2 diabetes in cases compared to referents, matched for sex and age, in relation to antigen levels of tPA (A), PAI-1 (B) and tPA/PAI-1 complex (C) above median value, BMI above 27 or the interaction of these variables.

Abbreviations: BMI: Body mass index; PAI-1: Plasminogen activator inhibitor-1; Ref: reference group without incident type 2 diabetes; tPA: tissue plasminogen activator; tPA/PAI-1: tPA/PAI-1 complex
In participants with normoglycaemic glucose levels at the time of examination elevated levels of PAI-1 antigen and tPA/PAI-1 complex, but not tPA antigen, showed significant interactions with BMI in the association with incident T2DM, as shown in Appendix II and Figure 17 A-B.

**Figure 17.** Age-adjusted binary logistic regression showing the risk of incident type 2 diabetes in cases with normal glucose levels compared to referents matched for sex and age. The plots show PAI-1 (A) or tPA/PAI-1 complex (B) above median value, and for age at time of participation. All associations were significant (p<0.05).

Abbreviations: BMI: Body mass index; PAI-1: Plasminogen activator inhibitor-1; Ref: reference group without incident type 2 diabetes; tPA: tissue plasminogen activator; tPA/PAI-1: tPA/PAI-1 complex
Individual changes in fibrinolytic factors, von Willebrand factor and C-reactive protein over a nine year period.
(Paper III)

Antigens of tPA, PAI-1 and tPA/PAI-1 complex, as well as VWF and CRP increased significantly over nine years. The increase in antigens of tPA, PAI-1 and the tPA/PAI-1 complex are highly significant but weakly correlated to one another and to VWF, and are inversely correlated to tPA activity. The increase in CRP was significantly correlated to the increase in all fibrinolytic factors and to the decrease in tPA activity, but most strongly to the increase in VWF.

There was a trend of increasing Δ-levels of VWF in both men and women across the four age groups. VWF increased significantly over nine years in all age groups except the youngest for both men and women, as shown in figure 18. Mean change, Δ1999-1990, was related to age groups in both men and women (p for trend= 0.001 and <0.001, respectively). The mean Δ1999-1990 was comparable between men and women (0.16 IU/mL vs 0.11 IU/mL, p=0.739).

Figure 18. VWF levels in 1990 and 1999 in men (n=158) and women (n=151) aged 25-64 in 1990.
Increased fibrinolytic antigen levels over nine years were seen in both men and women and in most age groups. The increase in $\Delta_{1999-1990}$ of tPA, PAI-1 and tPA/PAI-1 antigen concentrations was similar in all age groups. In contrast, the activity of tPA appears to be stable over nine years and across age groups. The antigen of PAI-1 increased significantly over nine years in all age groups of men and women, as shown in Figure 19. Mean change, $\Delta_{1999-1990}$, was not related to age groups ($p$ for trend= 0.151 and 0.613, for men and women, respectively). The mean $\Delta_{1999-1990}$ was comparable between men and women (8.41 ng/mL vs 8.32 ng/mL, $p=0.593$), resulting in an overall PAI-1 antigen level increase of 75% for men and 95% for women.

**Figure 19.** PAI-1 level increase over nine years in men (n=155) and women (n=149) aged 25-64 in 1990.
**PAI-1 increase over ten years in another population**

In the validation study the median PAI-1 activity level at baseline was 7.32 (IQR: 4.84-12.20) IU/mL and 12.42 (IQR: 7.28-22.25) IU/mL at follow-up (see **figure 20**). The intra-individual mean increase over ten years was 8.16 (95% CI: 4.07-12.95) IU/mL. The median tPA antigen level at baseline was 9.03 (IQR: 7.66-12.27) ng/mL and 12.71 (IQR: 9.31-16.35) ng/mL at follow-up, and the intra-individual mean increase over ten years was 3.03 (95% CI:1.51-4.55) ng/mL.

**Figure 20.** Comparison of tPA and PAI-1 antigen and activity levels at baseline and after nine years in the present study, and ten years in the validation study, respectively.
Additional results in paper III

Diurnal variation

The antigen and activity levels of tPA and PAI-1 are known to display a diurnal variation. This has previously not been investigated in tPA/PAI-1 complex. We found significant increasing trends in tPA activity levels from 7 a.m. to 2 p.m., as well as significant decreasing trends in PAI-1 antigen and tPA/PAI-1 complex levels, in both 1990 and 1999, as shown in Figure 21. No significant trend was found for tPA antigen.

Figure 21. Median and IQR for the fibrinolytic factors tPA activity and antigen, PAI-1 antigen and tPA/PAI-1 complex measured at different hours during the day in MONICA 1990 (n=804) and 1999 (n=808).
Due to diurnal variation only samples donated during the morning hours were selected for analyses in papers III and IV. **Table 4** shows the intra-individual differences in time of blood sampling in 1990 compared to 1999.

**Table 4.** Difference in time of blood sampling for each participant between 1990 and 1999, presented for all participants from Västerbotten and for those examined during the morning hours (paper III).

