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# **HEMOSTASIS AND MICROVASCULAR FUNCTION IN TYPE 1 DIABETES: EFFECTS OF TREATMENT WITH STATIN AND ASPIRIN**

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“I will help you to write your thesis, Mommy.  
Just tell me which keys to press on the computer.”

Tara, 3 years

# ABSTRACT

## Background

Patients with type 1 diabetes often develop microvascular complications and are at increased risk of premature cardiovascular disease. Women with type 1 diabetes lack the normal female protection against cardiovascular disease and may even be at higher risk compared with men with type 1 diabetes. Development of vascular complications may in part be explained by changes in hemostatic function in type 1 diabetes. Formation of a fibrin clot is the last step of the coagulation cascade and the structure of the formed clot reflects the environment in which it is formed. Tighter and less permeable fibrin clots are more resistant against degradation and are associated with cardiovascular risk factors and disease.

## Aims

The aims of this work were to investigate in vitro fibrin clot properties in adult patients with type 1 diabetes in relation to sex and microvascular complications (Paper I); treatment effects of high-dose atorvastatin on fibrin clot properties (Paper II) and skin microvascular reactivity (Paper III); and effects of aspirin on fibrin clot properties (Paper IV).

## Methods

The results are based on three studies: **Paper I**, a descriptive study involving 236 patients (107 females) with type 1 diabetes; **Papers II and III**, a randomized double-blind cross-over study in which 20 patients (10 females) with type 1 diabetes and dyslipidemia were treated daily with atorvastatin 80mg/day or placebo for two months; and **Paper IV**, a randomized cross-over study in which 24 patients (12 women) with good glycemic control and 24 patients (12 women) with poor glycemic control were treated with low (75 mg) and high (320 mg) doses of aspirin.

Fibrin clot properties were assessed by determination of the permeability coefficient (Ks) and by turbidimetric clotting and lysis assays. Thrombin generation was investigated by assessment of plasma levels of prothrombin fragment 1+2 and tissue factor-induced thrombin formation in vitro. Plasma fibrinogen concentrations were measured by means of the Clauss method. Circulating levels of platelet and endothelial microparticles were investigated by flow cytometry.

In Paper III, the effect of atorvastatin treatment on forearm skin microcirculation was investigated by way of laser Doppler perfusion imaging during iontophoresis of acetylcholine and sodium nitroprusside to assess endothelium-dependent and endothelium-independent microvascular reactivity. Various biochemical markers of endothelial function were also analyzed in this study.

## Results

**Paper I.** Fibrin clot properties in vitro did not differ between men and women with type 1 diabetes. Women had worse glycemic control and higher thrombin generation. In women, fibrinogen concentration was the only determinant of fibrin clot permeability, while age and glycemic control also influenced clot permeability in men. Females younger than 30 years had less permeable fibrin clots and prolonged lysis time compared with age-matched men. Tighter and less permeable fibrin clots were also found in patients with poor glycemic control and in patients with microvascular complications. Associations between fibrin clot properties and microvascular complications were independent of glycemic control.

**Paper II.** Treatment with high-dose atorvastatin (80 mg daily) was associated with increased fibrin clot permeability and reduced thrombin generation potential. In addition, reduced platelet microparticle concentrations and expression of prothrombotic antigens of platelet microparticles were found during atorvastatin therapy, indicating reduced platelet activation. These effects were independent of the lipid-lowering effects of atorvastatin.

**Paper III.** Impaired endothelial-dependent skin microvascular reactivity and glycemic control was observed during atorvastatin treatment, concomitantly with a tendency towards increased levels of circulating endothelial microparticles.

**Paper IV.** Treatment with aspirin at 75 mg daily had no effect on fibrin clot permeability, clot density or lysis time, while treatment with aspirin at 320 mg daily increased fibrin clot permeability and lag time in the turbidimetric clotting analyses. The beneficial effects of aspirin at 320 mg daily were more pronounced in patients with poor glycemic control.

## Conclusions

Men and women with type 1 diabetes and no history of macrovascular disease have similar fibrin clot properties in vitro. Microvascular complications in type 1 diabetes are associated with formation of more prothrombotic fibrin clots. High-dose (80 mg/day) atorvastatin treatment in patients with type 1 diabetes and dyslipidemia induces positive effects on hemostatic function, while the endothelial-dependent skin microvascular function and glycemic control were impaired. Treatment with high-dose (320 mg/day) aspirin affects fibrin polymerization and increases fibrin clot permeability, whereas treatment with low-dose (75 mg/day) aspirin has no effect on fibrin clot characteristics in patients with type 1 diabetes.

## LIST OF PUBLICATIONS

- I. Tehrani S, Jörneskog G, Lins P-E, Wallén HN, Antovic A. Fibrin clot properties and hemostatic function in men and women with type 1 diabetes. Manuscript.
- II. Tehrani S, Mobarrez F, Antovic A, Santesson P, Lins P-E, Adamson U, Henriksson P, Wallén H, Jörneskog G. Atorvastatin has antithrombotic effects in patients with type 1 diabetes and dyslipidemia. *Thromb Res*, 2010;126:e225-231
- III. Tehrani S, Mobarrez F, Lins P-E, Adamson U, Wallén H, Jörneskog G. Impaired endothelium-dependent skin microvascular function during high-dose atorvastatin treatment in patients with type 1 diabetes. *Diab Vasc Dis Res*, 2013;10:483-8.
- IV. Tehrani S, Antovic A, Mobarrez F, Mageed K, Lins P-E, Adamson U, Wallén H, Jörneskog G. A high dose of aspirin is required for positive influence on plasma fibrin network in patients with type 1 diabetes. *Diabetes Care*, 2012;35:404-408.

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## LIST OF ABBREVIATIONS

ACh	Acetylcholine
AU	Arbitrary units
AGEs	Advanced glycation end-products
ANOVA	Analyses of variance
BMI	Body mass index
CAT	Calibrated automated thrombogram
CI <sub>s</sub>	Confidence intervals
CV	Coefficient of variation
CVD	Cardiovascular disease
EMPs	Endothelial microparticles
ETP	Endogenous thrombin potential
F1+2	Prothrombin fragment 1+2
GFR	Glomerular filtration rate
HbA <sub>1C</sub>	Glycated hemoglobin
HDL	High-density lipoprotein
ICAM	Intercellular adhesion molecule
K <sub>s</sub>	Fibrin clot permeability coefficient
LDL	Low-density lipoprotein
MESF	Molecules of Equivalent Soluble Fluorochrome
MPs	Microparticles
PAI-1	Plasminogen activator inhibitor 1
PMPs	Platelet microparticles
SNP	Sodium nitroprusside
TAFI	Thrombin-activatable fibrinolysis inhibitor
TAT	Thrombin-antithrombin complex
TF	Tissue factor
TFPI	Tissue factor pathway inhibitor
tPA	Tissue plasminogen activator
VCAM	Vascular cell adhesion molecule
vWF	Von Willebrand Factor



# 1 INTRODUCTION

Vascular complications are common in patients with type 1 diabetes and may cause gradual loss of organ function. Microangiopathy affecting the retina, kidneys and nerves are typical manifestations of type 1 diabetes and may already develop within the first years after onset of disease. Disturbances in microvascular function are characterized by a paradoxical increase in microvascular flow and capillary pressure, which causes basement membrane thickening in the affected organs [Tooke 1996]. This sclerotic process limits the vasodilatory reserve and autoregulatory capacity of the microvasculature with increasing disease duration.

Retinopathy develops to some degree in most patients with type 1 diabetes and can be partially reversible if glycemic control is improved. Diabetic retinopathy is well-characterized and manifests as vascular microaneurysms and blot hemorrhages in the early stages, and findings of hard exudates, macular edema, neovascularization, vitreous hemorrhages and retinal detachment in the latter stages.

Diabetic nephropathy, also known as Kimmelstiel-Wilson syndrome, is characterized by diffuse glomerulosclerosis and is detected through leakage of proteins into the urine. The earliest clinical sign is increased glomerular filtration rate (GFR), while persistent microalbuminuria (defined as a urinary albumin excretion rate between 20-300  $\mu\text{g}/\text{min}$ ) is considered as incipient nephropathy. As the kidney damage progresses, it manifests as macroalbuminuria (urinary albumin excretion rate  $>300 \mu\text{g}/\text{min}$ ) and progressive kidney failure. Patients with albuminuria are at higher risk of cardiovascular disease (CVD) [Nathan 2005].

Neuropathy, a common diabetes complication, is not a distinct entity but consists of different clinical syndromes caused by disturbances in the peripheral and/or central nervous system. Sensory neuropathy in the feet causes numbness, dysesthesia and/or pain, and contributes to the development of chronic foot ulcers. Impaired capillary circulation and reduced oxygen supply to the nerves via the vasa nervorum is considered a contributing factor behind peripheral diabetes neuropathy [Fagerberg 1959]. Autonomic neuropathies, including foot deformities, gastroparesis, orthostatic hypotension, and erectile and urinary dysfunction, are also common complications in type 1 diabetes.

Autonomic neuropathy in the skin impairs the sympathetic regulation of thermoregulating arteriovenous shunts. Denervated shunts lose their normal contraction and stay open, which causes the blood to surpass the nutritional capillaries [Jörneskog 1995]. Increased microvascular arteriovenous shunting transforms the arterial blood pressure to the venous side, which contributes to impaired capillary circulation. Thus, while total skin microcirculation is normal, or even augmented, capillary circulation and tissue perfusion is markedly reduced. Other factors contributing to the chronic capillary ischemia in skin microcirculation and other vascular beds may be altered hemorheology caused by elevated fibrinogen

concentrations, and imbalance between endogenous vasodilators (e.g. nitric oxide) and vasoconstrictors (e.g. endothelin-1) at the precapillary level.

In addition to microvascular disturbances, type 1 diabetes is associated with accelerated progression of atherosclerosis and these patients are at increased risk of peripheral artery disease and premature cardiovascular morbidity and mortality [Laing 2003, Soedamah-Muthu 2006]. Coronary heart disease is the most common cause of death in type 1 diabetes, and the first cardiovascular event may occur before the age of 40 years. Epidemiological studies reveal the same event rates in both sexes, indicating loss of female protection against CVD. In fact, several studies have shown higher risks of cardiovascular morbidity and mortality among women compared with men with type 1 diabetes [Juutilainen 2008, Pensina 2009, Anand 2008]. The reason behind increased risk of CVD in women with type 1 diabetes is not fully known.

The DCCT/EDIC trial was the first study to demonstrate the importance of good glycemic control in prevention of micro- and macrovascular complications in type 1 diabetes [DCCT 1993, Nathan 2005]. Chronic hyperglycemia causes increased polyol pathway flux, i.e. accumulation of sorbitol within the cells as a result of elevated glucose levels. Sorbitol accumulation impairs important cellular functions. Hyperglycemia is also associated with increased formation of advanced glycation end-products (AGEs), which are pro-oxidant metabolic derivatives formed non-enzymatically by glycation of proteins, lipids or nucleic acids. AGEs interact with cell surface receptors and alter the function of intracellular and extracellular proteins. Accumulation of AGEs in vessel walls contributes to micro- and macrovascular complications in patients with diabetes [Huebschmann 2006]. Increased activation of protein kinase C is yet another mechanism contributing to the development of vascular complications in patients with diabetes. Protein kinase C activation is associated with changes in blood flow, basement membrane thickening, extracellular matrix expansion, increased vascular permeability, angiogenesis, cell growth and enzymatic activity alterations [Das Evcimen 2007].

In addition to the metabolic and hemodynamic changes, altered hemostatic functions are found with patients with type 1 diabetes and vascular complications [Targher 2011]. Importantly, some coagulation factors seem to predict the development and progression of both micro- and macrovascular complications in type 1 diabetes [Targher 2011], although the underlying mechanisms are unclear. The effect of type 1 diabetes on platelet activity, coagulation factors and fibrinolysis regulators has been investigated in a limited number of studies, sometimes with conflicting results. Fibrin clot properties in patients with type 1 diabetes have been investigated in only a few small studies [Jörneskog 1996, Hess 2012]. In the present work, fibrin clot properties and hemostatic function were studied in a larger group of patients with type 1 diabetes.

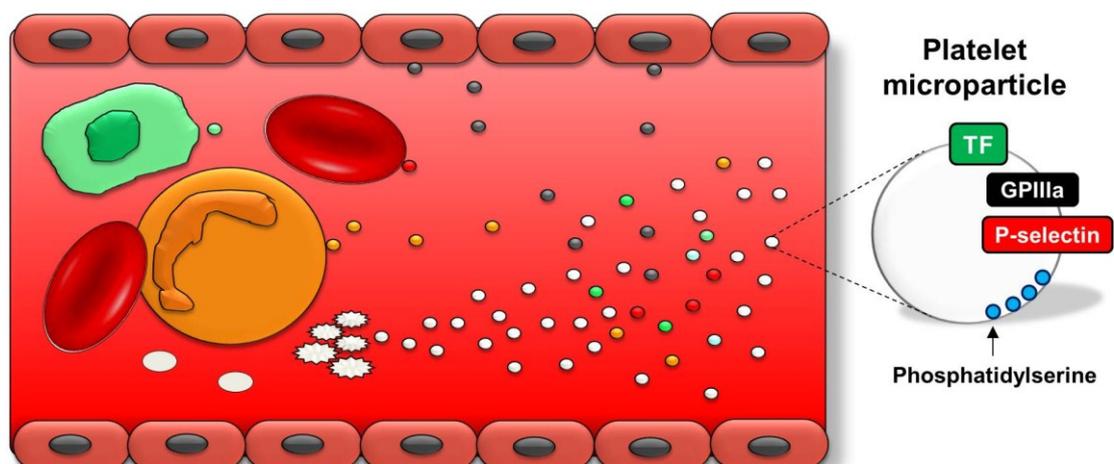
## 1.1 HEMOSTATIC FUNCTION IN TYPE 1 DIABETES

### 1.1.1 Platelet function

Platelets are small disk-shaped cell fragments in the circulation that play a key role in blood hemostasis. Upon activation, platelets change in shape, become more spherical and form pseudopods on their surface. Activated platelets also have highly increased expression of various receptors on their surface that enables aggregation and adherence to subendothelial collagen following vessel injury. The most abundant aggregation receptor, glycoprotein IIb/IIIa, is the receptor for fibrinogen and von Willebrand Factor (vWF), which are two proteins involved in formation of a platelet plug and fibrin clot at a site of vessel injury. Activated platelets expose and secrete prothrombotic proteins such as P-selectin, vWF and coagulation factors V and XIII from their  $\alpha$ -granules. They synthesize and secrete the vasoconstrictive eicosanoid thromboxane A<sub>2</sub>, which stimulates the activation of new platelets. The platelets also undergo changes that allow them to serve as a surface for assembly and activation of coagulation factors. One such change is increased expression of the anionic phospholipid phosphatidylserine on outer leaflet of the platelet membrane. Phosphatidylserine binds calcium-binding coagulations factors to the platelet surface as a result of its negative charge.

In addition to the above, activated platelets shed small vesicular buds (0.1-1  $\mu\text{m}$  in size) from their membranes into the circulation, as illustrated in Figure 1. These platelet microparticles (PMPs), as well as circulating MPs derived from erythrocytes, leucocytes and endothelial cells, transfer bioactive molecules between cells and are important mediators in inflammation, hemostasis and atherothrombosis. Being membrane-derived, MPs express the same antigens on their surface as their parental cells. PMPs are the most abundant MPs in the circulation, and usually act as procoagulants due to exposure of tissue factor (TF) and phosphatidylserine in a similar fashion as activated platelets [De Caterina 2013].

Figure 1. Circulating microparticles shed from activated cells. These membrane-derived vesicles express the same antigens as their parent cells and are often procoagulant.

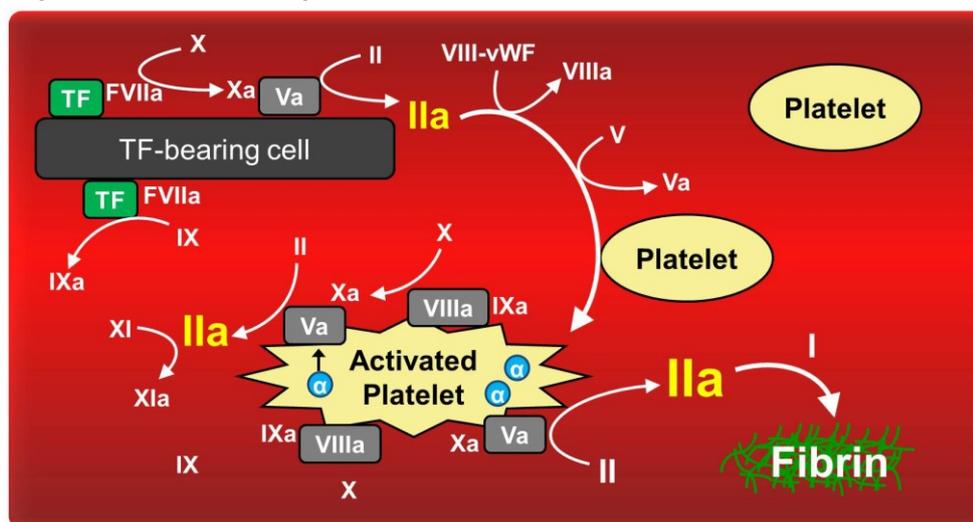


Patients with type 1 diabetes have increased platelet activity, demonstrated through an elevated platelet count, increased platelet aggregation and higher circulating levels of P-selectin and thromboxane A2 [El khawand 1993, Yngen 2004, Davi 2003, Hu 2004]. Increased platelet activity is found even in young patients with type 1 diabetes without any clinical sign of vascular complications [El khawand 1993, Davi 2003, Hu 2004]. In addition, patients with type 1 diabetes have higher levels of circulating PMPs and phosphatidylserine-expressing MPs compared with age-matched healthy controls, indicating increased platelet activation and MP-procoagulant activity [Sabatier 2002].

