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**STUDIES OF  
HAEMOSTASIS  
IN ACUTE CORONARY  
SYNDROMES  
AND DIABETES MELLITUS**

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**Long you live and high you fly  
And smiles you'll give and tears you'll cry  
And all you touch and all you see  
Is all your life will ever be.**

**Run, rabbit run.  
Dig that hole, forget the sun,  
And when at last the work is done  
Don't sit down it's time to dig another one.**

*Breathe, The Dark Side of the Moon  
By Pink Floyd*



## ABSTRACT

The pathophysiology of acute coronary syndromes (ACS) includes atherosclerotic plaque rupture and coronary thrombus formation. Antithrombotic treatment is effective but recurrent atherothrombotic or bleeding complications are not uncommon.

**Aim:** To study new markers and methods concerning haemostasis in ACS and conditions associated with high risk of this disease, in the search for laboratory tools that could help increase understanding of disease mechanisms and help to identify patients at risk.

**Methods and Results:** Eighty-seven patients suffering from ACS were investigated at admission (S1), after 24 h on standard antithrombotic treatment (S2), and six months after the acute event (S3). Sex- and age-matched healthy controls were also investigated. Thrombin generation *in vivo* was assessed by measurement of prothrombin fragment F1+2 in plasma and *in vitro* by using the calibrated automated thrombogram (CAT). Fibrinolysis was measured by assessment of PAI-1 and TAFI activity concentrations. The latter method was used as a result of a methods evaluation study. We also employed a global method developed by our group (Oh-index), to evaluate haemostasis. Oh-index gives a measure of fibrin formation and degradation capacity in plasma. Furthermore, a flow cytometric assay set up by our group was employed to measure platelet microparticles (PMP) in plasma formed upon platelet activation. In addition, we investigated ADAMTS13, an enzyme previously called von Willebrand factor (VWF)-degrading protease, and we also measured its substrate (i.e. VWF). The ACS patients, of whom more than half were high-risk patients (TIMI score  $\geq 4$ ), showed signs of inflammation and endothelial activation, as expected. Only the CAT method could detect hypercoagulability in the patients (increased peak thrombin concentration) and this finding was evident acutely and 6 months after the event. Thrombin generation *in vivo* (F1+2) or fibrin generation capacity in plasma did not indicate hypercoagulability at any time point. CAT, F1+2 and fibrin generation capacity were strongly reduced following initiation of antithrombotic treatment (S2), as expected. PAI-1 and TAFI levels were elevated, reflecting impaired fibrinolysis, but this was not observed with our method that assesses fibrin degradation capacity; rather, this method indicated increased fibrinolytic capacity at admission and this capacity was grossly increased after initiation of standard antithrombotic treatment (S2). ADAMTS13 activity and antigen concentrations were unchanged during and after ACS, but the VWF:ADAMTS13 ratio was significantly elevated in ACS patients and two different populations of patients with diabetes mellitus. The ACS patients had significantly elevated concentrations of PMP at admission, particularly PMP subpopulations with exposed P-selectin and tissue factor (TF). Concentrations of PMP decreased following initiation of antithrombotic treatment (S2), but in the subpopulations with exposed P-selectin and TF they remained significantly higher than in controls at 6 months (S3).

**Conclusions:** Our PMP data are in agreement with the concept of a dominating role of platelets in the pathophysiology of ACS, and PMP deserve to be studied in more detail in coronary artery disease, including their roles in the effects of treatment and relationships to coagulation, risk and prognosis. However, the data on coagulation and fibrinolysis obtained in this study indicate that there is not yet sufficient information to support the clinical use of markers to assess coagulation or fibrinolysis in individual patients.

## LIST OF PUBLICATIONS

This thesis is based on the following original papers, which will be referred to in the text by their Roman numerals I-IV.

- I. Skeppholm M**, Kallner A, Kalani M, Jörneskog G, Blombäck M, Wallén NH.  
ADAMTS13 and von Willebrand factor concentrations in patients with diabetes mellitus.  
*Blood Coagul Fibrinolysis*. 2009;20:619-26.
- II. Skeppholm M**, Wallén NH, Malmqvist K, Kallner A, Antovic J.  
Comparison of two immunochemical assays for measuring thrombin activatable fibrinolysis inhibitor concentration with a functional assay in patients with acute coronary syndrome.  
*Thromb Res*. 2007;121:175-81.
- III. Skeppholm M**, Kallner A, Malmqvist K, Blombäck M, Wallén NH.  
Is fibrin formation and thrombin generation increased during and after an acute coronary syndrome?  
*Thromb Res*. 2011;128:483–89.
- IV. Skeppholm M**, Mobarrez F, Malmqvist K, Wallén NH.  
Platelet derived microparticles during and after acute coronary syndrome.  
*Submitted*.

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## LIST OF ABBREVIATIONS

ACS	=	acute coronary syndromes
AMI	=	acute myocardial infarction
AUC	=	area under the curve
BMI	=	body mass index
CAD	=	coronary artery disease
CAI	=	coronary angiography
CAT	=	calibrated automated thrombogram
Cp	=	Coagulation profile
DAT	=	double antiplatelet treatment
DM	=	diabetes mellitus
ELISA	=	Enzyme-Linked Immunosorbent Assay
ETP	=	endogenous thrombin potential
Fp	=	Fibrinolysis profile
FRETs-	=	Fluorescence resonance energy transfer (FRET) assay
VWFF73	=	using a synthetic 73-amino-acid peptide of VWF
hsCRP	=	high sensitive C-reactive protein
IL-6/IL-1	=	interleukin-6, interleukin-1
LMH	=	low molecular weight heparin
MP	=	microparticles
NSTEMI	=	non ST-elevation myocardial infarction
Oh-index	=	Overall Haemostasis Index
OHP	=	Overall Haemostasis Potential
PAI-1	=	plasminogen activator inhibitor-1
PAOD	=	peripheral artery occlusive disease
PMP	=	platelet microparticles
Pro-BNP	=	pro B-type natriuretic protein
PS	=	phosphatidylserine
STEMI	=	ST-elevation myocardial infarction
TAFI	=	thrombin-activatable fibrinolysis inhibitor
TF	=	tissue factor
TFPI	=	tissue factor pathway inhibitor
TIMI	=	Thrombolysis In Myocardial Infarction (risk score)
VWF	=	von Willebrand factor
UFH	=	unfractionated heparin
ULVWF	=	ultra large von Willebrand factor

## LIST OF PHARMACEUTICALS

clopidogrel	=	platelet inhibitor
aspirin	=	platelet inhibitor
prasugrel	=	platelet inhibitor
ticagrelor	=	platelet inhibitor
bivalirudin	=	direct thrombin inhibitor
enoxaparin	=	low-molecular weight heparin
dalteparin	=	low-molecular weight heparin
warfarin	=	oral anticoagulant
tirofiban	=	GPIIb/IIIa inhibiting agent