<table>
<thead>
<tr>
<th></th>
<th>Västerbotten (n=566)</th>
<th>7.00-11.30 a.m. (n=344)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-1 hour</td>
<td>292 (52%)</td>
<td>211 (61%)</td>
</tr>
<tr>
<td>0-2 hours</td>
<td>474 (65%)</td>
<td>301 (87%)</td>
</tr>
<tr>
<td>0-3 hours</td>
<td>545 (78%)</td>
<td>334 (97%)</td>
</tr>
</tbody>
</table>

*Freezing in liquid nitrogen (-196°C) compared to -20°C*

The mean PAI-1 antigen levels were 7.81 ± 12.05 ng/mL for samples frozen at -20°C, and 8.22 ± 11.93 ng/mL for samples snap-frozen in liquid nitrogen, see **Figure 22**. The slope coefficient for comparison of methods was 0.97 (95% CI: 0.92-1.02) and the correlation coefficient, r, was 0.999 (R² = 0.998, p<0.001). Wilcoxon related-samples signed rank test showed no difference between methods (p=0.093).

**Figure 22.** Comparison of effect of freezing temperature on levels of PAI-1 antigen in plasma collected in Stablyte test tubes (n=10).
Individual PAI-1 increase over nine years relates differently in men and women to changes in anthropometric, glycaemic, inflammatory and lipid markers. (Paper IV)

A linear regression analysis, adjusted for age, was performed for Δ1999-1990 with PAI-1 antigen as the dependent variable and co-variates added separately, as shown in Figure 23. The PAI-1 increase in men and women showed different correlations patterns to the studied risk factors. In men ΔPAI-1 was related to Δweight, ΔCRP, ΔFPG, Δ2hPG and Δtriglycerides. In women ΔPAI-1 was related to Δweight, Δwaist circumference, Δhip circumference and ΔCRP.

Figure 23. Age-adjusted correlations between changes in PAI-1 and changes in measurements of anthropometry, blood pressure, inflammation, lipids and plasma glucose levels between 1990 and 1999, in men and women, respectively. Only significant correlations (p<0.05) are shown.

The multivariate regression analysis included the significant age-adjusted variables, for men and women, respectively. ΔCRP and ΔFPG remained significantly correlated to ΔPAI-1 in men. In women, only Δwaist circumference remained significant (see Table 5).
Table 5. Multivariate linear regression with variables significantly correlated with ΔPAI-1 antigen in the age-adjusted linear regression in men and women, respectively.

<table>
<thead>
<tr>
<th></th>
<th>MEN</th>
<th></th>
<th>WOMEN</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ΔPAI-1 antigen</td>
<td>R² = 0.306</td>
<td>β</td>
<td>P</td>
</tr>
<tr>
<td>Age 1990</td>
<td>-0.006</td>
<td>0.103</td>
<td>Age 1990</td>
<td>-0.003</td>
</tr>
<tr>
<td>ΔWeight</td>
<td>1.264</td>
<td>0.085</td>
<td>ΔWeight</td>
<td>0.005</td>
</tr>
<tr>
<td>ΔC-reactive protein</td>
<td>0.100</td>
<td>0.018</td>
<td>ΔC-reactive protein</td>
<td>0.091</td>
</tr>
<tr>
<td>ΔFasting plasma glucose</td>
<td>0.775</td>
<td>0.012</td>
<td>ΔWaist circumference</td>
<td>2.286</td>
</tr>
<tr>
<td>Δ2 hour plasma glucose</td>
<td>-0.006</td>
<td>0.963</td>
<td>ΔHip circumference</td>
<td>0.100</td>
</tr>
<tr>
<td>ΔTriglycerides</td>
<td>0.163</td>
<td>0.114</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

In MONICA 1990, PAI-1 antigen levels were found to be correlated to BMI, waist and hip circumference, and glucose, lipid and CRP levels in a similar way in both men and women (see Appendix III).
Discussion

General discussion

The changes in fibrinolytic factors have a complex relation with anthropometry and incident T2DM. Anthropometric variables, such as weight and waist and hip circumferences, are risk factors associated with both incident T2DM and cardiovascular disease [151, 152], but the association between changes in these variables and changes in fibrinolytic factors less well studied. Obesity is associated with the development of IFG, IGT and T2DM [150, 153]. Hyperglycaemia is associated with impaired fibrinolysis [116, 117], and impaired fibrinolysis is associated with the development of cardiovascular events [20, 22, 66, 154, 155]. Atherothrombosis is the main cause of death in patients with T2DM [156]. However, the pathophysiology and impact of a dysregulated fibrinolytic system in the development of T2DM is unclear. Also, the changes in fibrinolytic factors over time in men and women, and how long-term storage affect the samples used for laboratory analysis of fibrinolytic factors was unknown. The health examinations in MONICA and VIP also collect measurements and laboratory analyses making them ideal for the aim of studying fibrinolytic factors and the relation to changes in anthropometry and incident diabetes.

Strengths and limitations

Repeated cross-sectional studies

A cross-sectional study is an observational study that collects data from a population, or randomized subset of the population, on one specific occasion. The repeated cross-sectional study continues to collect data from the same population on later occasions.