### 1.1.2 Coagulation factors

Tissue factor (TF), or FIII, is considered to be the principal initiator of coagulation. It is an integral membrane protein expressed on various extravascular cells under normal conditions and it is structurally unrelated to the other coagulation factors. Following vessel injury, extravascular TF comes into contact with plasma and binds with high affinity to FVII, forming the TF-FVIIa complex. Circulating monocytes, endothelial cells and MPs can also express TF during inflammation and cellular activation. The TF-FVIIa complex catalyzes the conversion of inactive protease FX into its active form, FXa. Importantly, these processes occur at the surface of various cells in vivo, as described in the cell-based model of hemostasis (illustrated in Figure 2) [Hoffman 2001]. If FXa leaves the protected environment of the cell surface, it is rapidly inactivated by circulating TF pathway inhibitor (TFPI), secreted by endothelial cells. However, the FXa that remains on the cell surface combines with membrane-bound FVa to form the prothrombinase complex, which converts prothrombin into the active protease thrombin (FIIa). This action is caused by cleavage of prothrombin and release of prothrombin fragment 1+2 (F1+2), a peptide that can be quantified in plasma for assessment of thrombin formation in vivo. Although FXa can by itself catalyze the activation of prothrombin, the rate at which this reaction occurs is increased about 300 000-fold with formation of the prothrombinase complex.

Figure 2. Cell-based coagulation model.



The small amounts of thrombin generated on the surface of TF-bearing cells amplify the initial procoagulant signal by activating platelets, enhancing platelet adhesion to the site of injury, and by releasing FVIII from vWF in the circulation. The activated platelets release FV and FVa from their  $\alpha$ -granules. These reactions represent a positive feed-back loop, as FVa and FVIIIa serve as co-factors for the large-scale burst of thrombin generation, which is needed for conversion of fibrinogen to fibrin and formation of a fibrin clot. After thrombin-induced dissociation from vWF, circulating FVIIIa is rapidly inactivated by activated protein C. FVa is also inactivated by activated protein C and cleared from the circulation. Thrombin is inactivated by antithrombin, a glycoprotein synthesized in the liver that circulates in blood and binds to thrombin, forming the stable thrombin-antithrombin complex (TAT). Antithrombin also has the ability to inactivate the coagulation factors VIIa, IXa, Xa, XIa and XIIa. Thus, TFPI, protein C and antithrombin are the main inhibitors of coagulation.

Circulating levels of coagulation factors may differ between men and women. In a study on a healthy population, women had higher levels of prothrombin and FVII but lower levels of FV and TFPI compared with men [Brummel-Ziedins 2005]. Furthermore, in vitro investigation of TF-induced thrombin generation showed that women achieved higher thrombin levels at a faster rate, while total thrombin generated over time was similar in both sexes [Brummel-Ziedins 2005]. In the study, increasing age and BMI as well as the use of oral contraceptives were associated with increased thrombin generation. Interestingly, the influence of the individual coagulation factors on peak thrombin generation was less than 9% [Brummel-Ziedins 2005].

It seems that fairly well-controlled patients with type 1 diabetes have no, or less pronounced, alterations in coagulation pathways, whereas patients with poor glycemic control and/or vascular complications have increased coagulation activity [Carmassi 1992, Guisty 2000, Knöbl 1993, Ibbotson 1993, Lee 1993]. Higher circulating FVII levels have been found in patients with poor glycemic control compared with patients with good glycemic control and healthy controls [Carmassi 1992]. Elevated levels of FVII antigen, FVII coagulant activity and prothrombin fragment 1+2 have been shown in patients with proliferative retinopathy, but not in patients with absent/moderate levels of retinopathy, despite mean duration of diabetes of 18 years [Guisty 2000]. Plasma levels of FVII and antithrombin and coagulant activities of FVII and FVIII have been found to be progressively increased with increased albumin excretion rates, while levels in normoalbuminuric patients were similar to those in healthy controls [Knöbl 1993, Ibbotson 1993, Lee 1993]. Moreover, glycation of antithrombin is related to glycemic control in patients with diabetes [Ducrocq 1985], although the clinical relevance of increased antithrombin glycation is unclear, as activity levels of antithrombin in diabetes patients with poor glycemic control have been reported to be similar to those in healthy controls [Altunbas 1998].

### 1.1.3 Fibrinogen

Fibrinogen (Factor I) is one of the most abundant coagulation proteins present in plasma at a concentration of about 2.5 g/L (2-4 g/L) under normal conditions. This 340-kDa glycoprotein is made up of three pairs of polypeptide chains, A $\alpha$ , B $\beta$  and  $\gamma$ . Fibrinogen is mainly synthesized in the liver and has a biological half-life of about 100 hours in plasma. As an acute phase reactant, its concentration in blood increases rapidly in response to cytokine release from activated immune cells following an infection or tissue injury. Fibrinogen is a determinant of blood viscosity and plays also an important role in inflammation, atherogenesis and thrombogenesis through its interactions with platelets, leucocytes, monocytes and endothelial cells. In addition, fibrinogen is the precursor of fibrin and highly essential for blood coagulation. More than one million different variants of the fibrinogen molecule are estimated to be present in the circulation. Fibrinogen heterogeneity is in part caused by alternative mRNA splicing and posttranslational modifications, and this may affect the function of the protein. Importantly, elevated fibrinogen levels are an independent risk factor of CVD [Fibrinogen Studies Collaboration 2005].

Various factors have been identified as determinants of plasma fibrinogen concentrations in healthy individuals [Kamath 2003]: genetic polymorphisms may account for up to 50% of variations in fibrinogen concentrations; women have higher fibrinogen levels than men at all ages regardless of pregnancy or hormonal changes; fibrinogen levels generally increase with age and are positively related to body mass index (BMI); smoking increases fibrinogen concentration while regular physical activity decreases it. Thus, healthy women have a lower risk of CVD compared with men despite increased thrombin generation and higher fibrinogen concentrations.

Various methods are available to measure fibrinogen concentrations in plasma. The most widely used functional assay in most clinical laboratories is the Clauss method [Mackie 2003]. Elevated fibrinogen levels have been reported in patients with type 1 diabetes with and without vascular complications [Ganda 1992, Ceriello 1994, Carmassi 1992, Knöbl 1993]. Patients with pronounced microvascular complications, i.e. macroalbuminuria and proliferative retinopathy, have higher fibrinogen levels compared with patients without these complications [Jensen 1988, Knöbl 1993, Sjølie 1997]. Microalbuminuria in the large-scale EURODIAB study (n=2091) was, however, not associated with changes in fibrinogen concentrations [Greaves 1997]. Investigators have also reported similar fibrinogen levels in patients with type 1 diabetes compared with healthy controls despite disturbances in other parameters of hemostatic function in the diabetic group [El Khawand 1993, Jörneskog 1995]. These data suggest that changes in fibrinogen concentrations are not an early sign of hemostatic dysfunction, but may reflect a more advanced stage of hemostatic disturbance in type 1 diabetes.

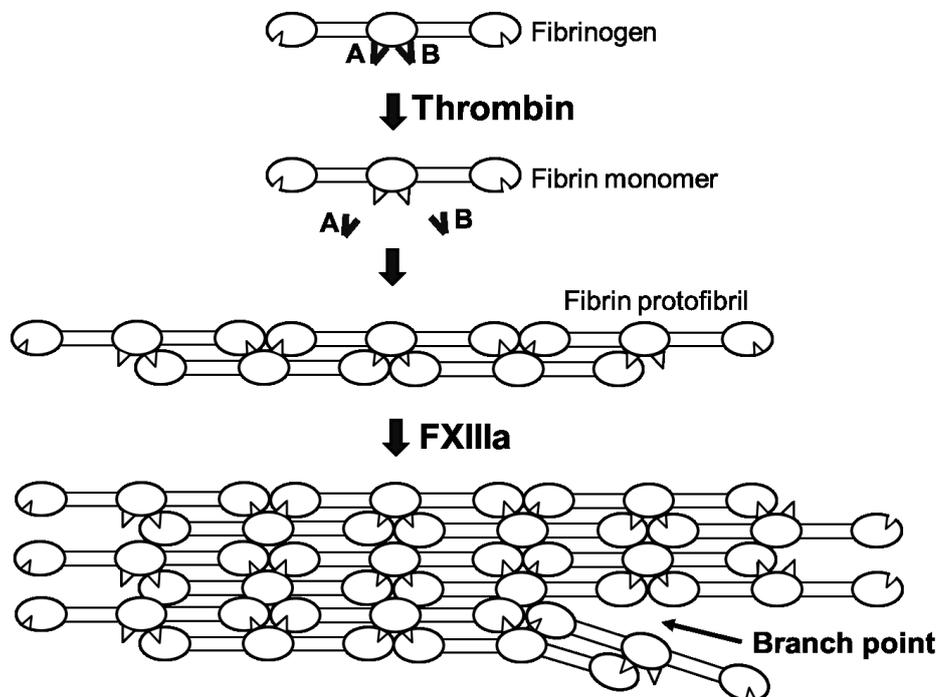
Although fibrinogen concentrations may be within the normal range in patients with type 1 diabetes, protein function can still be affected as a result of increased fibrinogen glycation in patients with diabetes [Lütjens 1985, Ardawi 1990]. Mass

spectrometry studies of fibrinogen incubated with glucose at physiological concentrations have recently revealed glycated lysines at two sites in the fibrinogen molecule [Svensson 2012]. Increased fibrinogen glycation may affect fibrin clot formation and degradation as discussed below.

#### 1.1.4 The fibrin clot

During blood coagulation, thrombin cleaves and releases two short peptides (fibrinopeptides A and B) from the central region of the fibrinogen molecule, and fibrin monomers are formed. The nomenclature for fibrinogen, ( $A\alpha$ ,  $B\beta$  and  $\gamma$ )<sub>2</sub>, arises from the localization of fibrinopeptides A and B at their parent chains ( $\alpha$  and  $\beta$ ). No peptides are cleaved from the  $\gamma$  chains by thrombin. Although fibrinopeptides A and B are small, their release has profound effects on the fibrinogen molecule as hidden binding sites, so called knobs, are exposed on the  $\alpha$  and  $\beta$  chains (see Figure 3). These knobs pocket into holes that are already exposed in the  $\alpha$  and  $\beta$  chains, allowing the fibrin monomers to interact with each other and to form insoluble oligomers that elongate into fibrin polymers, also called protofibrils when reaching a certain length. The protofibrils aggregate laterally into fibers, which then branch and in the presence of thrombin-activated FXIII form a three-dimensional cross-linked network, the fibrin clot (Figure 4). This cross-linking involves both  $\gamma$ - and  $\alpha$ -chains.

Figure 3. Schematic diagram of fibrin polymerization.

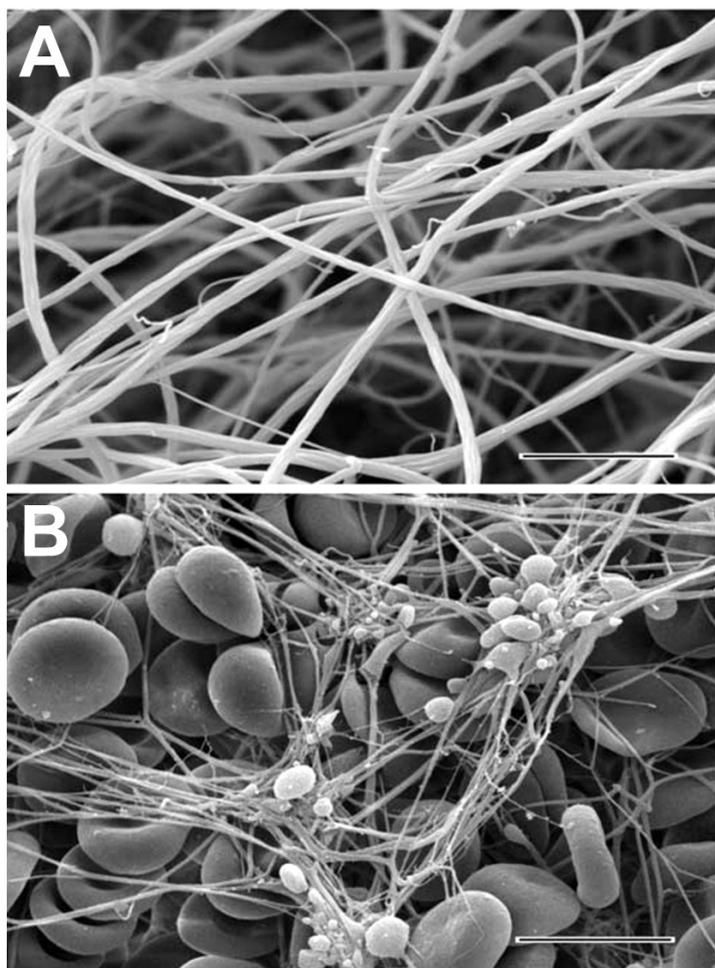


Thrombin cleaves fibrinopeptide A (primary) and B from fibrinogen, producing fibrin monomers, which aggregate via knob-hole interactions to make oligomers. The oligomers elongate to yield protofibrils, which aggregate laterally to make fibers. Three-dimensional cross-linked network is formed in the presence FXIIIa. At the bottom of the diagram, a branch point has been initiated by the divergence of two protofibrils.

During fibrin polymerization, lateral aggregation contributes to fiber thickness, while the number of branch points relates to the pore size in the forming clots. There is in general a balance between these two processes and as fiber diameter increases, the number of branch points decreases. Thus, clots made up of thicker fibers usually have fewer branch points and larger pores, while clots made up of thinner and highly-branched fibers have small pores [Weisel 2013].

Once the branching fibers form a space-filling, three-dimensional network structure, a clot exists. Clotting time or gel point, which is the commonly used term in chemistry indicating the appearance of a polymer network, occurs relatively early during this process, perhaps when only about 10% of the fibrinogen has been incorporated into the clot [Weisel 2007]. Thus, new fibers and branch points are still established after the gel point has been reached. The final fibrin clot structure can be characterized by assessment of fiber thickness, density, pore size and elasticity.

Figure 4. Scanning electron microscope images of fibrin clots.



A) Electron micrograph of clot formed by addition of thrombin to purified fibrinogen. Magnification bar, 5  $\mu\text{m}$ .

B) Electron micrograph of whole blood clot, made from freshly drawn blood with no additions. Aggregated platelets, erythrocytes and leukocytes are found in the meshwork. Magnification bar, 10  $\mu\text{m}$ .

Reprinted from *Advances in Protein Chemistry*, Volume 70, John W. Weisel, Fibrinogen and Fibrin, page 268, Copyright (2005), with permission from Elsevier.

Fibrinogen concentrations and hereditary or acquired variations in fibrinogen molecule are important for fibrin clot structure [Blombäck 1994, Weisel 2013]. Variations of fibrinogen structure due to genetic polymorphisms, splice variations, protein acetylation by aspirin and increased protein glycation in diabetes patients affect fibrin clot structure and function [Ariens 2013]. FXIIIa can incorporate various proteins into the fibrin chains. Incorporation of  $\alpha$ 2-antiplasmin reduces the susceptibility of clot degradation by plasmin inhibition [Fraser 2011], and incorporated vWF anchors platelets to the forming clot [Hada 1986]. Other factors influencing fibrin clot architecture are thrombin activation, environmental conditions in plasma such as pH and ionic strength, interactions with various cells and/or MPs, and the hydrodynamics of blood flow at the site of injury [Weisel 2013].

Fibrin clot architecture regulates the distribution of lytic enzymes and thereby the clot degradation rate [Lord 2011]. Tighter and more compact clots are lysed more slowly than clots with a loose structure [Gabriel 1992, Collet 2000]. It is thus the overall fibrin clot structure and density, rather than the thickness of individual fibers, that influences the lysis rate. Hence, all the processes that determine clot structure described above also modulate fibrinolysis [Collet 2003, Weisel 2013].

Investigation of fibrin clot properties in vitro has clinical implications, as tighter and more compact clots are formed in individuals with manifest CVD or conditions associated with an increased risk of atherothrombotic complications compared with those in healthy controls [Fatah 1996, Collet 2006, Rooth 2011, Collet 1999]. Assessment of fibrin clot permeability through percolation of a fluid through a fully hydrated clot characterizes average pore size and is a physiologically relevant measurement, as it indicates the accessibility of lytic enzymes to the clot [Lord, 2011]. This method was described by Carr et al. in 1977 and Blombäck and Okada in 1982 [Carr 1977, Blombäck 1982], and is based on Darcy's law, which is represented by a constitutive equation used to assess the flow of a fluid through a porous medium. It was described by Henry Darcy in 1856 [Darcy 1856]. Most laboratories currently measuring the fibrin clot permeability coefficient (Ks), also called Darcy's constant, use a variation of this method in which thrombin and calcium at certain concentrations are used to initiate fibrin polymerization. In previous studies, the expression "fibrin gel structure" was often used. Over time, the term "fibrin network structure" was applied instead, and this nomenclature was used in Papers II-IV of this thesis. However, since most researchers today use the term "fibrin clot structure", this nomenclature has been applied to the rest of this work, including Paper I.