# 1 INTRODUCTION

## 1.1 General background

Coronary artery disease (CAD) is still the leading cause of both morbidity and mortality in the Western world. It is a heterogeneous disease with respect to pathophysiology, presentation, prognosis as well as effects of treatment. The pathophysiological mechanisms of the atherosclerotic process in the arteries, which is the dominating cause of CAD, include inflammation, endothelial dysfunction/activation and neurohormonal activation. Rupture of an atherosclerotic plaque in a coronary artery is in most individuals the trigger of acute coronary syndromes (ACS), accounting for about 70% of fatal events (White 2008). ACS consists of two types of patients, those with unstable angina/non-ST-elevation myocardial infarction (UAP/NSTEMI) and those with ST-elevation myocardial infarction (STEMI). This thesis is focused on the former group, patients with UAP/NSTEMI, an unstable condition shown to have an even worse prognosis than STEMI (Terkelsen 2005).

Rupture or erosion of an atherosclerotic plaque exposes highly thrombogenic components, leading to activation of both platelets and the coagulation system, thereby initiating thrombus formation. Platelet hyper-reactivity as well as procoagulant states with hypercoagulation and/or hypofibrinolysis could also contribute to a thrombotic condition. The results of several large studies support the idea that ACS is a prothrombotic condition, since it is well documented that antithrombotic treatment with combinations of different antiplatelet drugs (Yusuf 2001; Wiviott 2007; Wallentin 2009) or antiplatelet drugs in combination with oral anticoagulating agents (Hurlen 2002) reduce the risk of mortality and recurrent ischaemic events. This has led to broad antithrombotic treatment regimes in ACS, especially during the acute phase. However, there is also evidence of beneficial effects of prolonged antithrombotic treatment and today ACS patients commonly receive double antiplatelet treatment (DAT) for up to 3–12 months after the acute event (Yusuf 2001). In selected patients there is even a need for so-called “triple therapy”, i.e. DAT in combination with warfarin, even though this treatment has not yet been documented in randomized trials.

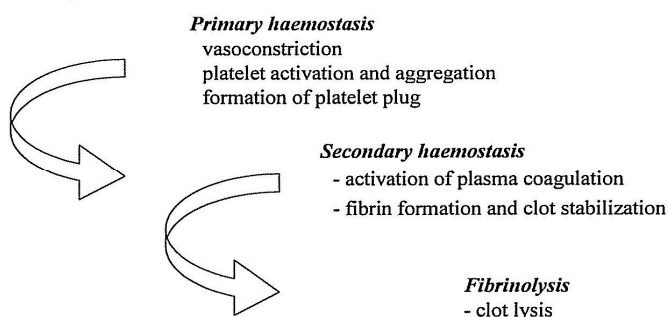
In spite of this intense improved antithrombotic treatment, ongoing activation of coagulation after ACS can be detected (Eikelboom 2002) and it seems to be more common in patients with recurrent ischaemic events (Eikelboom 2002, Oldgren 2001). Furthermore, the hypothesis that remaining platelet hyper-reactivity increases the risk of recurrent events (Snoep 2007; Krasopoulos 2008) is supported by the fact that more potent antiplatelet agents, e.g. prasugrel and ticagrelor, reduce the risk of complications related to an acute event (Wiviott 2007; Wallentin 2009). This indicates that there could exist a prolonged condition of both hypercoagulation and platelet hyper-reactivity after ACS and clearly there is a need for extended antithrombotic treatment in selected patients. This has led to expanded use and a market for antithrombotic agents. As a consequence, patients in an acute situation could be treated with three different antiplatelet agents (e.g. aspirin + clopidogrel + GPIIb/IIIa receptor blocker) at the same time and in combination with

anticoagulating agents (e.g. bivalurudin, LMH or UFH). This combined treatment with antithrombotic agents with effects on different pathways of haemostasis clearly carries an increased risk of bleeding. Several large studies have shown that both major and minor bleeding in the setting of ACS is linked to a poor prognosis (Eikelboom 2006; Budaj 2009). It is therefore important to search for tools that can help to identify individuals prone to bleeding.

For clinical use, score systems based on combinations of clinical factors and laboratory variables have been developed for determination of a patient's risk of thrombosis (Antman 2000; Eagle 2004) or bleeding (Subherwal 2009; Moscucci 2003). However, no risk score for prediction of bleeding has yet become established in clinical daily practice, at least not in Sweden. At present we lack simple clinically useful laboratory methods which can be used to identify ACS patients that are in a pronounced prothrombotic state, and distinguish them from individuals with an increased bleeding tendency. In the present project we have studied some new methods and markers of both coagulation and fibrinolysis, and platelet activation, in the search for possible tools for the assessment of haemostatic balance in ACS patients.

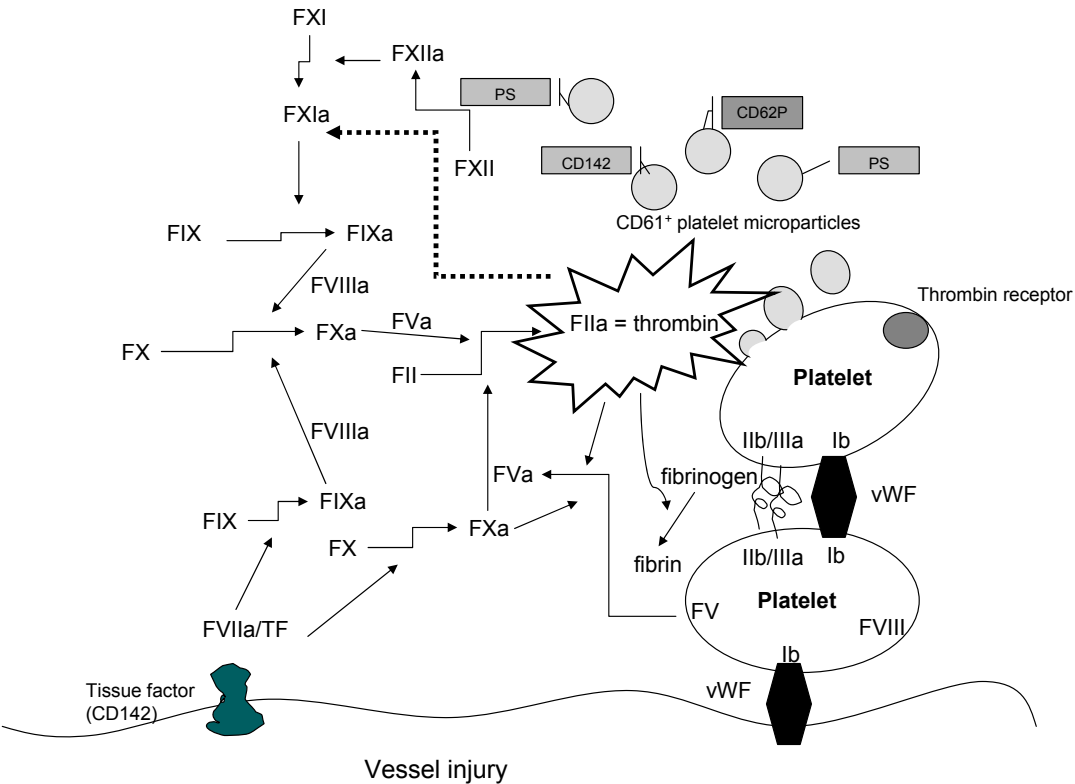
## 1.2 Haemostasis; assessment and disturbances