The major strength with the repeated cross-sectional design (used in paper I, III and IV) is that the individuals act as their own references, which decreases the effect of confounding factors such as genetic variation. Therefore, the changes affecting fibrinolytic factors, over the course of nine years for these particular individuals, will depend mainly on life style changes and increasing age.
A cross-sectional study, or several repeated cross-sectional studies, can only investigate the prevalence of exposure and outcome, and calculate relative risks. In health research one often requires a large population, which makes the design rather expensive. The cross-sectional design is also unsuitable for the study of rare diseases.

**Nested case-referent study**

A nested case-referent study is conducted within a cohort study, and includes the individuals who fall ill, compared with a matched sample of the rest of the cohort.

The prospective nested case-referent design (used in paper II) has several advantages. Firstly, at baseline examination all participants are free from diabetes and the risk of recall bias is avoided because the risk of under- or over-reporting in questions on behaviour is likely to be evenly distributed among cases and referents. Secondly, because samples were collected prior to the outcome measure the prospective design is less affected by reverse causality. Thirdly, this design is also cost- and time effective compared to cohort studies because only referents matched to the incident cases are selected for analyses, instead of the entire cohort.

The data in case-referent studies can be used to calculate odds ratios, but it cannot prove causality between the exposure and the event.

**Validity**

Validity addresses the degree to which a measurement or study is able to find the true value in order to reach the correct conclusion. Internal validity is the extent to which the results truly reflect the situation of the study population, and external validity is the extent to which the results are applicable to other populations [157].

Paper I investigated the effect of long-term storage and reagent kit modifications on levels of fibrinolytic factors. We found that analysis of all samples on the same occasion was important for the internal validity of the study. Thus, the samples in papers II and papers III-IV, respectively, were analysed on one occasion.

In paper II false positive cases were excluded by a thorough study of case records. There is still some probability of false negative cases because it has
previously been shown that many people with T2DM are undiagnosed [158]. It is reasonable to assume that the identified cases have more symptoms and a more progressed disease than undiagnosed cases of T2DM, and this might affect the associations.

There was no follow-up of the referents, which means that some referents might have developed T2DM after the health examination. These referents with undiagnosed T2DM would attenuate the differences between cases and referents, and might underestimate the association of fibrinolytic factors with future T2DM. Two age- and sex matched referents were randomly chosen from all possible referents. This is cost-effective, but might introduce a selection bias.

Cases and referents prioritized to other studies (cancer, myocardial infarction and stroke) were excluded, and this might limit the external validity. In addition, it is unknown whether cases and referents with no blood samples available for analysis of haemostatic factors constituted a selected group.

The Northern Sweden MONICA population of 1990 was a randomised sample of the population aged 25-64 years, thus representing the majority of the adult population in northern Sweden. To avoid the influence of diurnal variation in fibrinolytic variables only those who were examined between 7 a.m. and 11 a.m. in both 1990 and 1999 were selected for analysis in paper III. Only participants with complete data sets including fibrinolytic factors, and OGTT were included in paper IV. Out of the original 1,000 participants, 65% were randomised to OGTT, but only approximately 50% were able to perform an OGTT. Altogether, these restrictions might have affected the internal validity of the study in paper IV.

In terms of external validity the vast majority of those who participated in VIP and MONICA in papers I to IV were Caucasian. This limits the generalizability to populations of other ethnic origins.

Confounders

Confounding is defined as the effect of an external variable with the effects of the exposure and disease of interest. A potential confounder associates with the outcome in the absence of exposure, and with the exposure but not as a result of being exposed [157].

Levels of fibrinolytic factors are associated with BMI, lipid levels, physical activity, smoking, alcohol consumption, inflammation and genetic polymorphisms, all of which are possible confounders when studying the association of fibrinolytic factors with T2DM. To decrease the risk of bias
due to confounding factors, cases and referents were matched for age, sex and year of participation. To adjust for the impact of possible confounders in paper II we used multivariate analysis. In the repeated cross-sectional study design the same individuals act as their own referents and this minimizes the risk of bias due to confounding factors.

Results in paper II are based on one baseline examination and one blood sample. There is no control for intra-individual changes of exposure variables over time, which is a potential limitation.

**Gender considerations**

Men and women differ in terms of fat distribution [159] with men having more intra-abdominal fat and women having more peripheral fat [160]. In addition, women have a higher fat mass percentage than men [161] and lower postprandial metabolism [162]. The differences are explained in part by higher estrogen concentrations [163]. The accumulation of visceral fat has been shown to be associated with increased levels of PAI-1 [9, 34], which is in accordance with findings of higher PAI-1 levels in men [17, 18].

The relevance of menopause and menstrual cycles in relation to fibrinolysis has been debated. A review in 2012 concluded that most studies have found no cyclic effect of fibrinolytic factors [164]. In clinical trials the use of oral contraceptives over 6 cycles appear to lower antigen levels of tPA and PAI-1 [165, 166], and the use of hormone replacement therapy might also have an overall positive effect on fibrinolysis [167, 168]. The transition from pre- and early peri-menopause to post-menopause has not been found to be associated with significant differences in tPA or PAI-1 antigen levels [169]. In paper III, we were unable to detect any effect of menopausal state on the increase of tPA or PAI-1 antigen levels. Natural menopause has not been associated with increased risk of T2DM [170].

Smoking has been associated with dysfibrinolysis in some studies [23, 24, 57, 58, 60]. In Sweden, more women than men smoke and young women constitute the largest group of those who start smoking [171].