Patients with type 1 diabetes form tighter and less permeable fibrin clots in vitro compared with healthy individuals [Jörneskog 1996]. This seems to be in part a consequence of hyperglycemia *per se*, although the underlying mechanisms are unknown. An early investigation by Nair et al. showed that addition of glucose to normal plasma resulted in tighter and less permeable fibrin clots [Nair 1991]. Similarly, studies on purified fibrinogen have shown that addition of glucose at levels above the equivalent of normoglycemic values in vivo are associated with denser

fibrin clots [Dunn 2005]. It can be postulated that altered fibrin clot structure in diabetes patients is partly due to increased fibrinogen glycation, as purified fibrinogen from patients with type 2 diabetes is also associated with formation of denser and less permeable fibrin clots in vitro [Dunn 2005].

It should be noted that in the study by Dunn et al. cited above, glucose levels *below* the equivalent of normoglycemic values in vivo were also associated with less permeable fibrin clots [Dunn 2005], indicating that hypoglycemia also has a negative impact on hemostatic function. Indeed, induced hypoglycemia in both healthy individuals and patients with type 1 diabetes has been associated with platelet activation and increases in fibrinogen concentration, inflammatory markers and vascular adhesion molecules [Dalsgaard-Nielsen 1982, Gogitidze, 2010, Hanefeld 2013]. Furthermore, hypoglycemia activates the sympathoadrenal system and release of glucagon, cortisol and catecholamines [Trovati 1986]. Although catecholamine release is a physiological protective mechanism, its cardiovascular effects may be hazardous, especially in patients with vascular disease. Increased hypoglycemic episodes during intensive insulin treatment in various trials have been associated with increased risks of cardiovascular events and mortality [Hanefeld 2013].

### **1.1.5 Fibrinolysis**

Fibrinolysis is a complex system that prevents fibrin accumulation in vessels. As opposed to the coagulation cascade, which is designed to rapidly initiate and magnify thrombin generation and lead to the formation of a stable fibrin clot, the fibrinolytic system is slow and designed to resolve the clot within hours-days after its formation. This arrangement prevents blood loss during vessel injury in an acute manner, while allowing the damaged vessel time to heal before removing the clot. The slow nature of fibrinolysis is thus important to prevent further blood loss until vascular patency has been restored. Enhanced fibrinolysis is associated with bleeding disorders, whereas impaired fibrinolysis and delayed clot resorption prolong turbulent blood flow and stress the vascular wall.

The key components of the fibrinolytic system are plasmin, which is the protease that cleaves fibrin, tissue plasminogen activator (tPA), which promotes fibrinolysis, and its specific inhibitor, plasminogen activator inhibitor-1 (PAI-1). Plasmin is synthesized in an inactive form, plasminogen, in the liver. Plasminogen binds to lysine residues on fibrin and is incorporated within the clot. Damaged or activated endothelial cells increase their release of tPA into the circulation, which activates the fibrin-bound plasminogen. This conversion occurs efficiently only on the fibrin surface and free circulating plasmin is rapidly inactivated by  $\alpha_2$ -antiplasmin. The fibrin-bound plasmin is thus partially protected from inactivation and can stimulate further tPA release from the surrounding endothelial cells. Plasmin degrades the fibrin clot by cleaving specific peptide bonds and releasing soluble fragments, called fibrin degradation products, into the circulation. One such degradation product is D-dimer, which is clinically used in investigation for venous thromboembolism.

The most important fibrinolysis inhibitor is PAI-1, a serine protease produced by vascular endothelial cells, hepatocytes, adipocytes and activated thrombocytes. PAI-1 is an acute phase reactant and its plasma concentrations increase rapidly in response to infections or tissue injury. PAI-1 forms a stable complex with tPA and thereby blocks tPA-dependent plasmin generation. The interaction between PAI-1 and tPA is very rapid and has a reaction rate of  $10^7/M$  per second in healthy individuals [Wiman 1984]. Under basal conditions, tPA seems to be the limiting factor for this complex formation, and approximately 40% of tPA is linked to PAI-1 [Alessi 1990]. In addition to PAI-1, tPA also binds to other inhibitors such as  $\alpha_2$ -antiplasmin and complement 1 esterase inhibitor, although these reactions occur at much slower rates [Chmielewska 1983]. Just like fibrin-bound plasmin, which is protected from inactivation by  $\alpha_2$ -antiplasmin, tPA that binds to fibrin and plasminogen forms a ternary complex that protects it from inactivation by PAI-1 and other inhibitors.

Another important fibrinolysis inhibitor is  $\alpha_2$ -antiplasmin, which is synthesized in the liver and inactivates both circulating plasmin and fibrin-bound plasmin. Alpha2-antiplasmin can be incorporated within the clot by FXIIIa, where it increases the resistance against degradation. Thrombin-activatable fibrinolysis inhibitor (TAFI) is a glycoprotein also synthesized in the liver that binds to plasminogen in an inactive form. Upon activation by thrombin, TAFI removes lysine residues from fibrin and thereby prevents plasminogen binding at the fibrin surface. Thrombin-induced TAFI activation is relatively inefficient in the absence of thrombomodulin, an integral membrane protein mainly found on vascular endothelial cell membranes. When bound to thrombomodulin, thrombin greatly increases the rate of TAFI activation and also activates liver-synthesized protein C. Activated protein C together with its cofactor protein S, synthesized by endothelial cells, inactivate FVa and FVIIIa and thereby prevent thrombin formation. Thus, thrombomodulin both downregulates fibrinolysis by stimulating thrombin-induced activation of TAFI, and upregulates fibrinolysis by activating protein C, which leads to inhibited thrombin formation and thereby reduced TAFI activation. It has been suggested that activation of TAFI and protein C occur simultaneously, but the outcome of the overall effect is determined by the thrombomodulin concentration. Low thrombin-thrombomodulin concentrations stimulate TAFI activation, while high thrombin-thrombomodulin concentrations activate protein C, and TAFI activation is then reduced through inhibited thrombin generation [Monsier 2001].

Fibrinolysis activity can be determined in vivo through immunological and functional assays measuring antigen concentrations or activity levels of various fibrinolysis regulators in plasma, or by using global assays in vitro. Reduced fibrinolytic activity increases the risk of both atherothrombotic events and venous thrombosis [Dawson 1992]. Antigen and/or activity levels of tPA and PAI-1 are commonly measured in clinical studies. PAI-1 levels are elevated in conditions associated with chronic inflammatory states such as abdominal obesity, insulin resistance and dyslipidemia. Circulating levels of tPA-PAI-1 complex correlate well with PAI-1 activity levels and are also associated with BMI and triglyceride concentrations [Nordenhem 2005].

Elevated PAI-1 levels are common in type 2 diabetes, which is characterized by insulin resistance, while both increased and decreased PAI-1 levels have been reported in patients with type 1 diabetes [Sibal 2009, Vicari 1992, Huves 1999]. In a prospective study in patients with type 1 diabetes, PAI-1 and tPA-PAI-1 complex levels were not associated with CVD incidence, whereas elevated baseline levels of tPA-PAI-1 complex were associated in development of nephropathy during the study period [Bosnyak 2003]. Plasma tPA antigen levels mainly reflect tPA bound to various inhibitors, since only a small fraction of tPA is in a free and active form. Hence, tPA antigen is a measure of fibrinolysis inhibition rather than tPA activity. Elevated antigen levels of tPA, PAI-1 and tPA-PAI-1 complex are all associated with an increased risk of CVD in men and women [Thøgersen 1998, Wiman 2000]. In a healthy population, levels of tPA antigen and tPA-PAI-1 complex in men were found higher and increased with age until their 40s, while women had constantly low levels up to their 50s [Takada 1989]. PAI-1 activity levels were also lower in women than in men up to 50 years of age. After the age of 50s, levels of tPA antigen, PAI-1 activity and tPA-PAI-1 complex raised in women with increasing age while remaining unchanged in men. At the age of 60, levels of tPA antigen, PAI-1 activity and tPA-PAI-1 complex were similar in men and women. The enhanced fibrinolytic activity in healthy women menopause may in part explain their lower risk of CVD, despite their increased thrombin generation and higher fibrinogen concentrations.

Clot lysis time can be assessed during in vitro analyses of fibrin clot properties through addition of tPA together with thrombin and calcium to induce clot formation. Patients with type 1 diabetes have increased clot lysis times compared with age- and sex-matched controls [Ajjan 2013]. Increased fibrinogen glycation in diabetes affects the fibrin clot structure, and is associated with impaired fibrinolysis [Brownlee 1986, Dunn 2006]. Interestingly, it has been shown that glycation of the fibrinogen molecule occurs in the plasmin-binding region of the protein [Svensson 2012], and thus increased fibrinogen glycation may hypothetically impair fibrinolysis through interference with plasmin-fibrin interaction. In addition, reduced conversion of plasminogen to plasmin and impaired plasmin proteolytic activity has also been shown in patients with type 1 diabetes [Ajjan 2013]. Improved glycemic control in the study was associated with increased plasmin proteolytic activity [Ajjan 2013].

Interestingly, a study with a rat model of pulmonary embolism demonstrated that PAI-1 is incorporated within the fibrin clot [Reilly 1991]. Clot-bound PAI-1 inactivated tPA within the thrombus, making the clot more resistant to degradation in a similar fashion as  $\alpha_2$ -antiplasmin [Reilly 1991]. Increased PAI-1 incorporation in fibrin clots of patients with type 1 diabetes is a possible mechanism behind increased clot lysis time. However, no studies have yet been carried out to investigate incorporation of PAI-1 in fibrin clots of healthy individuals or patients with diabetes.

Table 1. Factors and regulators in coagulation and fibrinolysis processes in type 1 diabetes.

	<b>Actions</b>	<b>Plasma levels in type 1 diabetes</b>	<b>References</b>
Platelet	Procoagulant in active state	↑ activity	Yngen 2004
vWF	Binds to FVIII, mediates platelet adhesion	↑, especially in pat with microangiopathy	Blann 2004 Knöbl 1993
FI (fibrinogen)	Acute phase reactant Precursor of fibrin	↑ and normal levels Increased glycation	El Khawand 1993, Ganda 1992, Lütjens 1985
FII (prothrombin)	Its active form (FIIa, thrombin) activates platelets, FI, FV, FVII, FVIII, FXI, FXIII, TAFI, protein C	↑, especially in pat with proliferative retinopathy or microalbuminuria	Ceriello 1992 Guisty 2000 Gruden 1993
FIII (tissue factor)	Co-factor for FVIIa	↑ coagulant activity	Singh 2012
FIV (Calcium)	Required for coagulations factors to bind to phospholipid		
FV	Co-factor for FX with which it forms the prothrombinase complex		
FVI	Old name for FVa		
FVII	Binds to TF (FIII), activates FIX and FX	↑ in pat with poor glycemic control or prolif. retinopathy or in pat with increased AER	Carmassi 1992 Guisty 2000 Knöbl 1993
FVIII	Forms the tenase complex with FIX, activates FX	↑ in pat with increased AER	Ibbotson 1993
FIX	Forms the tenase complex with FVIII, activates FX		
FX	Activates FII (prothrombin), forms prothrombinase complex with FV	↓ levels	Ceriello 1990
FXI	Activates FIX		
FXII	Activates FVII, FIX		
FXIII	Cross-links fibrin, incorporates proteins into the clot		
Antithrombin	Inhibits FIIa, FVIIa, FIXa, FXa, FXIa and FXIIa	↓ levels have been reported. ↑ in pat with increased AER Increased glycation	Ceriello 1990 Lee 1993 Ducrocq 1985
Protein C	Inactivates FVa and FVIIIa	↑ in pat with increased AER	Knöbl 1993 Lee 1993
Protein S	Co-factor of protein C	↑ levels in pat with increased AER	Knöbl 1993 Lee 1993
Thrombomodulin	Co-factor of FIIa, with which it activates TAFI and protein C	↑ in pat with increased AER and in microangiopathy	Blann 2004
Plasminogen	Converts to plasmin, lyses fibrin	Increased glycation	Ajjan 2013
Alpha2-antiplasmin	Inhibits plasmin		
tPA	Activates plasminogen	Normal levels	Vicari 1992
PAI-1	Inactivates tPA	↑ and ↓ levels	Sibal 2009, Huvers 1999

AER, albumin excretion rate.

## **1.2 ENDOTHELIAL FUNCTION IN TYPE 1 DIABETES**

Endothelial cells line the inner layer of all blood vessels, forming a barrier between the vessel lumen and surrounding tissue. The endothelium is highly involved in various aspects of vascular biology, such as regulation of vessel tone, vessel permeability, inflammation, angiogenesis and blood hemostasis. These important functions are regulated through secretion and/or expression of vasoactive substances inducing vasodilatation (e.g. nitric oxide) or vasoconstriction (endothelin-1, angiotensin II and thromboxane A<sub>2</sub>), adhesion molecules for thrombocytes (vWF) and leucocytes (intercellular adhesion molecule, ICAM; vascular cell adhesion molecule, VCAM; and E-selectin), platelet activation (thromboxane A<sub>2</sub>), anticoagulants (TFPI), fibrinolytic factors (tPA, thrombomodulin and protein S) and fibrinolysis inhibitors (PAI-1).

Under normal conditions, the endothelium has anticoagulant and fibrinolytic properties, mainly through expression of thrombomodulin but also via secretion of TFPI, tPA and protein S. Thrombin that binds to thrombomodulin loses its procoagulant properties and is instead involved in fibrinolysis regulation through activation of protein C and TAFI, as discussed above. Upon vessel injury, endothelial cell activation promoting vasoconstriction, inflammation and coagulation is crucial to prevent blood loss and to enable vessel healing. These actions by the endothelial cells are thus part of its proper function triggered by vascular wall injury. However, a chronic shift of the actions of the endothelium towards reduced vasodilatation, proinflammation and procoagulant activities is a pathological state associated with development of micro- and macrovascular diseases.

### **1.2.1 Biomarkers of endothelial function**

Disturbed endothelial function is an early sign of micro- and macrovascular complications in type 1 diabetes. Endothelial dysfunction is assessed by quantification of various proteins secreted or expressed by endothelial cells. However, while some of these markers are almost exclusively synthesized by the endothelial cells (e.g. E-selectin), others are less specific (e.g. PAI-1) and interpretation of the data must therefore be carried out with caution. This is probably one of the reasons why studies often investigate a combination of biomarkers instead of a single marker in assessment of endothelial dysfunction. In addition, plasma levels of the various biomarkers may reflect different stages and/or features of endothelial dysfunction depending on the extent of vascular impairment and underlying pathophysiology.

Two biomarkers of endothelial function that have been in focus in recent years are circulating endothelial microparticles (EMPs) and endothelial progenitor cells. EMPs, like other MPs as described in section 1.1.1, are shed from the endothelial cell membrane upon activation or apoptosis. Circulating endothelial progenitor cells are stem cells with the ability to differentiate into endothelial cells and they are therefore important mediators of vascular repair.

Type 1 diabetes, cardiovascular risk factors and manifest CVD are all associated with increased plasma levels of most biomarkers of endothelial function, including VCAM, ICAM, E-selectin, vWF, tPA, PAI-1 and thrombomodulin [Constans 2006, Schram 2003, Targher 2005]. In addition, elevated circulating levels of EMPs and decreased levels of endothelial progenitor cells have also been reported in connection with CVD, cardiovascular risk factors and type 1 diabetes [Chironi 2009, Sen 2011, Sabatier 2002, Hörtenhuber 2013]. In patients with type 1 diabetes, levels of endothelial cell biomarkers are related to glycemic control and vascular complications [Schram 2003, Targher 2005, Hörtenhuber 2013]. Furthermore, induced hyperinsulinemic hypoglycemia in these patients is also associated with increased levels of VCAM, ICAM and E-selectin [Gogitidze 2010], indicating that hypoglycemia may also contribute to endothelial dysfunction and thereby accelerate the development of micro- and macrovascular complications in patients with type 1 diabetes, as mentioned in section 1.1.4.

### **1.2.2 Endothelial-dependent skin microvascular reactivity**

In addition to measurement of biomarkers of endothelial function in plasma, functional methods are frequently used for determination of endothelial-dependent vasodilatation after stimulation with vasoactive substances *in vivo*. Investigation of skin microcirculation is a reliable and non-invasive method to study microvascular endothelial function in a clinical setting and may be used as a model for generalized microvascular function [Roustit 2012, Rendell 1992, Holowatz 2008, Chang 1997]. Regulation of skin microcirculation is complex and involves multiple signalling pathways with integrated endothelial, neural and vascular smooth-muscle contributions. Iontophoresis of acetylcholine (ACh) and sodium nitroprusside (SNP) is used to assess the endothelium-dependent and endothelium-independent skin microvascular function, respectively [Roustit 2012]. Acetylcholine causes localized endothelium-dependent vasodilatation, although the contribution of nitric oxide, prostanoids, hyperpolarising factor and C-fiber nerves in mediating this response remains unclear. Nitroprusside is a direct donor of nitric oxide, which bypasses the endothelium and relaxes the vascular smooth-muscle cells. Disturbances in skin microvascular reactivity are found early after onset of type 1 diabetes, and are aggravated in patients with microvascular complications [Roustit 2012, Khan 2000].