The term *haemostasis* describes the physiological mechanisms that produce a localized thrombus at the site of a vessel injury, thereby preventing bleeding while maintaining blood flow in the damaged vessel. In normal conditions haemostasis is a balanced, ongoing process, but after a vessel injury with damaged endothelium the interactions between the balancing systems are markedly increased. Haemostasis includes the whole clotting process including platelet plug and clot formation as well as lysis, and is usually classified as follows:



**Figure 1:** Steps in haemostasis

Haemostasis is a very intricate and fine-tuned system in which several proenzymes, enzymes and enzyme inhibitors interact. All the factors necessary for coagulation and fibrinolysis are circulating in the blood and can be activated and inhibited as the need occurs. Vessel damage and intrinsic factors may trigger an imbalance in the system and cause uncontrolled bleeding or excessive coagulation. A schematic summary of the interactions is presented in Figure 2.



**Figure 2.** Schematic figure of haemostasis. The suffix “a” symbolizes an activated form of the factor. The GPIb and GPIIb/IIIa is the receptor for VWF and fibrinogen respectively. TF stands for tissue factor and PS for phosphatidylserine. CD142 and CD62P are synonymous with tissue factor and P-selectin respectively and CD61 is synonymous with GPIIIa.

Disturbances at several levels in the haemostatic system may result in a prothrombotic state. It is reasonable to hypothesize that some of the known risk factors of cardiovascular complications could exert their negative effects through interactions with the haemostatic system. Diabetes mellitus (DM) is a well-known risk factor as regards recurrent ischaemic events and cardiovascular death after myocardial infarction. There are indications that the increased risk is partly related to a proinflammatory and prothrombotic state induced by the metabolic disturbances associated with diabetes. For example, our group has shown that

patients with diabetes form a tighter fibrin network (Jörneskog 1996), and that potent glucose-lowering treatment reduces the tightness of the fibrin network, probably making it less “thrombogenic” (Jörneskog 2003). Furthermore, diabetes is a condition with disturbed platelet function (Ajjan 2006) and impaired endothelial function (Avogaro 2011), both contributing to a prothrombotic state. Indeed, hyperglycaemia in diabetes patients is associated with platelet activation (Yngen 2001; Razmara 2007).

During the past few years, several studies on different haemostatic markers in ACS have been conducted (see below), but few investigators have attempted to transfer the results for use in a clinical setting. As for many other cardiovascular biomarkers (troponin and pro-BNP excluded) there is a lack of information about their usefulness in individualized patient handling (de Lemos 2011). There is still a need for studies of the mechanisms behind haemostatic disturbances in ACS. With increased understanding, new possibilities to characterize patients and optimize and individualize treatment strategies may be revealed.

## **1.2.1 Coagulation**

### **1.2.1.1 *The cell-based model of haemostasis***

Tissue factor (TF) is considered to be the main physiological trigger of the coagulation cascade (formerly called the extrinsic pathway). TF is exposed to the circulation, for example after vessel injury, or presented by activated circulating blood cells/cell fragments and forms there after a complex with small amounts of circulating activated factor VII.

#### ***Initiation phase***

The TF/VIIa complex (the suffix “a” symbolizes an activated form of the factor) starts the “initiation phase” of the cascade by activating more factor VII as well as factor IX and factor X. Factor IXa in complex with factor VIIIa also contributes to the activation of factor X to Xa. Activated factor X complexes with factor Va and converts small amounts of prothrombin (factor II) into thrombin (factor IIa) by splitting of the prothrombin fragment F1+2 (F1+2).

#### ***Amplification phase***

The second step in the cascade takes place on phospholipid surfaces, mostly on activated platelets. The initial small amount of thrombin produced in the “initiation phase” is sufficient to activate platelets to release factor V, which in turn is activated by thrombin or factor Xa into factor Va. Circulating VWF/factor VIII complexes bind to activated platelets and more factor VIII is released and then activated into factor VIIIa by thrombin.

#### ***Propagation phase***

The third and final step includes formation of procoagulant complexes on the phospholipid surfaces of activated platelets. The “tenase” complex, i.e. factor IXa, factor VIIIa and calcium activates factor X into factor Xa. Thereafter the “prothrombinase

complex”, i.e. factor Xa, factor Va and calcium induce a thrombin burst. Thrombin also stimulates on-going coagulation by feedback activation of factor XI into factor XIa. Thrombin then finalizes “the thrombotic process” by converting fibrinogen into fibrin, and through activation of factor XIII. The latter factor cross-links the fibrin monomers and a stable network which stabilizes the platelet plug is formed.

#### **1.2.1.2 The contact pathway**

Thrombin can also be activated by the contact pathway (formerly called the intrinsic pathway) which starts off with factor XII being activated into factor XIIa. Activators of FXII *in vivo* could be collagen, polyphosphates released from dense granules of activated platelets or maybe the surface of the platelets, for example (Mackman 2010; Gailani 2007; Müller 2009). Factor XIIa then activates factor XI, which subsequently activates factor IX. The produced factor IXa then joins the pathway described above and activates factor X into Xa. This pathway has not been regarded as important *in vivo* but recent findings show that mice lacking factor XIIa are resistant to arterial thrombosis in combination with absence of bleeding problems (the latter in agreement with findings in humans), which makes this pathway an interesting target for future antithrombotic treatment (Cheng 2010).

#### **1.2.1.3 Coagulation inhibitors**

Importantly, there are also coagulation inhibitors balancing the reactions described above. Tissue factor pathway inhibitor (TFPI) rapidly inhibits the factor Xa produced during the initiation phase and thereafter inhibits the TF/factor VIIa complex by forming a quaternary complex with TF/factor VIIa and factor Xa. The main inhibitor of thrombin is antithrombin and the thrombin-antithrombin complex (TAT) is formed when antithrombin irreversibly binds to and inactivates thrombin. Antithrombin also inactivates factors IXa, Xa, XIa and XIIa. Thrombin bound to thrombomodulin activates protein C, which in combination with its cofactor protein S inactivates factor VIIIa and factor Va.