The differences between men and women presented above imply that fibrinolytic analyses should preferably be conducted separately for each sex. Therefore, the correlations in paper IV were calculated for men and women separately.
Considerations on laboratory measurements

Avoiding pre-analytic errors

Bias, which is defined as the effects caused by factors unrelated to the independent variable by errors (random variability), whether pre-, peri- or post-analytical, can affect all study results. It is, therefore, of utmost importance to reduce all known sources of bias and errors prior to conducting a study.

To reduce the effect of seasonal and diurnal variations participants were sampled during the same time of year (spring) and during the morning hours with 97% donating samples with a maximum difference of two hours between both sampling occasions in papers III and IV.

In paper II samples were collected in EDTA test tubes, whereas the MONICA samples used in paper III and IV were collected in Stabilyte test tubes. As previously shown, the choice of tubes might affect the results, with EDTA tubes yielding a higher tPA-PAI-1 complex concentration than citrate or Stabilyte tubes [145]. In our laboratory, samples collected in EDTA tubes appear to yield higher levels of PAI-1 antigen than Stabilyte tubes (data not shown), and this is the likely explanation for the differences in PAI-1 levels seen in paper II compared to papers III and IV. Nonetheless, because samples for both cases and referents in paper II were collected in EDTA tubes it is unlikely that this has affected the association found between PAI-1 and incident T2DM.

The plasma samples in papers I to IV were thawed and re-frozen between each analysis occasion, and this could negatively affect the accuracy and precision of the laboratory results. In paper I, we found a good agreement between all three analysis occasions in the tPA activity results, which suggests that such a bias is not present in the data. An effect on tPA by freeze-thawing, however, has been shown by Kluft and Metier who found a marginal effect of freeze-thawing on tPA antigen with a significant tPA antigen change of 4% at the 6th freeze-thaw cycle [79].

Peri-analytical consideration

In laboratory methods, accuracy is defined as how close measurements of a quantity are to that quantity's true value, and precision is the degree to which repeated measurements under the same conditions show the same results (repeatability). A valid method is both accurate and precise. The validity of the analytical kit is assessed by calculating CVs (see Table 3). The inter-assay CVs differed between the analysis of the TRIM and MONICA samples. The MONICA measurements used more than twice as many
reagent kits, but the CVs were within the range of CVs presented in other studies.

The removal of platelets, which contain large amounts of PAI-1, is achieved by centrifugation. It has been shown that in order to reach a platelet content of less than 10,000 per μL in plasma, centrifugation at 2000 x g for 13 minutes or more is required [172]. In papers I to IV the centrifugation of samples was 2000 x g, for 15 min at 4°C. Consequently, it is unlikely that the platelet content has affected the PAI-1 antigen results.

Blood samples were collected from the vein at the bend of the arm, and care was taken to avoid stasis. In a minority of participants a short-term stasis was required. Stasis for 20 minutes has been shown to affect fibrinolytic levels [173], but because one minute of stasis has been shown to have only a minor effect on low molecular weight proteins [174], this short-term stasis is not likely to have affected the fibrinolytic results.

For the analysis of VWF, we thawed the samples quickly (15 min in a 37°C water bath) and mixed the samples thoroughly. This was done to avoid agglutination of VWF multimers and subsequent errors in measuring VWF levels in the plasma samples.

In paper I, the effects of reagent kit modifications showed that the development of new reagent kits might affect the results. To avoid this source of bias, the same reagent kits were used in each paper.

Duplicate testing is a way to increase the power of detecting smaller differences with statistical significance. To preserve biobank samples, however, each laboratory analysis in papers I to IV was only performed a single time. For measurement of tPA antigen and tPA/PAI-1 complex levels a single specimen is sufficient to achieve a validity coefficient of 0.91, but two measurements of PAI-1 activity and antigen levels are required to achieve a similar validity coefficient [175].

Discussion of individual papers

Paper I

The biological samples collected at baseline in any given study are a finite resource that must be used as efficiently as possible. It would be convenient, cost saving and time saving to be able to re-use the results from the baseline analyses when conducting follow-up studies, e.g., by using a case-cohort design. In the case-cohort design, a random subset - the sub-cohort - is selected as a control or reference group that can be used for multiple
different case groups arising from the cohort in future follow-up studies [176, 177]. However, the results in paper I clearly show that the effect of analytical batch on the antigen measurements for tPA and PAI-1 is much higher than the effect of long-term storage, and because of this the case-cohort design is not appropriate when investigating tPA and PAI-1 antigen levels in long-term studies.

**Paper II**

Fibrinolytic factors, but not VWF, were significantly associated with incident T2DM. Average VWF concentrations are about 30% lower in participants with blood type O [75], but this was not controlled for as ABO blood group information was not available for this study.

In paper II the risk of incident T2DM was not studied separately for men and women. The additional statistical analyses stratified for sex showed similar pattern of associations between fibrinolytic factors and T2DM for men and women. However, in some models stratifying for sex decreased the statistical power and the significance was lost.