### **1.3 STATIN AND ASPIRIN TREATMENT IN TYPE 1 DIABETES**

Lipid-lowering treatment with statins (3-hydroxy-3-methylglutaryl-coenzyme A reductase inhibitors) is effective in primary and secondary prevention of CVD in non-diabetic patients and patients with type 2 diabetes, while prospective large-scale studies in patients with type 1 diabetes are absent [Kearney 2008]. Reduced occurrence of symptomatic venous thromboembolism has also been shown during statin treatment in healthy subjects with normal cholesterol levels [Glynn 2009]. Statins seem to exert potential antithrombotic effects that are independent of their lipid-

lowering properties, including reductions in thrombin generation and platelet activation [Undas 2005, Notarbartolo 1995]. Possible beneficial treatment effects of statins on hemostatic function have not previously been investigated in patients with type 1 diabetes. Furthermore, the impact of statin therapy on the microvasculature in patients with type 1 diabetes is unclear and while studies have failed to show any effects on microvascular function in type 1 diabetes [Zang 1995, Colhoun 2009, Sen 2002], beneficial effects of statin treatment on kidney function and retinopathy status have been shown in patients with type 2 diabetes [Fried 2001, Hommel 1992].

In recent years, it has been shown that statins impair glycemic control and even increase the risk of new-onset type 2 diabetes [Preiss 2011]. The underlying mechanisms behind statin interference with glucose homeostasis are unclear. It has been suggested that cholesterol-independent pathways may be involved, as statins not only block the synthesis of cholesterol but also inhibit the production of isoprenoid compounds through their action on HMG-CoA reductase. Isoprenoids serve as lipid attachments for post-translational modification of various intracellular proteins, a process that is essential for proper protein function and which enables attachment to the cell membrane [Danesh 2004]. Reduced isoprenylation of small guanosine triphosphatase (GTPase) proteins during statin therapy may cause downregulation of glucose transporter (GLUT) 4 expression at the cellular membrane and decreased insulin-mediated glucose uptake, which has been shown in adipocytes in vitro [Kostapanos 2010]. Indeed, dose-dependent impairment of insulin sensitivity during atorvastatin treatment has been reported in patients with hypercholesterolemia [Koh 2010]. Importantly, cardiovascular-protective effects of statin treatment in patients with diabetes have been established and seem to be independent of the type of diabetes [Kearney 2008]. Thus, the beneficial effects of statin therapy seem to outweigh the small risk of impaired glycemic control in patients with and without diabetes.

Low-dose aspirin therapy is one of the cornerstones in the management of CVD, although, the preventive effect seems to be reduced in patients with diabetes [Cubbon 2008]. Aspirin inhibits thromboxane A<sub>2</sub> production in platelets through irreversible acetylation of cyclooxygenase-1 (COX-1). Aspirin also influences the coagulation pathways through effects on thrombin generation, FXIII activation and fibrin clot structure [Undas 2007]. Aspirin treatment increases fibrin clot permeability in non-diabetic patients, possibly through acetylation of lysine residues on plasma fibrinogen [Antovic 2005, Williams 1998, Björnsson 1989], whereas the effect of aspirin on fibrin clot properties in patients with diabetes is unclear. It has been hypothesized that increased fibrinogen glycation in patients with diabetes may occur at the same lysine residues that are acetylated during aspirin treatment, and this competition might contribute to the reduced preventive effect of aspirin in CVD management in patients with diabetes. However, a recent mass spectrometry study by Svensson et al. concerning glycation and acetylation of the fibrinogen protein showed that aspirin and glucose bind to different lysine sites on the fibrinogen molecule and no interaction was found between the two compounds [Svensson 2012].

## 2 AIMS & HYPOTHESES

*The overall aims of this work were:*

- To study fibrin clot properties in adult patients with type 1 diabetes in relation to sex and microvascular complications (Paper I).
- To investigate the treatment effects of high-dose atorvastatin (80 mg daily) on fibrin clot properties and skin microvascular function in patients with type 1 diabetes and dyslipidemia (Papers II and III)
- To investigate the effects of low (75 mg daily) and high (320 mg daily) doses of aspirin on fibrin clot properties in patients with type 1 diabetes (Paper IV).

*We hypothesized that:*

- Female sex is associated with denser and less permeable fibrin clots in patients with type 1 diabetes.
- Patients with type 1 diabetes and microvascular complications have denser and less permeable fibrin clots compared with patients without microangiopathy.
- High-dose atorvastatin (80 mg/day) treatment has favourable effects on fibrin clot permeability and skin microvascular function in patients with type 1 diabetes.
- Treatment with high-dose aspirin (320 mg/day), as opposed to low-dose (75 mg/day) aspirin, is required to positively influence fibrin clot properties in patients with type 1 diabetes.

### 3 PATIENTS & METHODS

#### 3.1 STUDY DESIGN AND POPULATION

All patients were recruited from the Department of Endocrinology and Diabetology at Danderyd Hospital in 2009. Approximately 1290 adult patients with type 1 diabetes were regularly followed at this clinic at this time.

##### 3.1.1 Paper I

This study was an observational study with consecutive selection of patients with type 1 diabetes from January to December 2009. The patients were aged between 20 and 70 years and had no history of macrovascular disease. Pregnant women were excluded. A total of 236 patients (107 women, 129 men) were included. Medical records of all patients were checked for documentation of clinical nephropathy and neuropathy. Retinopathy status was determined through fundoscopic findings and categorized into three groups: a) no retinopathy, b) mild-moderate retinopathy, and c) severe retinopathy, i.e. laser treated severe non-proliferative retinopathy or proliferative retinopathy.

Patient characteristics are summarized in Table 2, and a complete table is to be found in Paper I (Appendices).

Table 2. Patient characteristics of subjects in Paper I.

	All patients n=236	Women n=107	Men n=129	p-value ♀ vs ♂
Age (years)	44 ±13	44 ±13	44 ±13	0.83
Diabetes duration (years)	23 ±14	22 ±14	23 ±14	0.81
Body mass index (kg/m <sup>2</sup> )	25.0 ±3.8	24.3 ±3.8	25.5 ±3.7	<b>0.02</b>
Systolic blood pressure (mmHg)	128 ±18	123 ±17	132 ±17	<b>&lt;0.001</b>
Microalbuminuria <sup>a</sup> (n, %)	58 (25)	30 (28)	28 (22)	0.37
Microangiopathy (n, %)				
- Retinopathy	143 (61)	65 (61)	78 (60)	0.97
- Nephropathy	29 (12)	16 (15)	13 (10)	0.54
- Neuropathy	43 (18)	17 (16)	26 (20)	0.57
HbA <sub>1C</sub> (%) (mmol/mol)	7.0 ±1.3 63 ±3	7.2 ±1.5 65 ±5	6.8 ±1.2 60 ±2	<b>0.03</b>
Serum lipids (mmol/L)				
- Total cholesterol	4.5 (4.0-5.0)	4.6 (4.2-5.0)	4.3 (3.9-4.9)	<b>0.02</b>
- LDL	2.6 (2.1-3.0)	2.6 (2.1-3.0)	2.5 (2.1-3.0)	1.0
- HDL	1.5 (1.2-1.8)	1.7 (1.3-2.0)	1.4 (1.1-1.6)	<b>&lt;0.001</b>
- Triglycerides	0.7 (0.5-0.9)	0.6 (0.5-0.9)	0.7 (0.5-0.9)	0.05

Data are presented as number of patients, means ±SD or medians (lower–upper quartiles). <sup>a</sup> Presence of microalbuminuria on the day of investigations.

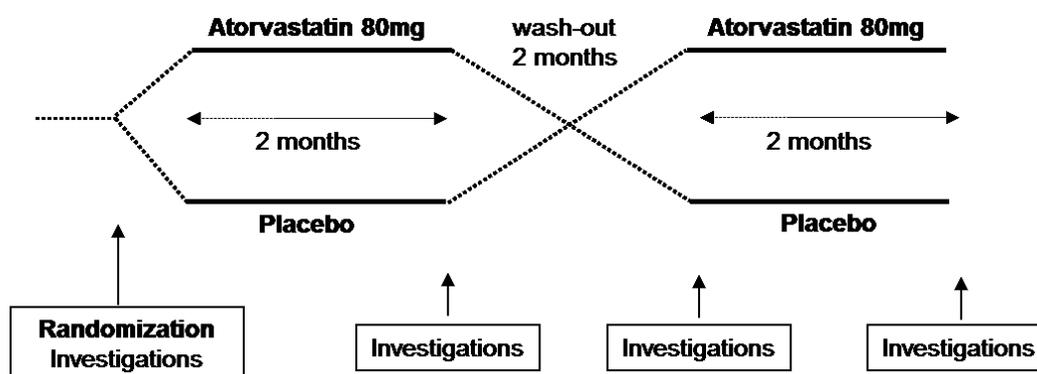
Mean age, diabetes duration, number of smokers and treatments with statins and antihypertensive drugs did not differ between men and women. Men had higher blood pressure and BMI, while women had worse glycemic control and higher HDL levels. The differences in blood pressure, BMI, HbA<sub>1C</sub> and HDL levels between the sexes were in accordance with the 2012 annual report from the Swedish National Diabetes registry [www.ndr.nu], in which 85-90% of all patients with diabetes in Sweden are registered.

Most patients (n=196) were treated with intermittent doses of short-acting insulin with meals and long-acting insulin analogs once or twice daily, while 39 patients (24 females) were treated with continuous subcutaneous insulin infusion. Of the 107 women, 15 women were using estrogen substitutes as oral contraceptives, 32 women were menopausal and 3 women were perimenopausal. The youngest menopausal woman was 50 years old, while the oldest menstruating woman was 54 years old.

### 3.1.2 Papers II & III

In this double-blind cross-over study, 20 patients (10 females) with type 1 diabetes and dyslipidemia, aged between 30 and 70 years, were randomized to treatment with 80 mg atorvastatin (Lipitor<sup>®</sup>, Pfizer) or matched placebo once daily for two months (Figure 5). Dyslipidemia was defined as elevated levels of plasma LDL (>2.5 mmol/L) and/or total cholesterol (>4.5 mmol/L). Patients with a history of macrovascular events were excluded. Investigations were performed at the start and end of the treatment periods, which were separated by a wash-out period of two months. The baseline characteristics of the patients are shown in Table 1 in Paper II in the appendices. Median age and mean diabetes duration were 44 and 23 years.

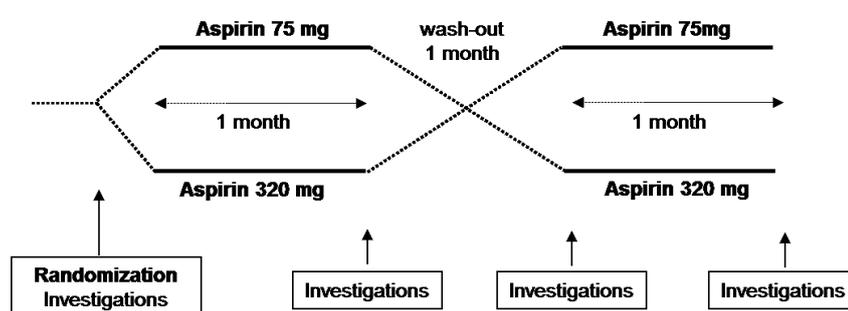
Figure 5. Flow chart of Paper II & III.



### 3.1.3 Paper IV

This was a cross-over study with randomization to treatment with 75 mg or 320mg aspirin (Trombyl<sup>®</sup>, Pfizer) once daily for four weeks, with a wash-out period of four weeks between the treatment periods (Figure 6). The study included 24 patients (12 women) with type 1 diabetes and good glycemic control (HbA<sub>1C</sub> <6.5%, 57 mmol/mol) and 24 patients (12 women) with type 1 diabetes and poor glycemic control (HbA<sub>1C</sub> >7.5%, 68 mmol/mol), aged between 30 and 70 years. Patients with a history of macrovascular events, previous aspirin treatment or ongoing treatments with non-steroidal anti-inflammatory drugs (NSAID) or anticoagulants were excluded. Investigations were performed at the start and the end of each treatment period.

Figure 6. Flow chart of Study IV.



Baseline characteristics of the 41 patients who completed the study are shown in Table 1 in Paper IV in the appendices. Mean age and median diabetes duration were 51 and 21 years. There were no significant differences between the patients with good vs poor glycemic control as regards mean age, diabetes complications or antihypertensive and statin treatments. The patients with good glycemic control, compared with the group with poor glycemic control, had longer diabetes duration (30 (19–43) vs 15 (10–29) years;  $p=0.01$ ), better lipid profiles and lower plasma fibrinogen levels ( $2.5 \pm 0.4$  vs  $2.9 \pm 0.7$  g/L;  $p=0.02$ ). The groups did not differ as regards age, diabetes complications, antihypertensive and statin treatments or baseline PMP concentrations.

## 3.2 CLINICAL INVESTIGATIONS

Height, weight and waist circumference of all patients were measured. Peripheral blood pressures were measured in a supine position after 20 minutes of rest. Systolic and diastolic arm blood pressures were determined by means of the Riva Rocci method.

Microalbuminuria was assessed by means of dipstick tests (Clinitek<sup>®</sup>, Bayer HealthCare LLC, USA) on urine samples of the same morning as other investigations. Signs of peripheral neuropathy in the feet were investigated by means of tests of vibration and superficial sensation, using a vibration fork (128 Hz) and a monofilament (Semmes-Weinstein 5.07), respectively. Medical records were checked for documentation of clinical nephropathy and neuropathy. The prevalence of retinopathy was determined through fundoscopic findings.

### 3.3 LABORATORY INVESTIGATIONS

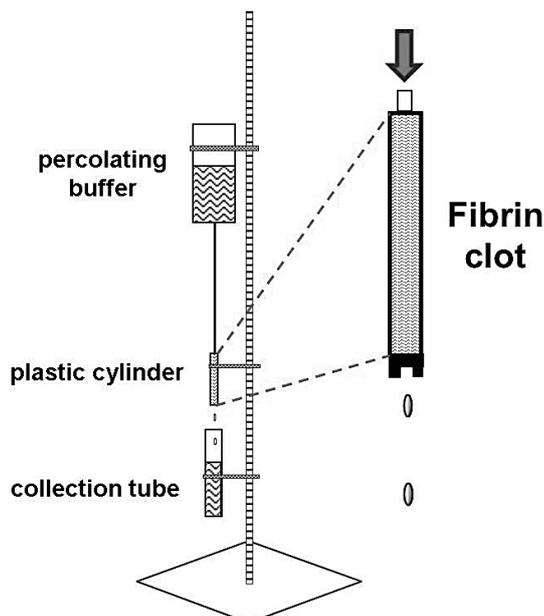
#### 3.3.1 Blood sampling

All patients arrived at the laboratory between 8 and 9 a.m. after a 10-hour fast. After an acclimatization period of at least 20 minutes, blood samples were collected through antecubital vein punctures into citrated or EDTA tubes, or tubes without anticoagulants. The citrated blood was immediately centrifuged at 2 000 *g* for 20 minutes at room temperature to obtain platelet-poor plasma. Plasma samples were stored in plastic tubes in aliquots of 0.5 mL at -70°C.

#### 3.3.2 Fibrin clot permeability

Fibrin clot permeability was investigated *in vitro* in citrated plasma, as described in detail by Blombäck et al. [1989, 1994]. The method was later modified, as described by He et al. in 2005 [He 2005]. In brief, citrated plasma samples were dialyzed and supplemented with CaCl<sub>2</sub> and thrombin to give final concentrations of 20 mmol/L and 0.2 U/mL, respectively. Fibrin clot permeability was determined following percolation of TRIS buffer through the formed fibrin clots at five different hydrostatic pressures, as illustrated in Figure 7. The permeation coefficient (Ks), Darcy's constant, which indicates the pore size of the fibrin clot structure, was calculated from the equation  $Ks = Q \times L \times \eta / (t \times A \times \Delta P)$ , where Q is the volume of liquid (in cm<sup>3</sup>), t is the time (in sec), L is the clot length (in cm),  $\eta$  is liquid viscosity (in dyne  $\times$  sec), A is the cross-sectional area (in cm<sup>2</sup>) and  $\Delta P$  is the differential pressure (in dyne). The unit of the resulting Ks is cm<sup>2</sup>. Low levels of Ks indicate reduced fibrin clot permeability. All measurements were performed in duplicate by an investigator blind to the origin of the samples. The inter-assay coefficient of variation was 9.5% [Antovic 2007].

Figure 7. Laboratory set-up for assessment of fibrin clot permeability.