#### **1.2.1.4 Coagulation in coronary artery disease**

Activation of the coagulation system in the context of ACS has been illustrated in several studies, e.g. by increased concentrations of F1+2, and/or TAT, both indicating increased thrombin generation *in vivo* (Merlini 1994; Undas 2009). High plasma tissue factor activity is linked to worse prognosis in CAD (Steppich 2009). It is also well known that elevated concentrations of fibrinogen are linked to an increased risk of cardiovascular disease (Maresca 1999), and elevated concentrations of fibrinogen could contribute to the building of a firmer fibrin clot (Silveira 2006). However, being an acute-phase reactant, plasma fibrinogen at elevated levels may reflect ongoing systemic inflammation, in addition to a primary hypercoagulable state.

### 1.2.2 Fibrinolysis

The fibrinolytic system dissolves and removes clots formed within the circulation. The proenzyme plasminogen accumulates in fibrin-rich clots and is activated into plasmin by tissue plasminogen activator (tPA), the main regulator of fibrinolysis, which is released from the vascular endothelium in the vicinity of the clot. Plasmin degrades fibrin into degradation products, i.e. d-dimers, which are formed from cross-linked fibrin only. The main inactivators of fibrinolysis are plasminogen activator inhibitor-1 (PAI-1), which inhibits tPA, and  $\alpha$ 2-antiplasmin, which inhibits plasmin. tPA is protected from inhibition by PAI-1 when bound to fibrin and at the same time its activation of plasminogen is facilitated by the fibrin surface. In addition, plasmin is partly protected from inhibition by  $\alpha$ 2-antiplasmin when bound to fibrin. These mechanisms localize fibrinolysis to the fibrin-rich thrombus.

#### 1.2.2.1 *Fibrinolysis in coronary artery disease*

The importance of the fibrinolytic system in the context of CAD is reflected by studies showing that high plasma concentrations of PAI-1 are independently related to reinfarction (Hamsten 1987) and that elevated PAI-1 activity (Leander 2003), as well as elevated plasma concentrations of tPA/PAI-1 complexes (Nordenhem 2005) are strongly associated with an increased risk of primary myocardial infarction (MI). However, data concerning the role of the fibrinolytic system in arterial thrombosis are not entirely consistent. PAI-1 is associated with inflammation and insulin resistance, both of which are risk factors of CAD and these associations might obscure the possible link between PAI-1 and CAD. Also, the results of tPA antigen are depending on the adjustments for other risk factors in the population studied (Meltzer 2009a). For example, in a prospective study of 3043 patients with stable CAD followed for 2 years, the associations with risk of ischaemic events disappeared as regards PAI-1 activity and PAI-1 antigen after adjustment for parameters reflecting insulin resistance, and as regards tPA antigen after adjustment for the combined variables reflecting insulin resistance, inflammation and endothelial cell damage (Juhan-Vague 1996).

Recently, elevated concentrations of  $\alpha$ 2-antiplasmin were shown to be associated with the risk of myocardial infarction independently of other cardiovascular risk factors (Meltzer 2010).

Elevated concentrations of d-dimer are seen in conditions with increased fibrin production followed by increased fibrinolysis and it is regarded mostly as a marker of coagulation and/or “fibrin turnover”. The usefulness of d-dimer as a diagnostic tool in CAD has been questioned (Gorog 2010).

#### 1.2.2.2 *Thrombin activatable fibrinolysis inhibitor (TAFI)*

An interesting inhibitor of fibrinolysis is the thrombin activatable fibrinolysis inhibitor (TAFI). TAFI, which in its active form is a carboxypeptidase, is synthesized as a proenzyme (pro-TAFI) in the liver and in megakaryocytes. TAFI may be present in the  $\alpha$ -

granules of the platelets and released upon activation (Mosnier 2003). As the name indicates, TAFI is activated by thrombin when complexed with thrombomodulin and it may therefore be viewed as a link between coagulation and fibrinolysis. TAFI decarboxylates partially degraded fibrin, a modification which limits the binding of plasminogen to fibrin in the thrombus, decreases the co-factor activity of fibrin on tPA, and results in reduced activation of plasminogen into plasmin. The decarboxylation of fibrin also causes decreased ability of partially degraded fibrin to protect the produced plasmin from  $\alpha$ 2-antiplasmin (Boffa 2007). TAFI thereby prevents fibrinolysis from proceeding into an acceleration phase, and if present above a threshold concentration it prevents fibrinolysis (Bouma 2006). However, if TAFI activity decreases below a certain threshold, fibrinolysis can accelerate.

Formation of TAFI, and TAFI activity, depend on the pro-TAFI concentration, the amount of pro-TAFI activated into TAFI, and the stability of produced TAFI. The active form of TAFI is relatively unstable, with a half-life of about 10 minutes at body temperature. After a conformational change, degradation is caused by proteolytic cleavage (e.g. by thrombin and plasmin) into an inactive form, TAFIi.

Increased TAFI concentrations in plasma have been associated with thrombotic conditions such as venous thromboembolism (Eichinger 2004) and ischaemic stroke (Leebeek 2005; Rooth 2007). In CAD, the data are contradictory. For example, patients with angiographically verified CAD have been reported to have increased concentrations of TAFI compared with healthy controls when samples were assessed by a functional method (Silveira 2000). Others found that patients who had suffered from a recent MI presented lower TAFI antigen concentrations compared with controls when measured by means of ELISA (Juhan-Vague 2002).

The study of TAFI can be difficult because of pre-analytical requirements and analytical conditions. Several different methods are available, with different advantages and disadvantages (Table 1.) (Willemse 2006). For instance, the three rapidly changing forms of TAFI present in plasma (i.e. pro-TAFI, TAFI and TAFIi) make the marker methodologically difficult to evaluate and the results of different studies hard to compare. Regarding the above-mentioned “threshold dependent” action of TAFI, methods for measuring only pro-TAFI might be less important than measurements of the actual TAFI concentration, or the degree of TAFI production from pro-TAFI (Heylen 2011). Furthermore, previously commonly used ELISAs have later been shown to be sensitive to the most common polymorphism of TAFI, the Thr325Ile polymorphism, resulting in the risk of obtaining falsely low TAFI concentrations. For example, Morange et al. first concluded that increased TAFI antigen concentrations were a risk factor for angina pectoris (Morange 2003), but after re-evaluation of the results using a gene-independent assay they found no such significant association (Morange 2005). The use of genotype-specific ELISAs also created the impression that polymorphisms were the major causes of variability in TAFI concentrations, something that later, however, has been reconsidered (Heylen 2011). In a prospective study on patients with angiographically proven CAD, Tregouet et al. found that concentrations of TAFIa/TAFIi measured by an ELISA were

independently associated with the risk of cardiovascular death (Tregouet 2009). In the same study, this association was not found for immunologically measured concentrations of “total TAFI” (according to the authors, reflecting mainly the proenzyme).