The mechanisms that link increased PAI-1 and tPA/PAI-1 complex levels to the development of T2DM remain unknown. In addition to fibrinolytic variables, the step-wise backward logistic regression analysis showed that BMI and triglyceride levels were significantly associated with incident T2DM (data not shown). This is consistent with previous findings in northern Sweden. Postprandial triglyceride levels, but not fasting, have been shown to be higher in diabetic subjects compared to controls matched for age, sex, and BMI (3.0 mmol/L in diabetic patients vs. 2.0 mmol/L in controls, \( p=0.028 \)) [178]. In another study on VIP participants, a BMI above 27 at baseline (OR: 10.8) was as effective as a 2hPG above 7.8 mmol/L and an FPG above 5.6 mmol/L (ORs 7.8 and 7.2, respectively) in predicting T2DM in this adult population [153].

The age-adjusted interaction showed that the combination of high PAI-1 antigen and BMI above 27 increased the risk of future T2DM (RERI: 9.12). After further adjustments, the RERI was 2.49 (model 3). In cases with normoglycaemia age-adjusted interactions between PAI-1 and BMI showed a RERI of 8.38. Thus, PAI-1 antigen and BMI are independently associated with the risk for future T2DM, and the combination had a synergistic effect on the risk that was also apparent in normoglycaemic cases.

It has also been shown that insulin induces PAI-1 secretion in a hepatoma cell line [119], thus it is plausible that the increased PAI-1 level is a reaction
to increased insulin production that will in time result in insulin resistance. However, in a study with young, non-diabetic, non-obese offspring of patients with T2DM, elevated PAI-1 activity had no association with plasma insulin concentrations [179], which contradicts that PAI-1 levels increase due to up-regulation of PAI-1 by insulin. When adding insulin levels to the adjustors in the multivariate logistic regression (model 3), the insulin levels were not significantly associated with incident diabetes. Adding insulin to the analysis did not change the significant association of PAI-1 (OR: 2.30, 95% CI: 1.13-4.68) with incident T2DM.

The results in paper II imply that the pathophysiological pathway linking high PAI-1 antigen with future T2DM is not solely explained by increased levels of glucose or insulin or by high BMI.

**Paper III**

**Considerable PAI-1 increase over nine years**

The mean Δ1999-1990 PAI-1 increase over nine years was 75% and 95% in men and women, respectively. This substantial increase made us consider analytical errors, and two other study populations were used for validation.

In the validation study, the intra-individual mean increase of PAI-1 activity and tPA antigen from baseline to follow-up was comparable to the mean Δ1999-1990 increase in the antigen levels of PAI-1 and tPA in paper III (see **Figure 20**). The activity of tPA did not change in either study. Both studies used Stabilyte plasma in the laboratory analysis. It is known that the activity and antigen levels of PAI-1 have a high correlation [180], and PAI-1 activity levels compared to antigen levels showed a high intra-individual correlation (0.89) in our laboratory. We conclude, therefore, that the increase in activity and antigen levels of PAI-1 are comparable over time in the two studies.

In MONICA 1990-1999 the antigen levels were similar to the activity levels measured ten years later in the validation study. In our laboratory, the PAI-1 antigen levels were 20% to 30% higher than the activity levels. If antigen had been measured in the validation study one would expect 20% to 30% higher levels, which would indicate that the increase in PAI-1 was consistent from 1990 to 2010.

The tPA antigen was measured in both MONICA and the validation study. The increase between 2000 and 2010 was comparable to the increase from 1990 to 1999, but started at a higher level. These findings support a continuous tPA antigen increase from 1990 to 2010 similar to what was seen for PAI-1.
We conclude that the substantial increase in PAI-1 activity in the validation study supports the conclusion that the increase in PAI-1 antigen observed in paper III was not an analytical error. Thus, there is a substantial PAI-1 antigen increase in the population over nine years. PAI-1 levels have been shown to be related to age, weight, inflammation, physical activity, alcohol consumption and smoking among others. The increase in PAI-1 might be caused by changes in these or other factors during the nine-year observation period. In paper IV we attempted to identify changes in anthropometric and biochemical factors that might explain the PAI-1 antigen increase.

**Paper IV**

To our knowledge, this is the first intra-individual study of changes in PAI-1 antigen and changes in factors related to the metabolic syndrome, over nine years.

The Δ1999-1990 change in PAI-1 antigen correlated to changes in weight and CRP in both sexes. For men, we also observed a correlation with changes in glucose and triglyceride levels. For women, on the other hand, there were correlations to changes in waist and hip circumference. Thus, there were different correlation patterns between men and women when studying changes over nine years compared to correlations at baseline.

We hypothesize that some explanation of the difference in correlation patterns between men and women depends on the difference in fat distribution. Women have a higher fat mass percentage than men [161] distributed as more peripheral fat, whereas men have a more intra-abdominal (visceral) fat distribution [160]. Men also tend to have higher triglyceride levels than women, and fat distribution appears to explain some of that difference as well [181]. Adipose tissue is assumed to be one of the main sources of PAI-1. If the accumulation or reduction of fat mass was different in men and women over nine years, this might explain the observed differences in correlations between men and women.

We expected that changes in anthropometric, glycaemic, inflammatory and lipid markers would explain a large part of the observed PAI-1 increase. However, the multivariate regression analysis showed that 70% to 80% of the change in PAI-1 antigen could not be explained by the studied variables. There are several possible explanations for this:

- Measuring weight, waist and hip circumference give some but limited information about the fat distribution. Advanced imaging technique might
be a better tool for determining the impact of change in fat distribution on changes in PAI-1 and other metabolic variables.