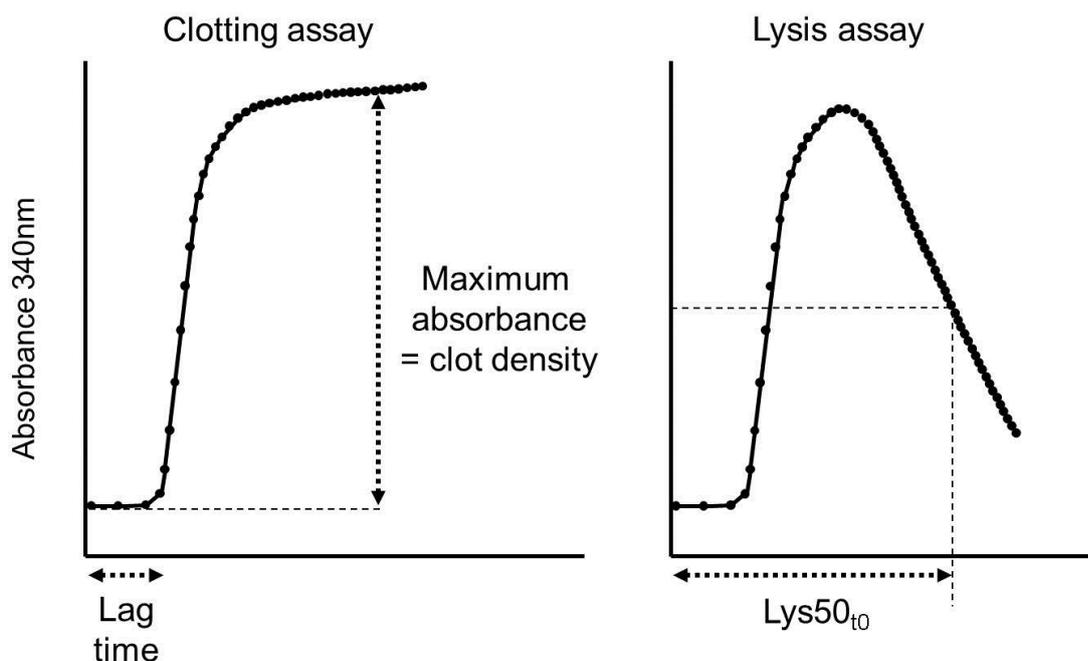


### 3.3.3 Turbidimetric clotting and lysis assays

Fibrin clot density and rates of fibrin polymerization and degradation were assessed by using turbidimetric clotting and lysis assays, respectively, according to the methods described by Carter et al. [2007]. In brief, 75  $\mu\text{L}$  of assay buffer (pH 7.4, 0.05 M TRIS-HCl, 0.15 M NaCl) was added to 25  $\mu\text{L}$  of citrated plasma (in duplicate) in a microtiter plate. Fifty  $\mu\text{L}$  of a mixture of thrombin (final concentration 0.03 U/mL) and  $\text{CaCl}_2$  (final concentration 7.5 mmol/L) was added to the plasma samples and absorbance at 340 nm was read every 18 sec (240 cycles for each sample) in a spectrophotometer. In the turbidimetric lysis assay, recombinant tPA (Technoclone) was added to TRIS-HCl buffer (final concentration of 83 ng/mL) before addition of thrombin and calcium.

Lag time, maximum absorbance and time to achieve 50% lysis were determined from the curves (Figure 8). Lag time represents the time at which sufficient protofibrils have formed to enable lateral aggregation and is defined as increased absorbance by 0.01 arbitrary unit (AU) above baseline level. Maximum absorbance reflects clot density and is defined as the highest absorbance value minus the absorbance at baseline. In the clotting assay, this variable is determined at the plateau phase at 30 minutes for all patients. Lysis time reflects fibrinolytic susceptibility, and is determined as the time to achieve a 50% fall in absorbance from the initiation of clot formation ( $\text{Lys}_{50_{t_0}}$ ). In our laboratory, the intra- and interassay coefficient of variances (CV) are 5% and 10% for lag time and 4% and 6% for maximum absorbance in normal pool plasma. The interassay CV for clot lysis time ( $\text{Lys}_{50_{t_0}}$ ) is 7%.

Figure 8. Determination of lag time, maximum absorbance and clot lysis time in the turbidimetric clotting and lysis assays.



### 3.3.4 Thrombin generation

Calibrated, automated thrombogram (CAT) assays were performed, as described by Hemker et al. [2006] and according to the instruction of the manufacturers of the equipment (Thromboscope BV, Maastricht, Holland). Briefly, 80  $\mu\text{L}$  of platelet poor plasma was mixed with 20  $\mu\text{L}$  of a platelet poor-plasma reagent containing TF and phospholipids at final concentrations of 5pM and 4  $\mu\text{M}$ , respectively. In addition, 80  $\mu\text{L}$  of the same reagent was mixed with 20  $\mu\text{L}$  of thrombin calibrator. A thrombin specific fluorogenic substance (2.5 mM), which is specifically cleaved by thrombin, was then added together with  $\text{CaCl}_2$  (100 mM) in Hepes buffer to start the reactions in the measurement and calibration wells. Fluorescence was subsequently measured every 30 sec by using a Fluoroscan Ascent fluorometer (Fluoroskan Ascent<sup>®</sup>, Thermo Scientific Vanta, Finland) and the following 4 variables were determined: 1) lag time, the time from start of analysis until detection of thrombin generation; 2) peak thrombin concentration; 3) endogenous thrombin potential (ETP), the area under the concentration-time curve; and 4) time to peak, the time from start of analysis until the peak thrombin value was achieved.

### 3.3.5 Microparticle analyses

In Study II, a MP-enriched pellet was obtained from platelet poor plasma. Plasma samples were thawed in water bath for 5 min and centrifuged for 45 min at 20 800 g at 10°C. The supernatant was then removed, leaving 50  $\mu\text{L}$  of a MP-enriched suspension, which was diluted with 450  $\mu\text{L}$  of TRIS-buffer and centrifuged again for 45 min at 20 800 g at 10°C. Again, 450  $\mu\text{L}$  of the supernatant was removed and the MP-enriched pellet was suspended in the remaining 50  $\mu\text{L}$  plasma. Twenty  $\mu\text{L}$  of the sample were then incubated for 20 min in darkness with phalloidin-Alexa 660 (Invitrogen, Paisley, UK) and CD42a-PE (glycoprotein IX, BD, Clone Alma-16), together with either CD61-FITC (GPIIIa, AbD Serotec, Clone Y2/51), CD62P-FITC (P-selectin, AbD Serotec, Clone AK-6) or CD142-FITC (TF, AbD Serotec, Clone CLB/TF-5). The PMP-gate was determined using forward scatter and CD42a expression. Expression of GPIIIa, P-selectin and TF was measured using a flow cytometric assay in which mean fluorescence intensities of antigen-positive particles were translated into MESF values (Molecules of Equivalent Soluble Fluorochrome). Reproducibility experiments using plasma samples from healthy individuals showed intra- and interassay CVs of <10% for CD62P and CD142 MESF [Mobarrez 2010].

In Study III and IV, a new flow cytometer was used (Beckman coulter Gallios<sup>®</sup>) and sample preparation was therefore modified. Frozen aliquots of platelet-poor plasma were thawed and centrifuged at 2000 g for 20 min at room temperature. The supernatant was then re-centrifuged at 13 000 g for 2 min. In Study III, 20  $\mu\text{L}$  of the samples were incubated for 20 minutes in dark with phalloidin-Alexa 660 (Invitrogen, Paisley, UK), lactadherin-FITC (Haematologic Technologies, VT, USA) and CD144-APC (VE-Cadherin, AH diagnostics, Stockholm, Sweden). Study IV had a similar

antibody protocol, however, CD42a-PE (Glycoprotein IX, Abcam, Cambridge, UK) was used instead of CD144.

The MP-gate was determined using Megamix beads (0.5  $\mu\text{m}$ , 0.9  $\mu\text{m}$  and 3.0  $\mu\text{m}$ , BioCytex, Marseilles, France). Microparticles were defined as particles of  $<1.0 \mu\text{m}$  in size, negative to phalloidin (cell-fragment marker) and positive to lactadherin. The EMPs in Study III were defined as MPs positive for CD144, and PMPs in Study IV were defined as MPs positive for CD42a. The results were presented as absolute numbers of MPs (microparticle counts  $\times$  standard beads/L)/standard beads counted (FlowCount, Beckman Coulter, CA, USA). The mean CV for EMP count assessed in 17 subjects on two separate days was 5% at our laboratory.

### 3.3.6 Skin microcirculation

Skin microcirculation was investigated through iontophoresis (see Figure 9), which is a non-invasive method for drug application through the skin using a small electric current [Jörneskog 2005]. Acetylcholine (ACh, Sigma-Aldrich AB, Stockholm, Sweden) and sodium nitroprusside (SNP, Hospira, Inc Lake Forest, USA), diluted in deionized water, were used to investigate endothelium-dependent and -independent microvascular reactivity. Electrode chambers (LI611 Drug Delivery Electrode Imaging, Perimed, Järfälla, Sweden) were attached to the volar side of the left forearm and filled with a small volume of either ACh (2%) or SNP (2%). A battery-powered iontophoresis controller (Perilont 382b, Perimed, Järfälla, Sweden) provided a direct current (0.1 mA for 60s) for drug iontophoresis. Acetylcholine was delivered using an anodal charge and SNP with a cathodal charge. Skin microvascular flux before, during and after iontophoresis was measured by means of laser Doppler perfusion imaging (Periscan PIM II, Perimed, Järfälla, Sweden) and expressed in AU. Each image has a duration of 36 seconds and consists of approximately 150 measuring points within a skin area of 11 mm in diameter. Baseline flux was averaged over four images. Microvascular flux was recorded continuously for 10 and 14 minutes after iontophoresis of ACh and SNP, respectively. Peak microvascular flux was determined. At our laboratory, the mean CV of peak microvascular flux after iontophoresis of ACh and SNP in seven healthy individuals on three separate days were 11 and 20%.

Figure 9. Iontophoresis for investigation of skin microcirculation



Figure shown with permission from Perimed AB, Järfälla, Sweden.

### 3.3.7 Biochemical analyses

Fibrinogen concentrations were assessed with the Fibri-Prest Automate<sup>®</sup> method (Clauss method, see reference from Mackie et al. from 2003) from Diagnostica Stago (reference range 2–4 g/L). Factor XIII (FXIII) antigen levels were determined through ELISA with antibodies from Enzyme Research Laboratories, South Bend, IN, USA: sheep anti-Human Factor XIII Purified IgG (capturing antibody) and sheep anti-Human FXIII Peroxidase Conjugate (detecting antibody), intraassay and interassay CV of 3.2% and 9.5%, respectively. Prothrombin fragment 1+2 (F1+2) was determined with Enzygnost F1+2 (monoclonal) ELISA assays from Siemens Healthcare Diagnostics Products GmbH, Marburg, Germany. Plasminogen activator inhibitor type 1 (PAI-1) activity was analyzed with TriniLIZE<sup>®</sup> PAI-1 activity ELISA assay from Trinity Biotech Plc. Bray, Ireland, with a detection range of 2.0–50 UI/mL according to the manufacturer. In order to improve the precision at lower PAI-1 levels, extra measuring points were added at the lower end of the calibration curve, as described by Ågren et al [2006]. Plasma levels of tPA-PAI-1 complex were assessed with TintElize<sup>®</sup> tPA-PAI-1 ELISA kits from Biopool, Umeå, Sweden (reference range 0.6-6.7 µg/L, 2.5 ±1.5 µg/L in 40 healthy donors). HbA<sub>1C</sub> levels were analyzed by the Mono S method using high-performance liquid chromatography (Variant II; Bio-Rad Laboratories, Hercules, CA, USA) and expressed in International Federation of Clinical Chemistry (IFCC) standardization of HbA<sub>1C</sub> values in mmol/mol.

### 3.4 STATISTICAL ANALYSES

Data are presented as mean values ±SD or 95% confidence intervals (CIs) for normally distributed data, and median values with lower-upper quartiles for skewed data. Data with skewed distributions were logarithmically converted and checked to be normally distributed after logarithmic transformation. Independent *t*-tests, Mann Whitney *U*-tests and one-way or two-way repeated measures analyses of variance (ANOVA) were used to compare variables between groups. In the cross-over studies (Papers II-IV), possible carry-over effects were investigated through assessment of the interaction between the treatment sequence and the investigated variables in repeated measures ANOVA. Linear and multiple forward stepwise regression analyses were used to estimate associations between variables. A probability (*p*) of less than 0.05 was considered statistically significant.

## 4 RESULTS & COMMENTS

### 4.1 MEN AND WOMEN HAVE SIMILAR FIBRIN CLOT PROPERTIES (PAPER I)

In the population of 236 patients (129 men, 107 women) with type 1 diabetes, there were no differences in fibrin clot permeability coefficient (Ks) or in the turbidimetric clotting and lysis analyses between the sexes, as shown in Table 3. Fibrinogen concentrations were similar in men and women. Women had higher FXIII levels. Women also had higher F1+2 levels, indicating higher thrombin generation in vivo, and higher peak levels of TF-induced thrombin generation in vitro. Higher thrombin generation in vitro in women, especially in females using oral contraceptives, has previously been shown in a healthy population [Brummel-Ziedins 2005]. Accordingly, women with estrogen substitution in our study (n=15) had higher peak thrombin generation in vitro, although they were on average 10 years younger than the rest of the females.

Plasma levels of tPA-PAI-1 complex were higher in men in our study, which also has been shown in healthy subjects of <50 years of age [Takada 1989]. In accordance with results in the healthy population, we found no differences in levels of tPA-PAI-1 complex in men and women of >55 years of age (n=54). Ks, fibrinogen and F1+2 levels did not differ between women with and without estrogen substitution, while tPA-PAI-1 complex levels were increased in women with estrogen substitutions.

Table 3. Hemostatic function in 236 patients with type 1 diabetes.

	All patients n=236	Women n=107	Men n=129	p-value (♀ vs ♂)
Ks (cm <sup>2</sup> ×10 <sup>-9</sup> )	10.7 ±4.0	11.0 ±3.9	10.5 ±4.1	0.43
Fibrinogen (g/L)	2.7 ±0.6	2.7 ±0.6	2.7 ±0.7	0.87
FXIII (AU/mL)	0.83 ±0.19	0.86 ±0.21	0.81 ±0.17	<b>0.047</b>
Turbidimetric analyses				
- Lag phase (sec)	305 (273-356)	310 (278-349)	305 (272-358)	0.58
- Max absorbance	0.36 ±0.10	0.37 ±0.09	0.36 ±0.10	0.54
- Lys50 <sub>10</sub> (sec)	1623 (1496-1794)	1663 (1524-1803)	1596 (1478-1770)	0.07
PAI-1 activity (U/mL)	2.2 (1.0-5.4)	1.8 (1.0-4.2)	2.5 (1.0-6.2)	0.19
tPA/PAI-1 (µg/L)	2.2 (1.7-3.0)	2.0 (1.6-2.7)	2.5 (1.9-3.9)	<b>&lt;0.001</b>
Fragment 1+2 (pmol/L)	220 (164-294)	242 (181-310)	204 (142-270)	<b>0.003</b>
CAT (5µM TF)				
- Lag time (sec)	160 (140-180)	150 (133-175)	170 (150-190)	<b>&lt;0.001</b>
- Peak (nM)	228 ±50	242 ±51	218 ±48	<b>&lt;0.001</b>
- Time to peak (sec)	352 ±69	328 ±62	369 ±68	<b>&lt;0.001</b>
- ETP (nM×min)	1292 ±223	1303 ±223	1284 ±223	0.54

Data are presented as means ±SD or medians (lower–upper quartiles).

## **4.2 YOUNG WOMEN FORM TIGHTER FIBRIN CLOTS (PAPER I)**

Subgroup analyses showed no differences in Ks levels between the sexes among patients <50 years of age (n=148) or among patients >50 years of age (n=69). However, younger women <30 years of age (n=17) compared with age-matched men (n=18) had lower Ks values ( $10.5 \pm 4.2$  vs  $14.1 \pm 5.1$   $\text{cm}^2 \times 10^{-9}$ ,  $p=0.03$ ) and prolonged clot lysis time (1673 (1528-1851) vs 1522 (1456-1583) sec,  $p=0.008$ ). The women in this group had higher levels of triglycerides, F1+2 and peak thrombin generation in vitro than corresponding men, while BMI and levels of fibrinogen and HbA<sub>1C</sub> did not differ between the sexes. No differences in clot density were found in this subgroup analysis. Ks levels, fibrinogen concentrations, thrombin generation in vivo/in vitro were not different between women <30 years with (n=6) or without (n=11) estrogen substitutes.

## **4.3 TIGHTER FIBRIN CLOTS IN PATIENTS WITH POOR GLYCEMIC CONTROL (PAPER I)**

Subgroup analyses were performed to compare fibrin clot properties in patients with good (HbA<sub>1C</sub> <6.5%, 57 mmol/mol) and poor (HbA<sub>1C</sub> >7.5%, 68 mmol/mol) glycemic control. The results are shown in Table 4. Treatments with antihypertensive drugs or statins did not differ between the two groups (data not shown). Higher triglyceride levels and lower HDL levels were found in patients with poor glycemic control (data not shown). Poor glycemic control was associated with formation of denser and less permeable fibrin clots and prolonged lysis time.

Notably, HbA<sub>1C</sub> levels seem to have a relatively small impact on fibrin clot properties, especially in women, as shown in the regression analyses (see section 4.4). Thus, the more prothrombotic fibrin clot properties observed in the group with poor glycemic control may be due to other factors, such as increased systemic inflammation or lipid abnormalities, or factors not investigated in our study.

## **4.4 TIGHTER FIBRIN CLOTS IN PATIENTS WITH MICROANGIOPATHY (PAPER I)**

Prevalences of retinopathy, nephropathy and neuropathy obtained from the medical records of the 236 patients and the presence of microalbuminuria on the day of investigation did not differ between men and women (Table 2). Clinical microangiopathy and microalbuminuria were associated with denser and less permeable clots, increased lysis time and higher fibrinogen concentrations (Table 5). These changes were more pronounced in patients with multiple microvascular complications (Figure 10). The associations between fibrin clot parameters and microvascular complications (separately or combined) were no longer significant after adjusting for fibrinogen concentrations, which could be expected since fibrinogen concentrations greatly influence fibrin clot properties, as shown in the regression analyses below.

Table 4. Hemostatic function of patients with good and poor glycemic control.