The conflicting results discussed above could partly be due to the use of different methods and the lack of common standardization, and obviously there is a need for a reference method. Like PAI-1, TAFI might be affected by inflammatory conditions and behave as an acute phase protein (Skeppholm 2009). This may also have implications for interpretation of the results.



Table 1. Method principles for TAFI.

Method	Performance/Analytes	Advantages	Disadvantages
<b>ELISAs</b>	Mono- or polyclonal antibodies (Abs) against the entire TAFI antigen (i.e. pro-TAFI, TAFI and TAFIa, TAFIa/TAFIi or pro-TAFI only.	-Easy to perform. -No activation of pro-TAFI needed.	-Different sensitivity for common polymorphisms. -Abs with varying reactivity towards different TAFI forms. -Insufficient standardization of measurement procedures and calibration.
<b>Activity-Based Assays (1–3 below)</b>	Based on TAFI decarboxylating a synthetic substrate. After <i>in vitro</i> activation the activity of TAFI is measured.	-The enzymatically activated TAFI concentration is measured. -Genome independent.	-Need for activation of pro-TAFI. -The instability of TAFI can lead to underestimation.
1. <i>HPLC-assisted assay</i>		- High detectability	-Time-consuming
2. <i>Spectrophotometric endpoint activity assay</i>		-Simple instrumentation	-Possibly less specific
3. <i>Kinetic spectrophotometric assay</i>		-Possibility to increase specificity	

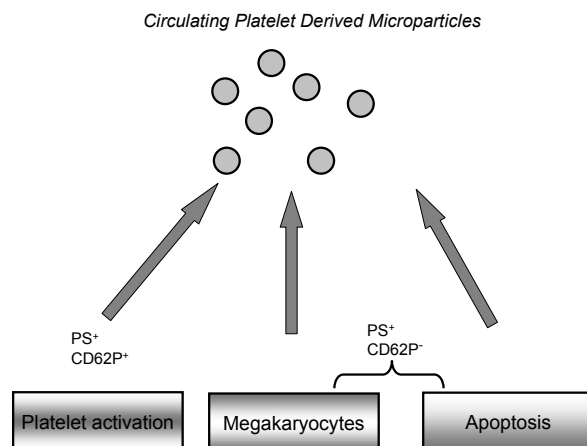
### 1.2.3 Platelet function

The significance of platelets in arterial thrombosis is undisputed, and as discussed above, increased platelet reactivity is associated with ACS. Studies have shown a link between high platelet reactivity on antiplatelet treatment and an adverse outcome both in CAD and in ACS patients (Gurbel 2007). However, platelet activation involves multiple pathways (Nurden 2011). At present, no single test captures all the aspects of platelet activation and it might be difficult to identify the most important step(s). Several methods are available but the poor predictability with regard to both cardiovascular complications and bleeding events in connection with the most commonly used platelet function methods (Breet 2010) has been a disappointment. Indeed, frequently used point-of-care assays are strongly user-dependent and require standardized sample handling and performance (Malmström 2010), something that may complicate the accomplishment of larger clinical trials. Grove et al have also demonstrated that the results of platelet function tests depend on the assay used (Grove 2010). The use of platelet function testing in clinical practice requires that methodological difficulties are overcome by careful standardization of methodology and the establishment of “best” cut-off levels. Evidence of improved outcome of a monitored therapy is a prerequisite for use in clinical practice. This has yet to be achieved.

#### 1.2.3.1 Platelet derived microparticles (PMP)

Activation of platelets includes membrane shedding of microparticles (MP) (VanWijk 2003). Circulating platelet derived microparticles (PMP) may therefore reflect the degree of platelet activation and might be used as a “diagnostic” tool. MP in general are phospholipid vesicles with a diameter of 0.1–1.0  $\mu\text{m}$  which express antigen from the parental cell. In this way it is possible to characterize their cellular origin. Although MPs can be derived from different cells, the majority of circulating MP originate from platelets. PMP are considered to be produced through three different pathways. These comprise *de novo* production from megakaryocytes, shedding during apoptosis and shedding as a result of platelet activation (Flumenhaft 2009) (Fig. 3). It is likely that PMP produced following platelet activation can expose and bind molecules like P-selectin and tissue factor (TF), while PMP originating directly from megakaryocytes in the bone marrow may not (Flaumenhaft 2009). Both P-selectin and TF are activity markers closely involved in the processes of inflammation and coagulation (Morel 2011). Furthermore, PMP, irrespective of origin, could expose negatively charged procoagulant phospholipids, mainly phosphatidylserine (PS), which provide an additional procoagulant surface that increases the catalytic efficiency of both tenase and prothrombinase complexes. TF acts synergistically with PS (Morel 2011; Key 2010), and the surfaces of PMP have been ascribed as possessing up to 50- to 100-fold greater procoagulant activity compared with activated intact platelets (Sinauridze 2007). Interestingly, recent studies show that far from all PMP express PS (Connor 2010; Perez-Pujol 2007), although the significance of these PS negative PMP (PS- MP) is unclear. Beside the haemostatic effects, there are data indicating that PMP may be directly involved

in the pathophysiology of atherosclerosis, e.g. by promoting adhesion of monocytes to the endothelium, which is an early step in the inflammatory process (Burnier 2009).



**Figure 3:** PMP may be produced through different pathways and express different markers.

Previous studies on ACS have shown elevated concentrations of circulating PMP among patients compared with controls (Matsumoto 2004, Bernal-Mizrachi 2003). Morel et al. reported that diabetes patients with acute MI and procoagulant microparticle levels above the median level of the study population suffered more cardiovascular complications at 6 months of follow-up (Morel 2004). However, the dynamics of circulating PMP during and after ACS have not been investigated previously. Whether PMP formation (and thus the plasma concentrations of PMP) follows the same pattern as platelet activation as measured by standard techniques has not been evaluated. Furthermore, given that PMP express different activation markers, investigation of different subpopulations of PMPs has the potential to give additional and better information on platelet activation status *in vivo* (van der Zee 2006). There is also a lack of information regarding the potential effects on PMP of antithrombotic treatment given in the acute and convalescent phases of ACS, although some previous investigations have shown reduced concentrations of PMP during antiplatelet treatment in the context of heart failure (Serebruany 2003), and diabetes and hyperlipidaemia (Nomura 2004). In summary, the many possible biological effects of microparticles (Morel 2006) in combination with the possibility that PMP in plasma reflect platelet function *in vivo*, make it worthwhile to perform further studies on PMP during and after an ACS.