- Levels of physical activity were obtained from questionnaires which have limited accuracy and precision. The correlations between changes in physical activity and PAI-1 will therefore be difficult to show.

- This study was conducted over nine years, which increases the probability of finding significant changes over time. On the other hand, change in lifestyle (i.e., food consumption, alcohol habits, stress and socioeconomic factors) and co-morbidity during the study period might explain a part of the change of PAI-1 levels. We did not retrieve data on these factors. Hence, we were not able to control for them.

- Other factors that are related to PAI-1 levels but are not controlled for in our study might have changed over nine years. Such factors have been described in other studies e.g., insulin, pro-insulin, free fatty acids and leptin [17, 182].

**Do increased PAI-1 antigen levels predict diabetes?**

PAI-1 is a potential determinant of incident T2DM. Over nine years, the levels of PAI-1 antigen increased markedly in the general population. The pathophysiological background to this increase and the association with T2DM needs to be further investigated separately for each sex.

Current determinants for the risk of future T2DM include elevated glucose levels and BMI. In paper II, high PAI-1 levels were shown to precede elevated glucose levels in those who later developed T2DM. We also found an interaction with BMI above 27 that further increased the risk of developing T2DM. It is plausible that a combination of BMI and high PAI-1 levels could be an earlier predictor of future T2DM than elevated glucose levels.

The laboratory analysis kits available for the measurement of PAI-1 antigen or activity are only designed for research purposes. In order to analyze PAI-1 clinically and to establish cut-off levels for individuals at high risk of future T2DM a standardized method is required.

If cut-off levels for PAI-1 are able to discriminate individuals with high risk of future T2DM, it would be possible for individuals to decrease levels of PAI-1, e.g., through weight loss or metformin treatment. Metformin has been shown to decrease PAI-1 and FPG levels as well as the incidence of T2DM.
It is also known that the development of T2DM can be prevented in high-risk individuals who comply with lifestyle interventions [184].

**PAI-1 and time trends in anthropometric measurements and glucose levels in northern Sweden**

Globally, the prevalence of T2DM is estimated to increase from 2.8% in the year 2000 to 4.4% in 2030 suggesting an ongoing global diabetes epidemic [185]. We know that PAI-1 antigen increased significantly between 1990 and 1999 (paper III) and that increased PAI-1 levels are associated with incident T2DM (paper II). The question, then, is whether the observed PAI-1 antigen increase predicts a change in the risk factor burden for T2DM in northern Sweden.

Recently, a study on trends in glucose distribution in northern Sweden was published using data on MONICA participants up to 2009. The prevalence of T2DM was unchanged, but FPG and 2-hPG were shown to have increased. The prevalence of IGT doubled in women and tripled in men [186].

Time trends in FPG and 2-hPG levels, and the prevalence and 10-year cumulative incidence of T2DM, have also been studied in participants in the VIP. Between 1990 and 2007 the mean FPG level increased by 0.5 mmol/L. The prevalence of T2DM increased by 44% in men and 17% in women during the same study period. A higher prevalence and incidence of T2DM was found in subjects with low levels of education [185].

In paper IV, there was no increase in FPG between 1990 and 1999. In women, 2hPG increased significantly and in men there was a non-significant increase. The PAI-1 antigen increase during the same study period was substantial in both men and women. Whether or not this PAI-1 increase precedes the increase in FPG and 2hPG cannot be determined from our data. However, in paper II high PAI-1 levels were shown to precede elevated glucose levels in the prediction of future T2DM. Future studies should evaluate whether the increased PAI-1 levels forebode a T2DM epidemic.

The additional results in paper II also indicate an interaction between increasing levels of PAI-1 antigen and BMI. In the USA increasing BMI has been followed by increasing incidence of T2DM. The BMI increase in Sweden occurred some ten years later than that in the US but it has followed the same pattern as in the US [187]. However, people born in the 1940s have a lower BMI increase than those born in the 1930s, which could mean that the trend is slowing down [188]. In paper IV, the anthropometric measurements (weight, and waist and hip circumferences) increased over nine years in both men and women. The relation of the changes in these anthropometric variables and the change in PAI-1 were weak and differed between men and
women. It is reasonable to assume that other life style and socioeconomic changes that were not studied in paper IV might help explain the increase in PAI-1.

**Future research**

**PAI-1 increase in northern Sweden**

Is there a continuing intra-individual increase in PAI-1 antigen levels after 1999? To study this, plasma from individuals who have participated in the VIP on several occasions (e.g., at 30, 40, and 50 years of age) could be analysed. A third examination of the individuals examined in MONICA 1990 and 1999 is also a way to study the intra-individual long-term change in PAI-1.

**Observational study**

Has the PAI-1 antigen increase seen during the 1990s resulted in a higher incidence of T2DM today? In 2014, the 6th MONICA survey will be conducted along with new health examinations including T2DM prevalence. This will enable us to estimate the time trends for T2DM prevalence from 1986 through to 2014.