	Good glycemic control n=104	Poor glycemic control n=80	p-value between groups
Distribution ♂ vs ♀	59/43	37/43	0.2
Age (years)	45 ±12	44 ±14	0.7
Diabetes duration (years)	23 ±16	20 ±13	0.2
BMI (kg/m <sup>2</sup> )	24 ±3	26 ±4	<b>0.02</b>
Tobacco use (n, %)	24 (23)	29 (36)	<b>0.04</b>
HbA <sub>1C</sub> (%)	5.8 ±0.5	8.4 ±1.0	<b>&lt;0.001</b>
hsCRP (mg/L)	0.8 (0.4-1.6)	1.4 (0.7-3.7)	<b>&lt;0.001</b>
Fibrinogen (g/L)	2.5 ±0.5	2.9 ±0.7	<b>&lt;0.001</b>
Ks (cm <sup>2</sup> ×10 <sup>-9</sup> )	11.3 ±3.8	9.8 ±4.1	<b>0.008</b>
Turbidimetric analyses			
- Lag phase <sub>c</sub> (sec)	311 (273-352)	301 (274-362)	0.7
- Maximum absorbance	0.34 ±0.08	0.39 ±0.11	<b>0.001</b>
- Lys50 <sub>t0</sub> (sec)	1551 (1469-1681)	1710 (1574-1903)	<b>&lt;0.001</b>
PAI-1 activity (U/mL)	1.6 (0.8-4.7)	3.2 (1.6-8.1)	<b>0.004</b>
tPA/PAI-1 (µg/L)	2.2 (1.6-2.8)	2.5 (1.9-4.2)	<b>0.008</b>
Fragment 1+2 (pmol/L)	219 (165-308)	223 (166-296)	0.5
CAT (5µM TF)			
- Lag time (sec)	160 (140-180)	164 (150-190)	<b>0.008</b>
- Peak (nM)	228 ±50	232 ±49	0.6
- Time to peak (sec)	342 ±65	359 ±67	0.1
- ETP (nM×min)	1298 ±226	1308 ±215	0.8

Data are presented as number of patients, means ±SD or medians (lower–upper quartiles).

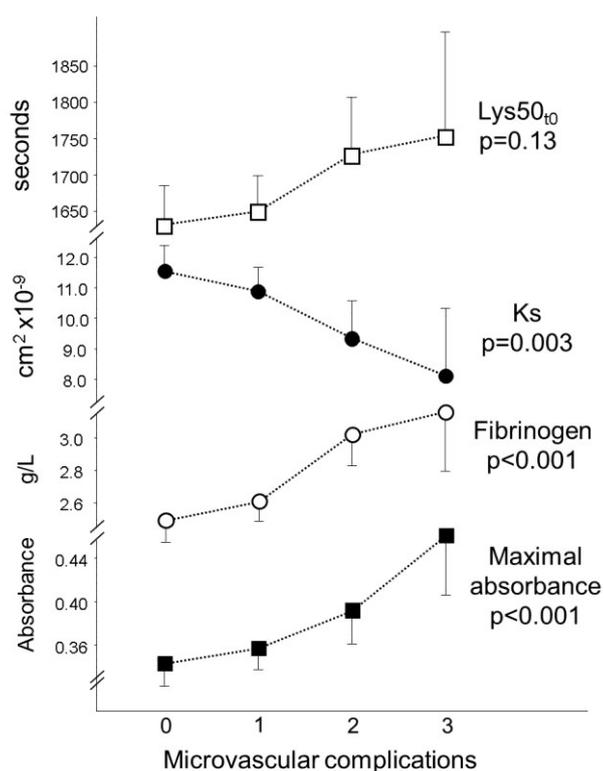
Higher HbA<sub>1C</sub> levels were found in patients with microalbuminuria, nephropathy and neuropathy, respectively, compared with patients without the respective complications. The prevalence of retinopathy (present or absent) was not associated with significant differences in HbA<sub>1C</sub> levels, while patients with severe retinopathy had higher HbA<sub>1C</sub> levels compared with patients with mild-moderate retinopathy (7.5 ±1.5% vs 6.8 ±1.1%, p=0.005), as expected. After adjusting for HbA<sub>1C</sub> levels, fibrin clot properties were still associated with retinopathy, nephropathy and neuropathy.

Table 5. Hemostatic function in patients (n=236) with and without clinical microangiopathy.

	Without	With	p-value
<b>Retinopathy</b> (n, %)	93 (39)	143 (61)	-
- Fibrinogen (g/L)	2.5 ±0.5	2.8 ±0.7	<b>0.01</b>
- Ks (cm <sup>2</sup> ×10 <sup>-9</sup> )	11.3 ±4.2	10.3 ±3.8	0.08
- Maximum absorbance	0.35 ±0.08	0.38 ±0.10	<b>0.02</b>
- Lys50 <sub>t0</sub> (sec)	1609 (1480-1794)	1636 (1498-1794)	0.51
<b>Nephropathy</b> (n, %)	205 (87)	29 (12)	-
- Fibrinogen (g/L)	2.6 ±0.6	3.2 ±0.9	<b>&lt;0.001</b>
- Ks (cm <sup>2</sup> ×10 <sup>-9</sup> )	11.0 ±4.1	8.8 ±2.8	<b>0.004</b>
- Maximum absorbance	0.35 ±0.09	0.43 ±0.11	<b>&lt;0.001</b>
- Lys50 <sub>t0</sub> (sec)	1608 (1478-1784)	1710 (1618-1945)	<b>0.002</b>
<b>Neuropathy</b> (n, %)	193 (82)	43 (18)	-
- Fibrinogen (g/L)	2.6 ±0.6	2.9 ±0.7	<b>0.008</b>
- Ks (cm <sup>2</sup> ×10 <sup>-9</sup> )	11.1 ±4.1	9.1 ±3.2	<b>0.002</b>
- Maximum absorbance	0.36 ±0.09	0.39 ±0.11	<b>0.03</b>
- Lys50 <sub>t0</sub> (sec)	1617 (1498-1794)	1627 (1489-1800)	0.73
<b>Microalbuminuria<sup>a</sup></b> (n, %)	173 (73)	58 (25)	-
- Fibrinogen (g/L)	2.6 ±0.6	2.9 ±0.7	<b>0.003</b>
- Ks (cm <sup>2</sup> ×10 <sup>-9</sup> )	11.1 ±3.9	9.7 ±4.0	<b>0.03</b>
- Maximum absorbance	0.35 ±0.09	0.39 ±0.10	<b>0.02</b>
- Lys50 <sub>t0</sub> (sec)	1609 (1496-1767)	1683 (1486-1869)	0.17

Data are presented as number of patients, means ±SD or medians (lower–upper quartiles). <sup>a</sup> Presence of microalbuminuria on the day of investigation.

Figure 10. Fibrin clot properties and plasma fibrinogen concentrations in relation to microvascular complications in 236 patients with type 1 diabetes.



Data are presented as means and 95% CIs. “0” indicates no microvascular complications (n=83), “1” indicates either retinopathy, nephropathy or neuropathy (n=102), “2” indicate a combination of two complications (n=39), and “3” indicates a combination of retinopathy, nephropathy and neuropathy (n=12). Values of p refer to interaction between the investigated variables and number of microvascular complications (ANOVA repeated measures).

#### 4.5 FIBRIN CLOT PROPERTIES ARE DETERMINED BY FIBRINOGEN LEVELS (PAPER I)

Regression analyses concerning the 236 patients showed that Ks was inversely related to fibrinogen levels ( $r=-0.6$ ,  $p<0.001$ ), hsCRP ( $r=-0.4$ ,  $p<0.001$ ), age ( $r=-0.3$ ,  $p<0.001$ ), BMI ( $r=-0.3$ ,  $p<0.001$ ), FXIII ( $r=-0.3$ ;  $p<0.001$ ), triglycerides ( $r=-0.2$ ,  $p=0.003$ ) and HbA<sub>1C</sub> ( $r=-0.2$ ,  $p=0.003$ ). No associations were found between Ks and diabetes duration or cholesterol levels. Stepwise multiple regression analysis revealed fibrinogen and age as independent determinants of Ks, together accounting for 38% of its variability, while addition of data on BMI, HbA<sub>1C</sub>, triglycerides, hsCRP and FXIII to the model did not change the power of predicting Ks.

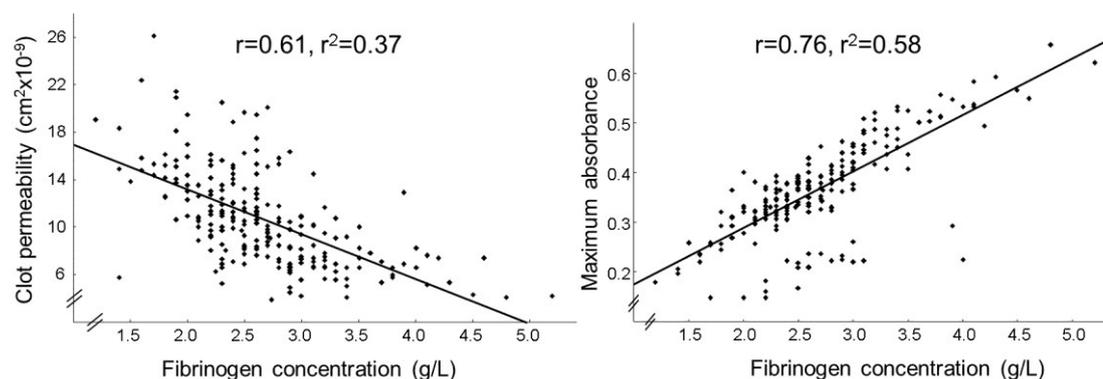
Linear regression analyses performed in men and women separately showed similar correlations between Ks and fibrinogen, BMI and FXIII in both groups. However, in men, Ks decreased with age and increasing levels of HbA<sub>1C</sub> and triglycerides, while these parameters had no effect on Ks values in women. In fact, plasma fibrinogen was the only predictor of Ks in women in the multiple regression analyses.

Furthermore, Ks was inversely related to plasma levels of tPA-PAI-1 complex ( $r=-0.4$ ,  $p<0.001$ ), while no associations were found between Ks and PAI-1 activity and F1+2. Levels of the female sex hormones FSH and estrogen did not correlate with Ks.

As expected, fibrin clot permeability was correlated with clot density ( $r=-0.5$ ,  $p<0.001$ ) and lysis rate (Lys50<sub>t0</sub>,  $r=-0.4$ ,  $p<0.001$ ). As mentioned in the Methods section, clot density reflects fiber thickness, which is determined by lateral aggregation during fibrin polymerization, while fibrin clot permeability reflects the average pore size, which depends on the number of branch points in the forming clot. The inverse correlation between clot density and clot permeability is thus in line with the statement that as fiber diameter increases, the number of branch points decreases and clots with larger pores are formed. Notably, however, the correlation coefficient between clot permeability and clot density is  $-0.5$ , meaning that these two variables are not in direct relation to each other.

Fibrinogen levels were positively correlated with clot density ( $r=0.8$ ,  $p<0.001$ ) and fibrinolytic rate (Lys50<sub>t0</sub>,  $r=0.6$ ,  $p<0.001$ ) and negatively correlated with clot permeability ( $r=-0.6$ ,  $p<0.001$ ). These results confirm that the fibrinogen concentration is an important determinant of fibrin clot properties. Furthermore, the fibrinogen concentration seems to have a greater impact on clot density ( $r^2=0.58$ ) than it has on clot permeability ( $r^2=0.37$ , Figure 11).

Figure 11. Correlation between fibrinogen concentrations and fibrin clot permeability and density, respectively.



#### 4.6 DETERMINANTS OF FIBRINOGEN CONCENTRATIONS (PAPER I)

Considering the great influence of fibrinogen concentration on fibrin clot properties, regression analyses were also performed with fibrinogen as the dependent factor. The results showed that fibrinogen concentrations were positively related to BMI ( $r=0.4$ ,  $p<0.001$ ), HbA<sub>1C</sub> ( $r=0.3$ ,  $p<0.001$ ), triglycerides ( $r=0.3$ ,  $p<0.001$ ), age ( $r=0.2$ ,  $p<0.001$ ), systolic and diastolic blood pressures ( $r=0.2$ ,  $p=0.002$  for both) and platelet count ( $r=0.2$ ,  $p<0.001$ ). Smokers had higher fibrinogen levels compared with non-smokers ( $2.8 \pm 0.6$  vs  $2.6 \pm 0.6$  g/L,  $p=0.03$ ).

Stepwise multiple regression analyses showed that BMI, age, HbA<sub>1C</sub> and triglycerides (in that order) are independent determinants of plasma fibrinogen, and account for 25% of its variability. Moreover, fibrinogen levels correlated with other inflammatory markers hsCRP ( $r=0.5$ ,  $p<0.001$ ) and white blood cell count ( $r=0.3$ ,  $p<0.001$ ). Linear regression analyses in men and women separately showed that fibrinogen concentrations increased with age in men but not in women, while systolic and diastolic blood pressure were correlated with fibrinogen levels only in women.

#### 4.7 ATORVASTATIN TREATMENT HAS ANTITHROMBOTIC EFFECTS (PAPER II)

The randomized cross-over study in 20 patients with type 1 diabetes and dyslipidemia showed increased fibrin clot permeability after two months of treatment with high-dose atorvastatin treatment (80 mg per day), while no changes were found during the placebo period (Figure 12). Fibrinogen concentrations were not changed during the atorvastatin or placebo treatments (Table 6). In vitro thrombin generation over time (ETP) decreased during atorvastatin therapy, while peak levels, time to peak and lag time remained unchanged. Levels of F1+2, indicating in vivo thrombin generation, were not changed during atorvastatin treatment.

Figure 12. Fibrin clot permeability coefficient (Ks) during atorvastatin and placebo treatments.

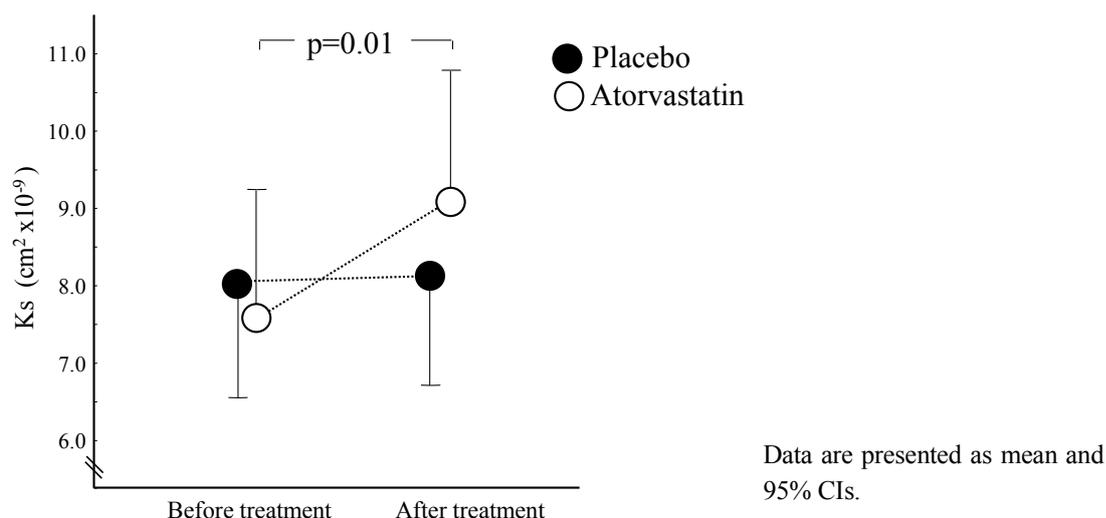


Table 6. Treatment effects of atorvastatin and placebo for 2 months.

	Atorvastatin 80mg	Placebo	p-value between treatm
Ks (cm <sup>2</sup> × 10 <sup>-9</sup> )	7.6 ± 3.1 → 9.1 ± 3.4	8.1 ± 3.0 → 8.1 ± 2.9	0.052
Fibrinogen (g/L)	2.7 (2.5–2.9) → 2.8 (2.4–3.0)	2.7 (2.3–3.0) → 2.8 (2.5–3.2)	0.3
F1+2 (pmol/L)	139 (96–193) → 118 (89–163)	168 (121–261) → 131 (88–212)	0.6
CAT (5μM TF)			
- Lag time (sec)	160 (140-167) → 160 (133-187)	160 (140-173) → 160 (140-192)	0.6
- Peak (nM)	293 ± 40 → 281 ± 41	284 ± 45 → 302 ± 40	<b>0.002</b>
- time to peak (sec)	310 (281-347) → 320 (266-360)	326 (281-353) → 340 (280-373)	0.2
- ETP (nM × min)	1659 ± 271 → 1537 ± 252	1641 ± 275 → 1705 ± 275	<b>&lt;0.001</b>

Data are presented as mean ± SD or median (lower–upper quartiles). Values of p refer to interaction between treatment effects of atorvastatin and placebo.

The concentration of PMPs and expression of glycoprotein IIIa, TF and P-selectin on PMPs decreased during atorvastatin therapy, as shown in Table 2 in Paper III (Appendices). As described earlier, these proteins promote platelet aggregation/activation and activation of coagulation. Decreased PMP concentrations and expression of these procoagulant antigens on the PMPs during atorvastatin treatment indicate decreased platelet activation.