### 1.2.4 Endothelial-related factors

The vascular endothelium is particularly affected by atherosclerosis. The von Willebrand factor (VWF) is considered to reflect endothelial dysfunction and is also mechanistically interesting in ACS. VWF mediates platelet adhesion and aggregation and acts as a carrier protein for factor VIII. VWF is produced as monomers in megakaryocytes as well as in the endothelium, and is subsequently polymerized and secreted as haemostatically active large multimers. Mature VWF and its pro-peptide are released into plasma in a 1:1 proportion. VWF can be secreted constitutively but can also be stored in Weibel–Palade bodies in endothelial cells or in  $\alpha$ -granules in platelets. The stored VWF is then released on demand, for example when endothelial cells or platelets are stimulated by thrombin. VWF multimers circulate in a ‘folded’ form with important epitopes hidden (Sadler 1998). Under high shear stress the molecule is stretched and unfolded and binding sites important for the haemostatic function of the molecule are exposed (Shim 2008).

When unfolded, VWF also exposes binding sites for a metalloprotease, ADAMTS13 (a disintegrin-like and metalloprotease with thrombospondin type I motifs), formerly called VWF cleavage protease, which degrades VWF into smaller fragments (Blombäck 2007). This limits the haemostatic effect of VWF and prevents widespread effects of the larger forms of the molecule within the vasculature. ADAMTS13 appears to be produced in the liver but reports also indicate production from platelets and endothelium (Liu 2005; Shang 2006). Thrombin and plasmin can inactivate ADAMTS13 by cleavage and thereby regulate its activity at the site of thrombus formation (Crawley 2005). Indeed, low ADAMTS13 activity has been linked to the severe disorder thrombotic thrombocytopenic purpura (TTP) (Hovinga 2004), a condition with widespread microthrombotic complications. ADAMTS13 is a target for diagnosis and treatment in this disease (Mörtzell 2011).

#### 1.2.4.1 *Von Willebrand factor (VWF) and ADAMTS13 in coronary artery disease*

Elevated plasma concentrations of VWF are frequently present in ACS (Schumacher 2002) and linked to an increased risk of recurrent events (Montalescot 1998). Previous studies on ADAMTS13 in CAD have shown partly contradictory results. There have been reports of lower levels of ADAMTS13 antigen concentrations in patients with acute MI (Matsukawa 2007) as well as lower ADAMTS13 activity in stable angina (Yoo 2003). In a case-control study, Bongers et al. found lower concentrations of both ADAMTS13 activity and antigen concentrations in young patients sampled 1–3 months after first acute ischaemic events of different aetiologies (Bonger 2009). The “low ADAMTS13 pattern” was most pronounced in the subgroup of CAD patients. In contrast, Chion et al. (Chion 2007) found no clear difference in ADAMTS13 antigen concentrations between controls and patients investigated more than 6 months after an MI, but increased ADAMTS13 antigen concentrations were associated with an increased risk of myocardial infarction.

#### 1.2.4.2 *Von Willebrand factor (VWF) and ADAMTS13 in diabetes mellitus (DM)*

Patients with diabetes mellitus, a major risk factor of CAD, are also known to have elevated VWF concentrations. Long-term studies on these patients have shown a link between high VWF concentrations and the development of both microvascular and macrovascular disease (Stehouwer 1995; Stehouwer 1992) and mortality (Standl 1996). The mechanisms behind elevated VWF concentrations in DM are unclear. However, one study on diabetic patients showed only slightly elevated concentrations of the VWF propeptide compared with mature VWF (van Mourik 1999). This could indicate that the patients had decreased degradation of mature VWF rather than an increased release. Others have found higher proportion of high-molecular-weight VWF fragments in urine from diabetic patients compared with urine from healthy controls (Silveira 1992). This led to the hypothesis that increased VWF concentrations in prothrombotic and metabolic disorders might be partly due to impaired activity of ADAMTS13.

#### 1.2.4.3 *Method principles for ADAMTS13*

ADAMTS13 antigen concentrations can be measured by immunological assays, but there have been methodological difficulties in determining the activity of the enzyme. Different methods have been described (see Table 2.), several which are complicated and not suitable for routine use. A commonly used *in vitro* test for VWF function can be used as an indirect method of ADAMTS13 activity, since it is ADAMTS13 that reduces the activity of VWF factor by degradation. In the ristocetin cofactor activity (R:CoF) assay the contribution of VWF to platelet aggregation in the presence of ristocetin is measured. VWF in plasma does not bind to platelet receptors unless it is structurally modified. Such a modification occurs when VWF binds to ristocetin.

A step forward was made when the assay based on the method described by Kokame et al. (Kokame 2005) became commercially available. It involves the use of a small fluorogenic labelled substrate for ADAMTS13 called “FRETS-VWF73”. This substrate is a VWF fragment consisting of 73 amino acids. Use of this low-molecular-weight synthetic peptide might not properly reflect the entire interaction between circulating VWF and ADAMTS13 (Gøtze 2008). However, the assay has several advantages. It is easy to perform, has a high specificity for ADAMTS13, and is able to detect decreased activity due to different causes.

**Table 2. Assays of ADAMTS13 activity.**

<i>Methods</i>	<i>Principles</i>
<b>VWF multimer structure analysis</b>	Electrophoretic determination of degradation products of high-molecular-weight VWF produced by ADAMTS13 activity.
<b>Immunological detection of VWF fragments</b>	Determination of specific VWF fragments produced by ADAMTS13 activity.
<b>Flow assay</b>	Determination of the degradation of ultra-large VWF - platelet strings at the surface of cultured endothelial cells.
<b>Indirect methods</b>	Determination of VWF activity concentration by binding to collagen or production of platelet aggregation (ristocetin cofactor activity). Decreased ADAMTS13 activity results in increased VWF activity and accumulation of VWF multimers.
<b>Methods using short synthetic VWF peptides as substrates</b>	Direct determination of ADAMTS13 activity concentration by measuring fluorescence produced during degradation of the substrate.

Modified from Manea et al. 2007 and Götze et al. 2008.

### **1.2.5 Global markers**

Mostly, single biochemical markers have been used for studies of haemostasis. However, these markers reflect only one aspect of the complex haemostatic state and at a given point of time. Moreover, the results are often conflicting. With regard to single markers of fibrinolysis in CAD there are about as many published articles showing positive associations as those reporting no associations between fibrinolysis and the disease (Gorog 2010). From a clinical point of view it is desirable to obtain results that better mirror the situation *in vivo*, i.e. by addressing the balance between coagulation and fibrinolysis by using functional methods.