**Prospective study (TRIM2: 2001-2013)**

Does the dramatic increase in PAI-1 antigen levels in the 1990s affect the predictive ability of PAI-1 antigen for the risk of developing T2DM? To confirm that fibrinolytic variables associated with future T2DM show the same pattern today as it did from 1989 to 2001, a new TRIM study should be conducted. It would be preferable to conduct a larger study including all municipalities in Västerbotten and with all variables analysed separately for men and women.

**Intervention**

Lifestyle intervention is known to delay or prevent the development of T2DM, and weight loss has been shown to decrease levels of PAI-1 activity [184]. Randomising individuals with high PAI-1 antigen levels to lifestyle intervention or to usual care could help us to determine whether a decrease in PAI-1 would affect the incidence of T2DM. However, the results from such
a study design would not discriminate between the effects of PAI-1 or weight change on the incidence of T2DM. PAI-1 has been shown to interact with BMI in the prediction of T2DM. In order to investigate a causal relationship between PAI-1 and T2DM we need to study whether a decrease in PAI-1 would affect the incidence of T2DM independently of a change in weight. To achieve this, a specific PAI-1 antagonist is required. Such antagonists have been developed but are not yet available for interventional use [189].

Comparison of test tubes used for PAI-1 antigen assessment

Stabilyte test tubes are recommended for tPA and PAI-1 activity measurements, and are also suitable when measuring PAI-1 antigen. In our laboratory it has been noticed that EDTA plasma tends to yield higher levels of PAI-1 antigen than Stabilyte plasma. A comparison of the use of different test tubes on the measureable levels of PAI-1 antigen is required to establish a stable gold standard. The CTAD tube, which contains sodium citrate with the addition of theophylline, adenosine and dipyridamol, is known to inhibit platelet activation and should also be included in such a study.
Conclusions

- Modifications of reagent kits affect the analytical results of tPA and PAI-1 antigen levels. Long-term storage time had a significant, but practically irrelevant, impact on plasma antigen levels of tPA, PAI-1 and tPA/PAI-1 complex. Consequently, analysis of all samples on the same occasion is preferable.

- Elevated levels of tPA, PAI-1 and tPA/PAI-1 complex precede incident T2DM after adjustments for traditional diabetes and cardiovascular risk markers. VWF was not associated with incident T2DM. PAI-1 was associated with incident T2DM, even in normoglycaemic subjects.

- There was an intra-individual longitudinal increase in the antigen levels of tPA, PAI-1, tPA/PAI-1 complex, VWF and CRP over 9 years in both men and women. The increase in PAI-1 antigen levels was the most pronounced.

- In men, the intra-individual increase in PAI-1 antigen over nine years was related to changes in weight, CRP, triglycerides, FPG and 2hPG levels. In women, the PAI-1 increase correlated to changes in weight, waist and hip circumference and CRP levels. No correlations with blood pressure were found. The multivariate models explained 20% of the variation in ΔPAI-1 in women and 30% in men.
Tack!


Tack till min huvudhandledare Lars Johansson, för att du alltid mött mig med ett öppet sinne, en förstående och humoristisk attityd, samt för stöd och utbildning som jag behövt för att ta mig vidare. Du har fått mig att känna mig som en medarbetare, och inte ”bara” en doktorand.


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APPENDIX I. Age-adjusted logistic regression results for risk of incident type 2 diabetes for cases with BMI above or below 27 in interaction with fibrinolytic factors above or below median value. ORs with 95% CIs per increment of 1 SD. Interaction numbers in **bold** typing indicate statistically significant results.

<table>
<thead>
<tr>
<th>BMI</th>
<th>Fibrinolytic factor</th>
<th>N cases / N referents</th>
<th>OR adjusted for age</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMI*tPA</td>
<td>Below 27</td>
<td>Below median</td>
<td>22 / 136</td>
</tr>
<tr>
<td></td>
<td>Below 27</td>
<td>Above median</td>
<td>23 / 30</td>
</tr>
<tr>
<td></td>
<td>Above 27</td>
<td>Below median</td>
<td>27 / 60</td>
</tr>
<tr>
<td></td>
<td>Above 27</td>
<td>Above median</td>
<td>82 / 44</td>
</tr>
<tr>
<td></td>
<td>Relative excess risk</td>
<td>5.44 (-1.94-12.84)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Attributable proportion</td>
<td>0.44 (0.08-0.81)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Synergy index</td>
<td>1.93 (0.92-4.07)</td>
<td></td>
</tr>
<tr>
<td>BMI*PAI-1</td>
<td>Below 27</td>
<td>Below median</td>
<td>20 / 128</td>
</tr>
<tr>
<td></td>
<td>Below 27</td>
<td>Above median</td>
<td>24 / 38</td>
</tr>
<tr>
<td></td>
<td>Above 27</td>
<td>Below median</td>
<td>28 / 69</td>
</tr>
<tr>
<td></td>
<td>Above 27</td>
<td>Above median</td>
<td>81 / 36</td>
</tr>
<tr>
<td></td>
<td>Relative excess risk</td>
<td>9.13 (1.69-16.56)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Attributable proportion</td>
<td>0.62 (0.39-0.84)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Synergy index</td>
<td>2.93 (1.49-5.78)</td>
<td></td>
</tr>
<tr>
<td>BMI* tPA/PAI-1 complex</td>
<td>Below 27</td>
<td>Below median</td>
<td>19 / 146</td>
</tr>
<tr>
<td></td>
<td>Below 27</td>
<td>Above median</td>
<td>15 / 30</td>
</tr>
<tr>
<td></td>
<td>Above 27</td>
<td>Below median</td>
<td>30 / 50</td>
</tr>
<tr>
<td></td>
<td>Above 27</td>
<td>Above median</td>
<td>91 / 44</td>
</tr>
<tr>
<td></td>
<td>Relative excess risk</td>
<td>9.59 (0.98-18.20)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Attributable proportion</td>
<td>0.54 (0.28-0.80)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Synergy index</td>
<td>2.32 (1.25-4.32)</td>
<td></td>
</tr>
</tbody>
</table>
APPENDIX II. Age-adjusted logistic regression calculated for cases with normal glucose levels at the time of sampling and their referents matched for age, sex and year of participation. ORs with 95% CIs per increment of 1 SD for risk of incident T2DM for cases with BMI above or below 27 in interaction with haemostatic factors above or below median value. Interaction numbers in **bold** typing indicate statistically significant results.