As expected, the levels of total cholesterol and LDL decreased during atorvastatin treatment from 4.86 ± 0.45 and 3.15 (2.80–3.35) to 3.05 ± 0.3 and 1.40 (1.20–1.70) mmol/L (p < 0.001 for both). At the same time, the levels of triglycerides decreased from 0.72 (0.58–0.97) to 0.46 (0.38–0.68) mmol/L (p < 0.001), while HDL levels remained unchanged. The changes in lipid levels were not correlated with changes in Ks, PMPs or expression of glycoprotein IIIa, P-selectin and TF on PMPs.

#### **4.8 STATIN-INDUCED IMPAIRMENT OF GLYCEMIC CONTROL (UNPUBLISHED DATA)**

Increased HbA<sub>1C</sub> levels (from 7.5 ±0.9 to 7.8 ±1.1%, p<0.001) were observed after the two-month treatment period with high dose (80 mg/day) atorvastatin. As described in the Introduction (section 1.3), statin-induced impairment of glycemic control has been shown previously and may be explained by increased insulin resistance. The lipophilicity of the different statins seems to be important in this context, since statins with lipophilic properties, such as atorvastatin and simvastatin, are associated with impaired insulin sensitivity while a hydrophilic statin such as pravastatin reportedly improves insulin sensitivity [Baker 2010]. In the few previous studies on the effects of statin therapy (simvastatin at 20 mg/day and atorvastatin at 40 mg/day) in patients with type 1 diabetes, no significant changes in glycemic control were found [Jialal 2007, Dogra 2005]. The discrepancy between our results and those of these studies may be ascribed to the higher dose of atorvastatin used in our study. Indeed, dose-dependent impairment of insulin sensitivity during atorvastatin treatment has been shown previously [Koh 2010].

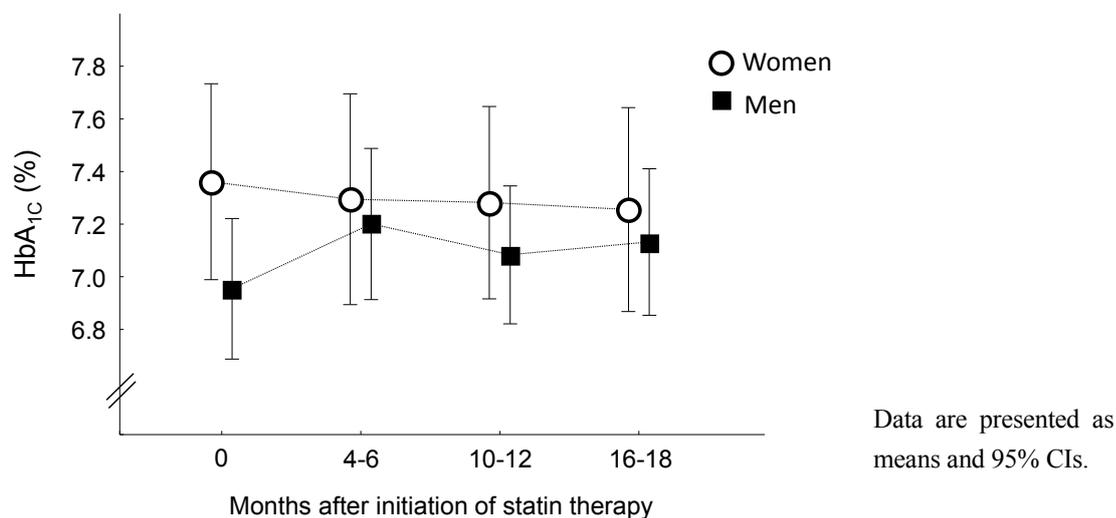
We performed a retrospective cohort study in order to investigate the long-term effects (up to 18 months) of statin treatment on glycemic control in patients with type 1 diabetes. This study included all patients with type 1 diabetes and on-going statin treatment who were followed at the out-patient clinic at the Department of Endocrinology and Diabetology at Danderyd Hospital in January 2010. Of the 1276 registered patients, 399 patients were undergoing statin treatment at the time of the investigation and were included in the study. Information regarding date when statin treatment was started, type and dose of statins used as well as lipid and HbA<sub>1C</sub> levels before and after initiation of statin therapy were obtained from medical records.

Repeated HbA<sub>1C</sub> analyses at 4-6, 10-12, and 16-18 months after initiation of statin therapy were found in 106 patients (36 women). Mean age and diabetes duration in this population were 57 ±10 and 30 ±13 years. Eighty-three patients were treated with intermittent doses of short- and long-acting insulins, and 23 patients were treated with continuous subcutaneous insulin infusion. Most patients (85%) were treated with simvastatin at 10 or 20 mg/day, while 8% were treated with atorvastatin at doses of 20 or 40 mg/day and 5% with pravastatin at 20 mg/day. In 74% of the patients, the type and dose of statin was not changed during the first 18 months.

Figure 13 shows HbA<sub>1C</sub> levels before and up to 18 months after initiation of statin treatment in the 106 patients. In men (n=70), HbA<sub>1C</sub> levels increased from 7.0 ±1.2 to 7.2 ±1.3 (p=0.008) during the first 4-6 months of treatment, while corresponding values in women remained unchanged. After 18 months, HbA<sub>1C</sub> levels in both men and women were unchanged compared with baseline values. Thus, these results indicate that introduction of statin treatment at low/moderate doses does not cause any long-term deterioration of glycemic control, at least when observed for up to 18 months.

Of note is the fact that women had worse glycemic control compared with men, which was also seen in the larger population of Study I and is in accordance with data in the 2012 annual report from the Swedish National Diabetes registry [www.ndr.nu].

Figure 13. HbA<sub>1C</sub> levels in 70 men and 36 women before and up to 18 months after initiation of statin treatment.

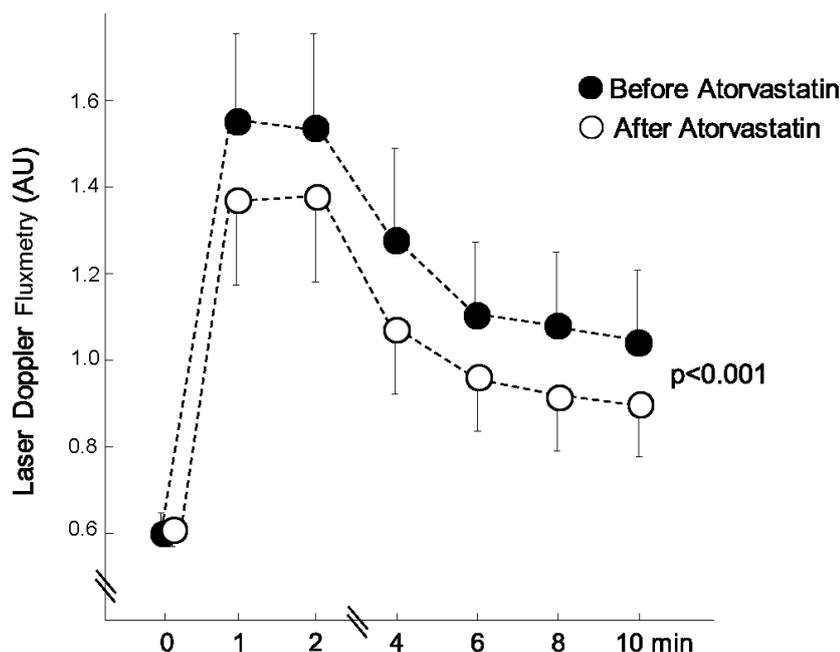


#### 4.9 HIGH-DOSE ATORVASTATIN IMPAIRS SKIN MICROVASCULAR REACTIVITY (PAPER III)

Reduced ACh-mediated flux measured continuously before, during and up to 10 minutes after iontophoresis was observed during atorvastatin treatment ( $p < 0.001$ ; Figure 14), which indicates an impairment in endothelial-dependent function in skin microcirculation. Similarly, ACh-mediated peak flux was lowered during atorvastatin treatment ( $p = 0.03$ ), while no changes were found in endothelium-independent (SNP) microvascular flux during atorvastatin or placebo treatment, as shown in Table 2 in Paper III (Appendices). ACh-mediated peak flux did not correlate with age, diabetes duration, plasma lipids, HbA<sub>1C</sub> levels or clinical signs of microangiopathy (retinopathy, neuropathy and nephropathy).

Levels of the endothelial biomarkers, vWF antigen, endothelin-1 and thrombomodulin, were not changed during atorvastatin or placebo treatment, while EMP levels tended to increase during atorvastatin treatment ( $p = 0.056$ , see Table 2 in Paper III in the appendices). No correlations were found between endothelial-dependent (ACh) peak flux and the endothelial markers, including EMPs. No carry-over effects were found as regards the endothelial biomarkers or skin microvascular reactivity before and after ACh and SNP iontophoresis.

Figure 14. Acetylcholine-mediated skin microvascular reactivity before and after atorvastatin treatment.



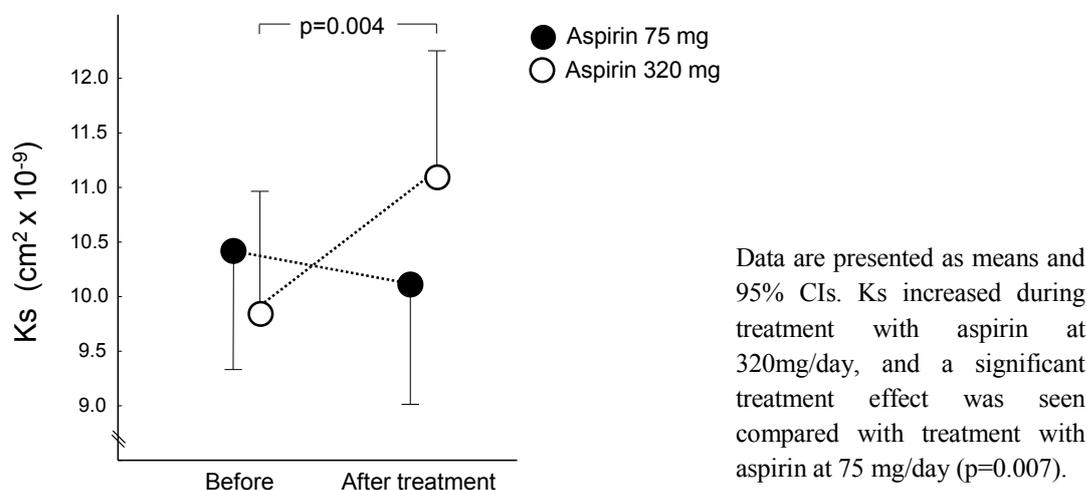
Data are presented as means and 95% CIs. ACh-mediated (endothelium-dependent) microvascular reactivity was reduced during atorvastatin treatment ( $p < 0.001$ , repeated measures ANOVA).

The trend towards increased EMP levels in this study is in accordance with the results of significantly increased EMPs during treatment with atorvastatin (80 mg/day) in patients with peripheral arterial occlusive disease [Mobarrez 2012]. However, interpretation of the changes in circulating EMP levels during statin treatment is difficult, as the involvement of EMPs in vascular function is unclear. In vitro studies have revealed both beneficial effects of EMPs on endothelial cell survival and repair [Dignat-George 2011], and direct impairment of endothelium-dependent vasodilatation via mechanisms involving diminished production and/or bioavailability of nitric oxide [Brodsky 2004]. Inverse relationships between EMP levels and endothelium-dependent coronary and brachial vasodilatation have been demonstrated [Koga 2005, Amabile 2005, Werner 2006], while associations between EMP levels and microvascular reactivity have not previously been studied. Importantly, the origin of the circulating pool of EMPs is unclear and we can therefore only speculate upon whether the tendency towards increased EMP levels in our study could be associated with our finding of impaired endothelium-dependent reactivity in skin microcirculation.

#### 4.10 HIGH-DOSE IS ASPIRIN REQUIRED TO AFFECT FIBRIN CLOT PROPERTIES (PAPER IV)

In the 41 patients who completed this cross-over study, no changes in fibrin clot permeability (Ks) were found during treatment with aspirin at 75mg/day, while Ks increased from  $9.8 \pm 3.3$  to  $11.0 \pm 3.4 \text{ cm}^2 \times 10^{-9}$  ( $p=0.004$ ) during treatment with aspirin at 320mg/day (Figure 15). Similarly, turbidimetric clotting assays showed increased lag time ( $603 \pm 144$  to  $640 \pm 142 \text{ s}$ ,  $p=0.01$ ) during treatment with aspirin at 320mg/day, while no changes were found during treatment with aspirin at 75mg/day. Clot density and lysis time were not changed during treatment with aspirin at either 75 or 320 mg/day. No changes were found in PMPs, plasma fibrinogen, HbA<sub>1C</sub> or lipid levels during treatment with aspirin.

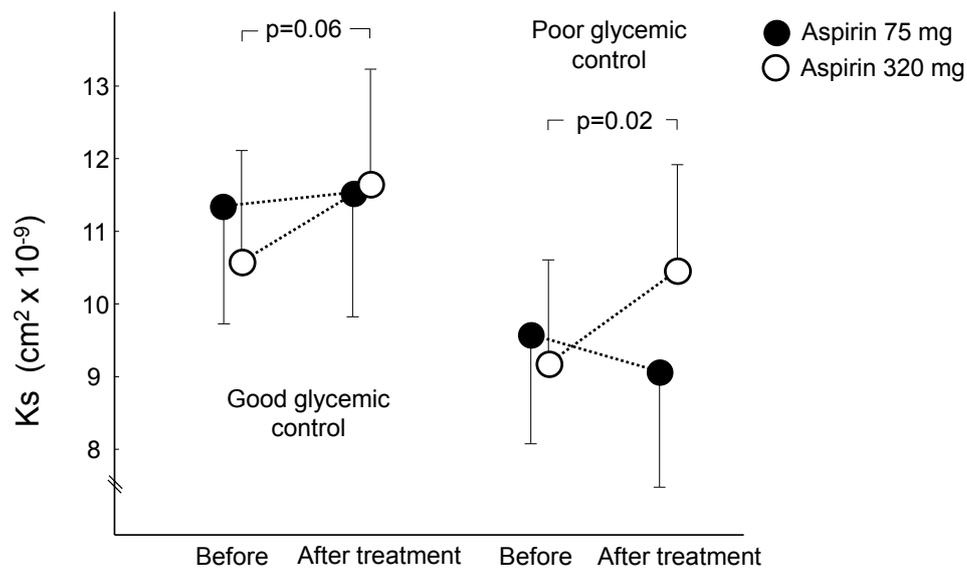
Figure 15. Fibrin clot permeability coefficient (Ks) during treatment with aspirin 75mg and 320 mg/day.



Subgroup analyses were performed to compare treatment effects of aspirin in patients with good (HbA<sub>1C</sub> <6.5%, 57 mmol/mol) and poor (HbA<sub>1C</sub> >7.5%, 68 mmol/mol) glycemic control. Baseline fibrin clot parameters indicated denser and less permeable clots and longer clot lysis time in patients with poor glycemic control, although these differences did not reach statistical significance (data not shown). Similar analysis among the 236 patients in Study I showed more prothrombotic fibrin clot properties with statistically significant differences between patients with good and poor glycemic control (see Results section 4.2).

During treatment with high-dose aspirin (320 mg/day), Ks levels increased in patients with poor glycemic control ( $p=0.02$ ), while they tended to increase in patients with good glycemic control ( $p=0.06$ ; Figure 16). In the turbidimetric assays, lag time increased during treatment with high-dose aspirin in patients with poor glycemic control ( $578 \pm 147$  to  $627 \pm 114 \text{ sec}$ ;  $p=0.02$ ), while no significant changes were found in clot density and lysis time in either patient group during treatment with low or high doses of aspirin.

Figure 16. Fibrin clot permeability coefficient (Ks) during treatment with aspirin at 75mg/day and 320 mg/day in patients with good and poor glycemic control, respectively.



Data are presented as means and 95% CIs. No significant treatment effect was found in patients with good glycemic control, whereas Ks increased during treatment with aspirin at 320 mg/day in patients with poor glycemic control (p=0.02).

Thus, high-dose aspirin treatment induced more permeable fibrin clots, especially in the patients with poor glycemic control, while clot density and lysis time remained unaffected. These results are consistent with early findings reported by Williams et al. [1998] showing significantly increased fibrin clot permeability despite no changes in clot lysis time following 3 weeks of daily treatment with aspirin at 75 mg/day in healthy individuals. Unexpectedly, treatment with aspirin at 320 mg/day in healthy subjects had no effect on clot permeability.

Increased lag time observed during treatment with aspirin at 320 mg/day in patients with poor glycemic control in the present study indicates inhibited fibrin polymerization in the initial phase of clot formation, since the fibrin oligomers need to grow to a sufficient length to be able to aggregate laterally and form fibrin fibers (which is the point when absorbance increases during turbidimetric assays). This may be an effect of acetylation of the fibrinogen molecule, which in patients with type 1 diabetes seems to require a higher dose of aspirin, as aspirin at 75 mg/day did not affect the lag time.

## 5 SUMMARY & DISCUSSION

### 5.1 PAPER I

The two main findings in this Study are that fibrin clot properties *in vitro* are not different between men and women with type 1 diabetes and no history of CVD, and that microvascular complications are associated with more prothrombotic fibrin clots in patients with type 1 diabetes. Altered fibrin clot properties observed in patients with microvascular complications are not surprising since common factors such as impaired glycemic control and increased fibrinogen concentrations affect both these entities. Investigation of a possible causal relationship between altered fibrin clot properties *in vitro* and diabetic microangiopathy would be of interest in future studies.