### 1.2.5.1 Methods focused on coagulation

*Activated partial thromboplastin time (aPTT)* and *prothrombin time (PT)* are used in two routine assays with a detectable fibrin clot as an end point. Their usability in detecting prothrombotic states and monitoring new antithrombotic agents is not optimal (Antovic 2008). *Thrombin clotting time (TCT)* reflects the conversion of fibrinogen into fibrin after adding thrombin to a plasma sample, and when low or moderate thrombin concentrations are used the observed clotting time largely depends on the fibrinogen concentration and quality in the sample. A general problem with methods based on the formation of a detectable fibrin clot is that the clot occurs as soon as when 3–5% of the total amount of thrombin is produced. Haemostatic reactions later on in the process may thus not be detected (Antovic 2008).

An assay which is generally considered to be a “true global method” is *thromboelastography (TEG)*. This method monitors clot formation, clot stability and clot dissolution (Chandler 1995). It is performed with whole blood, which makes it possible to study interactions between platelets and plasma coagulation, but this may also be a disadvantage, since the method has to be performed within hours after blood sampling, and frozen-thawed samples cannot be used.

One commonly used test is the thrombin generation test modified by Hemker et al., herein called the calibrated automated thrombogram (CAT) (Hemker 2003). This includes the endogenous thrombin potential (ETP) and related parameters (lag phase, peak thrombin, time to peak of thrombin) and is commercially available. This method is claimed to detect higher ETP in thrombotic disorders, e.g. deep vein thrombosis and stroke (ten Cate-Hoek 2008; Faber 2003). Furthermore, patients with previous myocardial infarction have been found to have significantly higher peak thrombin concentrations compared with patients with stable CAD (Orbe 2008). Taken together, this may indicate that an individual’s tendency to generate thrombin after stimulation (in the test tube) could be relevant to the pathophysiology of thrombotic disorders. However, even though this method gives information about the greater part of the clotting system, it cannot be regarded as a true global assay since it does not measure the final step of coagulation, i.e. fibrin formation, and it does not assess fibrin degradation.

The *fibrin network* formed in the final step of coagulation can be studied via turbidometric and permeability analyses. These can provide information about the fibrin network characteristics, the fibre mass/length ratio and the permeability coefficient of the fibrin gel *in vitro* (Blombäck 1994; He 2005). Studies have shown a tighter fibrin network among young men with myocardial infarction (Fatah 1996). Other thrombotic conditions associated with a tighter fibrin network include diabetes, stroke and venous thromboembolism (VTE) (Jörneskog 1996; Rooth 2011; Undas 2011). Using real-time confocal microscopy techniques *in vitro*, Collet et al. showed that clots with a tight fibrin network and with smaller pores are more difficult to lyse (Collet 2000).

### **1.2.5.2** *Methods focused on fibrinolysis*

The euglobulin clot lysis time (ECLT) method in plasma and the dilute whole blood clot lysis time method were previously used to assess the overall fibrinolytic system. However, during precipitation of the euglobulin fraction (in the ECLT method), plasma inhibitors of fibrinolysis, for example both PAI-1 and antiplasmin, are removed and the method does not take into account elevated concentrations of these inhibitors. The dilute whole blood clot lysis time method excludes the important connection to coagulation through TAFI and factor XIII, i.e. due to reduced thrombin generation caused by the use of citrate in the assay, (Meltzer 2009b). A modified plasma-based method, prolonged clot lysis time (CLT) (Lisman 2001), has been reported to be associated with an increased risk of venous thrombosis (Lisman 2005) and an increased risk of a first myocardial infarction among younger men (Meltzer 2009b).

### **1.2.5.3** *Measurement of overall haemostasis*

The most common principle of global methods is to measure thrombin generation, clot formation or fibrin degradation. However, haemostasis is an integrated function of two counteracting systems, coagulation and fibrinolysis. We have speculated that an individual's risk of bleeding and thrombosis would be better estimated if the balance between these two opposing systems could be estimated. He & Blombäck developed the Overall Haemostasis Potential (OHP) method (He 1999). This assesses the area under the fibrin aggregation curve formed when both coagulation and fibrinolysis are triggered by adding a low concentration of thrombin, a platelet reagent (phospholipids) and tPA to a recalcified plasma sample. The OHP method has been used to study several conditions involving disturbed haemostasis (He 2001; Antovic A 2003; Antovic JP 2003) and treatment with antithrombotic agents (He 2001; Antovic A 2002; Antovic JP 2002). It has been further developed in order to assess the dynamics of coagulation and fibrinolysis, and has been renamed the Overall Haemostasis Index (Oh-index) (see Methods section and He et al. 2007). In brief, in this method changes in optical density (OD) are measured, which reflect the rate of fibrin formation and degradation. The Oh-index has been shown to be sensitive to various factor deficiencies as well as to the influence of anticoagulating agents (He 2007).



## 2 AIMS

The overall aims of this study were:

- ◆ To study possible mechanisms behind thrombotic complications in CAD and diabetes mellitus, a strong risk factor for CAD, by using new methods and markers
- ◆ To elucidate the contribution of a disturbed balance between coagulation and fibrinolysis, and increased platelet activation, to a prothrombotic state in CAD
- ◆ To search for tools to identify patients at an increased risk of thrombosis or bleeding

## 3 MATERIAL AND METHODS

### 3.1 Patients and healthy controls

#### 3.1.1 Study I

We investigated plasma samples from two groups of patients with diabetes mellitus. Group one consisted of twenty-three patients with type 1 diabetes (DM) but with no medical history of cardiovascular events in spite of median diabetes duration of 38.5 years. Group two consisted of sixty-five patients with diabetes and peripheral artery occlusive disease (DM(+))PAOD) in combination with chronic foot ulcers. These patients were originally selected for other investigations by our group and studies on patients from group two have previously been published (Kalani 2003, Kalani 2007).

Age- and gender-matched subjects served as controls for the DM and DM(+))PAOD patients.

#### 3.1.2 Studies II–IV

The patients participating in Studies II–IV originated from a well-defined study population consisting of a total of 87 patients with ACS, of which 21 % had UAP and 79 % had NSTEMI.

For distribution of the participants in Studies II–IV, see Table 3.

Inclusion criteria were anginal chest pain at rest in combination with either

- 1) electrocardiographic signs of myocardial ischaemia such as typical ST-segment depression or persistent T-wave inversion or
- 2) elevated levels of troponin I, indicating myocardial necrosis (plasma concentration > 0.10 µg/L, i.e. above the 99th percentile in a healthy reference population).

The exclusion criteria were ST-elevation myocardial infarction (STEMI), on-going treatment with warfarin, malignancy, severe liver or kidney disease, severe inflammatory disease or surgery in the previous three months.

The protocol for investigations and blood sampling is shown in Fig. 4. In Study II an additional sample was collected three months (T) after the acute event.