<table>
<thead>
<tr>
<th>BMI*factor</th>
<th>N cases / N referents</th>
<th>OR adjusted for age</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>BMI*tPA</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Below 27</td>
<td>Below median</td>
<td>10 / 122</td>
</tr>
<tr>
<td>Below 27</td>
<td>Above median</td>
<td>6 / 46</td>
</tr>
<tr>
<td>Above 27</td>
<td>Below median</td>
<td>11 / 24</td>
</tr>
<tr>
<td>Above 27</td>
<td>Above median</td>
<td>32 / 34</td>
</tr>
<tr>
<td><strong>Measures of biological interaction</strong></td>
<td></td>
<td>5.64 (-0.77-12.06)</td>
</tr>
<tr>
<td><strong>BMI*PAI-1</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Below 27</td>
<td>Below median</td>
<td>6 / 112</td>
</tr>
<tr>
<td>Below 27</td>
<td>Above median</td>
<td>9 / 56</td>
</tr>
<tr>
<td>Above 27</td>
<td>Below median</td>
<td>13 / 33</td>
</tr>
<tr>
<td><strong>Measures of biological interaction</strong></td>
<td></td>
<td>8.38 (1.92-14.83)</td>
</tr>
<tr>
<td><strong>BMI*complex</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Below 27</td>
<td>Below median</td>
<td>9 / 130</td>
</tr>
<tr>
<td>Below 27</td>
<td>Above median</td>
<td>7 / 38</td>
</tr>
<tr>
<td>Above 27</td>
<td>Below median</td>
<td>9 / 24</td>
</tr>
<tr>
<td>Above 27</td>
<td>Above median</td>
<td>34 / 34</td>
</tr>
<tr>
<td><strong>Measures of biological interaction</strong></td>
<td></td>
<td>9.34 (0.88-17.80)</td>
</tr>
</tbody>
</table>
**APPENDIX III.** Spearman correlation coefficients between PAI-1 antigen in 1990 or 1999 and other variables available in MONICA.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Survey year</th>
<th>PAI-1 antigen 1990</th>
<th>PAI-1 antigen 1999</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Men</td>
<td>Women</td>
</tr>
<tr>
<td><strong>Age</strong></td>
<td>1990</td>
<td>0.23*</td>
<td>0.17</td>
</tr>
<tr>
<td></td>
<td>1999</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Waist</strong></td>
<td>1990</td>
<td>0.55**</td>
<td>0.63**</td>
</tr>
<tr>
<td></td>
<td>1999</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Hip</strong></td>
<td>1990</td>
<td>0.34**</td>
<td>0.50**</td>
</tr>
<tr>
<td></td>
<td>1999</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Weight</strong></td>
<td>1990</td>
<td>0.34**</td>
<td>0.52**</td>
</tr>
<tr>
<td></td>
<td>1999</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>BMI</strong></td>
<td>1990</td>
<td>0.48**</td>
<td>0.55**</td>
</tr>
<tr>
<td></td>
<td>1999</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Systolic blood pressure</strong></td>
<td>1990</td>
<td>0.32**</td>
<td>0.07</td>
</tr>
<tr>
<td></td>
<td>1999</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Diastolic blood pressure</strong></td>
<td>1990</td>
<td>0.32**</td>
<td>0.11</td>
</tr>
<tr>
<td></td>
<td>1999</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>FPG</strong></td>
<td>1990</td>
<td>0.30**</td>
<td>0.35**</td>
</tr>
<tr>
<td></td>
<td>1999</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>2hPG</strong></td>
<td>1990</td>
<td>0.24**</td>
<td>0.30**</td>
</tr>
<tr>
<td></td>
<td>1999</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Chol</strong></td>
<td>1990</td>
<td>0.09</td>
<td>0.25*</td>
</tr>
<tr>
<td></td>
<td>1999</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Trig</strong></td>
<td>1990</td>
<td>0.45**</td>
<td>0.41**</td>
</tr>
<tr>
<td></td>
<td>1999</td>
<td></td>
<td></td>
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**Significant (2-tailed) at the 0.01 level, * Significant (2-tailed) at the 0.05 level.