Regarding the influence of sex on fibrin clot properties, our hypothesis was that women would have tighter and less permeable fibrin clot structures *in vitro* compared with men, since the increased risk of CVD associated with diabetes is higher in women than in men. However, no differences in fibrin clot properties were found between the sexes. This result could actually agree with the loss of female protection against CVD in patients with diabetes, given the assumption that assessment of fibrin clot structure in a healthy population would show less prothrombotic fibrin clot properties in women than in men. Since no such study has yet been presented, we cannot draw such conclusion.

Interestingly, FXIII antigen levels were increased in women with type 1 diabetes. Since FXIIIa incorporates  $\alpha_2$ -antiplasmin and other proteins within the clot, it could be speculated that women with type 1 diabetes may have a preponderance to form fibrin clots that are more resistant to degradation *in vivo*, despite similar clot characteristics *in vitro*.

Fibrin clots in younger females with type 1 diabetes were less permeable and more resistant to degradation than in age-matched men. Although this observation was made in a small population of 35 individuals, it suggests that more prothrombotic fibrin clots are formed in younger females. If our data were to be confirmed in a larger study, it could be hypothesized that the increased risk of CVD in women with type 1 diabetes is explained by a metabolic imbalance during adolescence, resulting in a prothrombotic profile that affects their future cardiovascular risk. This “metabolic memory” could thus influence the future risk of vascular complications even after hemostatic function is restored, in a similar fashion as described in the DCCT study in which past hyperglycemia could be a risk factor as regards future vascular complications [White 2008]. Indeed, it has been shown that pre-adolescent girls and adolescent females with type 1 diabetes require higher insulin doses per kg body weight [Mortensen 1998], and have higher BMI, worse glycemic control and more lipid abnormalities [Schwab 2010], compared with corresponding males. The increased metabolic imbalance in females with type 1 diabetes may be a result of higher insulin resistance in girls compared with boys. In support of this theory is the fact that healthy girls seem to be less insulin sensitive than healthy males, and that decreased insulin sensitivity in females seems to

be compensated by increased insulin secretion, in order to maintain blood glucose at normal levels [Hoffman 2000]. Interestingly, a recent study demonstrated that early onset of type 1 diabetes (before 14 years of age) was associated with markedly higher mortality rates from future ischemic heart disease in women than in corresponding men [Harjutsaldo 2013]. This result suggests that the accelerated progression of atherosclerosis in patients with type 1 diabetes, which starts early in childhood, may be more pronounced in girls than boys. Altogether, the results of these studies agree with the hypothesis of metabolic deterioration in females with type 1 diabetes during childhood and/or adolescence, which may affect their future cardiovascular risk.

Plasma PAI-1 activity levels were within the lower reference values in both men and women in the patients with type 1 diabetes in the present study and not different between the sexes, while lower PAI-1 activity levels have been reported in healthy women compared with age-matched men [Takada 1989]. Levels of tPA-PAI-1 complex were also within the lower reference range in both men and women. Men with type 1 diabetes had higher levels of tPA-PAI-1 complex compared with women in our study, which also has been shown in a previous study on healthy subjects of <50 years of age [Takada 1989]. As mentioned in the Introduction (section 1.1.5), it has been suggested that PAI-1 may be incorporated in the forming clots and thus, although the levels of PAI-1 activity and tPA-PAI-1 complex in plasma in this study population were relatively low, patients with type 1 diabetes may hypothetically have an increased PAI-1 incorporation within the clots which could increase resistance of clot degradation. As FXIIIa incorporates various proteins in the clot, increased FXIII levels in women with type 1 diabetes in the present study could be associated with higher PAI-1 incorporation.

## **5.2 PAPERS II & III**

In Study II we showed that treatment with atorvastatin at a high dose (80 mg/day) is associated with increased fibrin clot permeability in patients with type 1 diabetes. Notably, the fibrinogen concentrations remained unchanged during the same treatment period. Since fibrinogen levels seem to affect clot density more than pore size in the forming clots (see Results, section 4.5), it can be speculated that the positive effects on fibrin clot structure during atorvastatin treatment are primary due to reduced branching points (larger pore size) rather than increased lateral aggregation (thicker fibers) during fibrin polymerization. In addition, reduced thrombin generation potential and indication of reduced platelet activity was found during atorvastatin treatment in patient with type 1 diabetes. These beneficial results are in accordance with previous findings of statin treatment effects in different populations [Undas 2005, Notarbartolo 1995]

Unexpectedly, skin endothelial-dependent microvascular reactivity was reduced during atorvastatin therapy. Since endothelial-independent vasoreactivity remained unchanged during the same treatment period, our data reflect an impaired endothelial function in skin microcirculation following high-dose atorvastatin treatment. Treatment with

atorvastatin was also associated with impaired glycemic control. Strong relationships between ACh-mediated (endothelial-dependent) skin microvascular reactivity and insulin sensitivity have previously been described in the literature [Serné 1999]. Hence, a causal relationship between impaired glycemic control and endothelium-dependent skin microvascular dysfunction during high-dose atorvastatin treatment may exist, despite no significant correlations found between these two variables in the regression analyses of the present study. Importantly, impaired skin microvascular reactivity during high-dose atorvastatin treatment may reflect deteriorated microvascular function in other organs as skin microcirculation seems to be a marker of generalized microvascular function [Roustit 2012, Rendell 1992, Holowatz 2008, Chang 1997]. Our findings thus motivate further investigations on the effects of high-dose (lipophilic) statins on microvascular function and diabetic microangiopathy.

### **5.3 PAPER IV**

In this study we showed that low-dose (75 mg/day) aspirin treatment has no effect on fibrin clot properties in patients with type 1 diabetes and with no history of CVD, while treatment with high-dose (320 mg/day) aspirin was associated with prolonged clotting time (lag time) and more permeable fibrin clots, especially in patients with poor glycemic control. The fact that high-dose aspirin had a greater effect on fibrin clot properties in patients with poor glycemic control does not support our idea of a possible competition between glycation and acetylation on the fibrinogen molecule. Accordingly, Svensson et al., recently revealed that glucose and aspirin bind to different lysine residues on the fibrinogen molecule in a mass spectrometry study [Svensson 2012]. Furthermore, the lack of effect on clot density during aspirin treatment, while lag time and clot permeability were increased in our study and in a study by Williams et al. [1998], suggests that modification of fibrin clot structure during aspirin therapy is mainly due to reduced fiber branching.

The observed lack of effect of low-dose aspirin on fibrin clot properties in the present study may contribute to aspirin treatment failure in patients with diabetes mellitus. The positive effects of high-dose aspirin on fibrin clot characteristics *in vitro*, especially in patients with poor glycemic control who are at increased risk of CVD, suggest that these patients might benefit from aspirin treatment at a higher dose than the recommended treatment at 75 mg/day. Our data thus suggests that treatment with aspirin at 320 mg/day instead of 75 mg/day should perhaps be considered in certain patients with type 1 diabetes in which effective antiplatelet therapy is crucial, for instance patients who recently have received a coronary stent. However, increased risk of bleeding and unwanted effects on protective prostaglandins when aspirin is given at a higher dose also needs to be considered. Especially since the low PAI-1 levels frequently found in patients with type 1 diabetes may increase their proneness to bleeding.

## **5.4 LIMITATIONS**

Two considerations are important to note in this work. Firstly, the principal aim was to investigate fibrin clot properties and other hemostatic functions in patients with type 1 diabetes. Patients with a history of macrovascular events were excluded in order to avoid the influence of treatments with vasoactive and anticoagulant drugs on the investigated variables. Therefore, the results cannot be extrapolated to patients with type 1 diabetes and CVD. Secondly, fibrin clot properties were investigated in platelet-poor plasma from venous blood sampling using a certain concentration of thrombin, and thus the influence of in vivo thrombin generation, platelet activation and possible interaction of endothelial and blood cells on fibrin clot formation in vivo was excluded.

## 6 CONCLUSIONS

- Men and women with type 1 diabetes and no history of macrovascular disease have similar fibrin clot properties in vitro.
- Patients with type 1 diabetes and microvascular complications form more prothrombotic fibrin clots in vitro, suggesting that fibrin clot properties are associated with microvascular function.
- High-dose (80 mg/day) atorvastatin treatment in patients with type 1 diabetes and dyslipidemia is associated with increased fibrin clot permeability, while endothelial-dependent skin microvascular function is impaired.
- Treatment with high-dose aspirin (320mg/day) affects fibrin polymerization and increases the fibrin clot permeability, whereas treatment with low-dose (75 mg/day) aspirin has no effect on fibrin clot characteristics in patients with type 1 diabetes.

## 7 SVENSK SAMMANFATTNING

Typ 1-diabetes är en livslång sjukdom som ofta debuterar i barndomen eller tidiga tonåren, men kan förekomma i alla åldrar. Sjukdomen kännetecknas av att bukspottkörteln inte kan producera insulin, det hormon som krävs för att kroppen ska kunna använda sig av sockret (**glukos**) i blodet. Utan insulin svälter således kroppen och blodsockernivån stiger. Behandlingen består i att tillföra kroppen insulin dagligen under resten av livet. Innan upptäckten av insulin 1921 hade sjukdomen en dödlig utgång.

Typ 1-diabetes utgör cirka 10-15% av all diabetes i Sverige enligt årsrapporten från Nationella Diabetesregistret år 2012, vilket innebär att upp till 0.6% av befolkningen lider av sjukdomen. Dessa patienter drabbas ofta av småkärlsjuka, vilket leder till ögonbottenförändringar (**retinopati**) och en försämrad syn, nedsatt njurfunktion (**nefropati**) och nervskador (**neuropati**). Dessutom har patienter med typ 1-diabetes en ökad risk för hjärtkärlsjukdomar (**kardiovaskulära sjukdomar**) och kan drabbas av hjärtinfarkt redan före 40-års åldern. Normalt har kvinnor en lägre risk för hjärtkärlsjukdomar jämfört med män, men kvinnor med diabetes tycks förlora detta skydd, och har minst lika hög risk att drabbas av hjärtkärlsjukdomar som män. Orsaken till detta är okänd och har hittills varit dåligt studerad.

Den höga frekvensen av kärlsjukdomar hos patienter med typ 1-diabetes förklaras till viss del av deras förhöjda blodsockernivåer. Orsaken är dock multifaktoriellt och andra faktorer, så som genetiska orsaker, rubbningar i fettbalansen (**dyslipidemi**) och nedsatt känslighet för insulin i olika vävnader (**insulinresistens**) bidrar också till utveckling av kärlkomplikationer. Det har även visats att patienter med typ 1-diabetes har förändrad **hemostas**, kroppens förmåga att bilda och bryta ned blodproppar, vilket har betydelse för deras ökade risk för kärlsjukdomar. En välfungerande hemostas med förmåga att snabbt kunna bilda blodproppar (**koagulation**) är viktig för att förhindra blödningar vid sår- och vävnadsskador, och det är också viktigt att kroppen så småningom bryter ned den bildade blodproppen för att återställa blodflödet igen. Nedbrytning av blodproppen, s.k. **fibrinolysen**, är en långsam process som kan ta flera timmar-dagar, vilket ger det skadade blodkärlet en chans att läka.

Vid proppbildning omvandlas ett protein i blodet, **fibrinogen**, till olösligt form, **fibrin**, vilket bildar trådar som korsbinder till varandra och formar ett fint nätverk. Strukturen på detta **fibrinnätverk** varierar beroende på olika faktorer i blodet och påverkar kroppens förmåga att bryta ned den bildade proppen. Blodproppar med ett tätt och kompakt fibrinnätverk löses upp långsammare jämfört med blodproppar som har lös och lucker struktur. Fibrinnätverkets struktur kan undersökas **in vitro** (utanför kroppen; ”i glaset”) via ett vanligt blodprov, och blodproppens täthet och permeabilitet kan bestämmas med hjälp av särskilda metoder. Tidigare studier har visat att patienter med hjärtkärlsjukdomar har ett tätare och mindre genomsläppligt fibrinnätverk jämfört med friska kontrollpersoner. Det har även i ett fåtal små studier visats att patienter med typ 1-diabetes har ett tätare fibrinnätverk jämfört med friska kontroller. Bildning av tätare blodproppar med långsammare nedbrytning hos patienter med typ 1-diabetes kan vara ytterligare en bidragande orsak till deras ökade risk för hjärtkärlsjukdomar.

Syftet med denna avhandling har varit att närmare undersöka hemostas hos patienter med typ 1-diabetes, med särskilt fokus på fibrinnätverkets struktur. Vi undersöker fibrinnätverket genom bestämning av permeabilitetskoefficienten (**Ks**) och via **turbidimetrisk metod**, som mäter ljusabsorption i blodproppen, vilket är ett mått på dess täthet.

Avhandlingen består av fyra delarbeten:

**Delarbete 1** inkluderade 236 patienter (107 kvinnor) med typ 1-diabetes. Patienter med hjärtkärlsjukdom har exkluderats från studien, då dessa patienter behandlas med mediciner som påverkar fibrinnätverkets struktur. Huvudsyftet med studien var att undersöka om fibrinnätverkets struktur skiljer sig mellan män och kvinnor med typ 1-diabetes. Resultaten visade att fibrinnätverkets permeabilitet, täthet och nedbrytningshastighet inte skiljer sig mellan könen. Vi kunde däremot visa att patienter med småkärlsjuka (i ögonbottnar, njurar eller nervbanor) har ett tätare och mindre genomsläppligt fibrinnätverk. Resultaten har sammanställts i ett manuskript-

**Delarbete 2** inkluderade 20 patienter (10 kvinnor) med typ 1-diabetes och förhöjda blodfetter. Syftet med studien var att undersöka om blodfettssänkande medicin med **atorvastatin** (80mg dagligen) påverkar fibrinnätverkets struktur. Atorvastatin är en **statin**, en medicingrupp som förebygger hjärtkärlsjukdom och som används för att sänka blodfetter hos individer med förhöjda värden samt hos patienter med genomgången hjärtinfarkt eller stroke. Tidigare studier har visat att statiner inte bara sänker blodfetterna utan även kan motverka proppbildningsförmågan. Samtliga 20 patienter i denna studie behandlades med atorvastatin eller placebo under två månader. Resultaten visade att fibrinnätverkets permeabilitet ökade under behandling med atorvastatin, och har publicerats i tidskriften *Thrombosis Research* 2010.

**Delarbete 3** baseras på resultat från samma studie (atorvastatin-studien) som beskrevs ovan. Syftet med detta delarbete var att undersöka hur behandling med atorvastatin påverkar hudens **mikrocirkulation**. Studier har visat att hudens mikrocirkulation, det vill säga blodflödet i de allra minsta blodkärlen i huden, återspeglar kärlfunktionen i andra organ, så som mikrocirkulationen i ögonbotten. Vår hypotes var att behandling med atorvastatin har en positiv påverkan på mikrocirkulationen, men resultaten visade tvärtom en negativ effekt på hudens mikrocirkulation och även en försämrad blodsockerkontroll. Denna potentiellt skadliga effekt av atorvastatin är oklar, och kan mycket väl bero på den höga dos av atorvastatin (80 mg) som använts i studien. Kanske höga doser av statiner ska användas med större försiktighet hos patienter med typ 1-diabetes, med anledning av dessa resultat. Resultaten har publicerats i tidskriften *Diabetes & Vascular Disease Research* 2013.

**Delarbete 4** inkluderade 48 patienter med typ 1-diabetes: 24 patienter (12 kvinnor) med bra blodsockerkontroll och 24 patienter (12 kvinnor) med dålig blodsockerkontroll. Patienterna behandlades med låg- (75 mg) respektive hög-dos (320 mg) av acetylsalicylsyra (ASA) under fyra veckor. Behandling med låg-dos (75 mg) ASA används för att förebygga hjärtkärlsjukdom hos patienter med genomgången hjärtinfarkt eller stroke. Patienter med diabetes tycks dock ha en sämre effekt av

behandlingen. Orsaken till denna s.k. ASA-resistensen är oklar. Tidigare studier har visat att behandling med 75 mg ASA har gynnsam effekt på fibrinnätverkets struktur hos friska individer. Syftet med denna studie var att undersöka effekten av låg- respektive hög-dos ASA på fibrinnätverkets struktur hos patienter med bra- respektive dålig blodsockerkontroll. Hypotesen var att den högre dosen av ASA behövs för att påvisa effekter på fibrinnätverkets struktur hos patienter med typ 1-diabetes samt att patienter med bra blodsockerkontroll har en bättre effekt av behandlingen jämfört med patienter med dålig blodsockerkontroll. Resultaten visade att behandling med ASA 75 mg inte alls påverkade fibrinnätverkets struktur, medan behandling med ASA 320 mg ökade permeabiliteten i fibrinnätverket. Denna positiva effekt av hög-dos (320 mg) ASA på fibrinnätverkets struktur var mest uttalad hos patienter med dålig blodsockerkontroll. Resultaten har publicerats i tidsskriften *Diabetes Care* 2012.

Sammanfattningsvis visar resultaten i denna avhandling:

- Att fibrinnätverkets struktur hos patienter med typ 1-diabetes inte skiljer sig mellan könen.
- Att patienter med småkärlssjuka har tätare och mindre permeabelt fibrinnätverk.
- Att behandling med hög-dos (80 mg) atorvastatin har en positiv påverkan på fibrinnätverkets struktur, men samtidigt försämras blodsockerkontrollen och hudens mikro-cirkulation.
- Att behandling med låg-dos (75 mg) ASA inte påverkar fibrinnätverkets struktur hos patienter med typ 1-diabetes, medan behandling med ASA 320 mg har en gynnsam effekt på fibrinnätverket.

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