*I. Sample I (SI): at admission to the coronary care unit.*

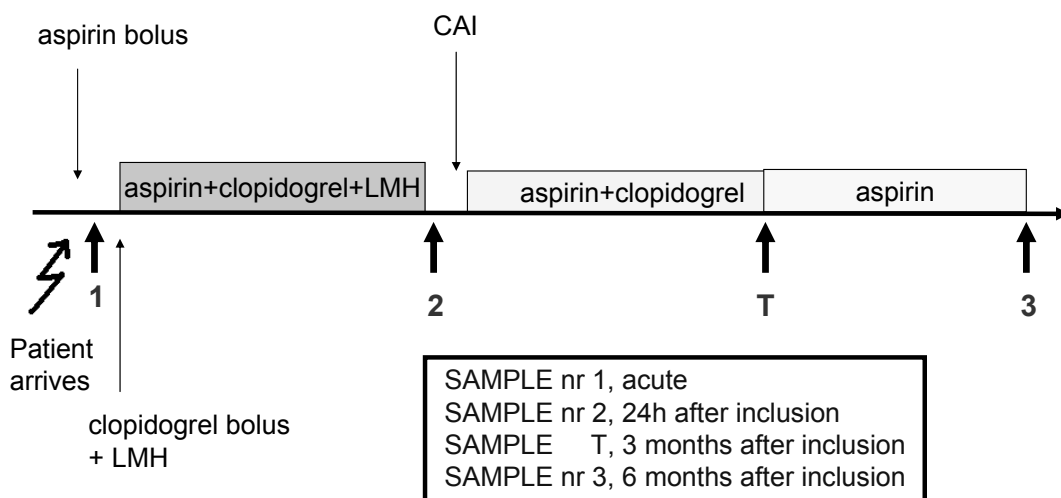
All patients had received an aspirin bolus (500 mg orally) in the ambulance or at the emergency unit, and 40 % had on-going treatment with aspirin. None of the patients was on clopidogrel or any other additional antithrombotic drug. Thirty per cent of the patients were on statins.

## II. Sample 2 (S2): within 24 h of admission and before coronary angiography.

All patients had received aspirin and a clopidogrel bolus (300 mg) had been given to 95% of them. Subcutaneous injections of low-molecular-weight heparin (LMH), i.e. enoxaparin at 1 mg/kg body weight twice daily (n=51, i.e. 59%) or dalteparin at 120 IU/kg body weight twice daily (n=36, 41%) had been given to all patients. Only 8% were on a GPIIb/IIIa-inhibiting agent (tirofiban).

## III. Sample 3 (S3): six months after admission.

Most (82%) of the patients were on aspirin (75 mg daily), 6% on a combination of aspirin and clopidogrel, 2% on clopidogrel alone, 1% on LMH and 9% on warfarin treatment. The majority (93%) of patients were treated with statins. The reasons for treating patients with warfarin or LMH were atrial fibrillation, pulmonary embolus, valvular prosthesis or left ventricular thrombus.



**Figure 4:** The protocol for investigations and blood sampling.

Compared with RIKS-HIA data (The Register of Information and Knowledge about Swedish Heart Intensive care Admissions ([www.ucr.uu.se/swedeheart](http://www.ucr.uu.se/swedeheart))) from our hospital the patients of the present study were found to be representative of the general ACS population of the Danderyd catchment area.

In Study III the patients were partitioned into risk groups on the basis of the Thrombolysis In Myocardial Infarction (TIMI) risk score, an established risk score system used to predict recurrent events (Antman, 2000). We used a cut-off defining high risk patients as those with a score of  $\geq 4$  points and low risk patients as those with a score of  $<4$ , since the study population was relatively small. This modified TIMI risk score has previously been used by others (Lee 2005).

### 3.1.3 Healthy controls

Sixty-one age- and gender-matched subjects were chosen as controls for Studies I–IV. Before inclusion, all control subjects had a medical investigation including measurements of blood pressure, and blood sampling for determination of fasting glucose and lipid concentrations. Electrocardiography was also performed. None of the controls had a history of cardiovascular disease, diabetes, malignancy, severe inflammatory disease or liver- or renal insufficiency or used antihypertensive, lipid-lowering, antithrombotic or anti-inflammatory drugs (e.g. aspirin).

**Table 3. Distribution of the participants in Studies II–IV.**

	<i>Patients</i>	<i>Controls</i>
Study II	36	14
Study III*	87	61
Study IV	51	61

\* Due to limited access to patient samples the Oh-index was measured in 65 patients and 55 controls, and CAT data obtained in 49 patients and 53 controls.

### 3.1.4 Blood sampling

Venous blood sampling was always performed through direct venipuncture with no or minimum stasis. In Study I blood was collected between 8 and 9 a.m. after a 20-min supine rest and after an overnight fast.

In Studies II–IV, S3 blood samples and most of the S2 samples were taken in the morning in a fasting state after 15 min of rest, whereas S1 sampling in patients was at admission in the acute situation.

In healthy controls blood samples were always taken in the morning in a fasting state.

Blood was drawn into vacuum tubes containing trisodium citrate (0.13 mol/L, pH 7.5). The tubes were immediately centrifuged for 20 min (2000–2200  $\times$  g, room temperature), and plasma was dispensed into plastic tubes and frozen at -70 °C.

## 3.2 Methods

### 3.2.1 ADAMTS13

In studies I and III we measured ADAMTS13 concentrations in plasma using the TECHNOZYM ADAMTS13 kit (Technoclone GmbH, Vienna, Austria). This is a commercial assay based on the method described by Kokame et al. (Kokame 2005) and uses a small fluorogenic substrate for ADAMTS13 called “FRETS-VWF73”; a VWF-fragment consisting of 73 amino acids (see above). Plasma is incubated in microwells

coated with an antibody specific for ADAMTS13 (mAb) which is thus attached to the wall of the well. After a washing procedure the substrate is added. This consists of both a fluorescent part and a part that extinguishes fluorescence. If the substrate is degraded by ADAMTS13 fluorescence is emitted and detected. The rate of fluorescence development is used to kinetically measure the ADAMTS13 activity, incubated at 30 °C and assayed at 360/460 nm. The antigen concentration is measured in the same samples by incubation with peroxidase labelled mAb towards ADAMTS13. After activation of the added peroxidase substrate the antigen concentration can be assayed at 360/460 nm.

ADAMTS13 activity and antigen concentrations are reported in relation to that of a pool of plasma from >100 healthy individuals. According to the manufacturer the reference interval for ADAMTS13 antigen concentration is 75-110% and for ADAMTS13 activity concentration 50-110% to that of the pool.

### 3.2.2 TAFI

We compared two sandwich enzyme-linked immunosorbent assays (ELISAs) for measurements of TAFI antigen concentrations, with a new functional chromogenic assay for the determination of TAFI activity concentration (see below). All three methods are commercially available:

(1) The ELISA TAFI Antigen assay (referred to as the Haemochrom method) measures total TAFI antigen (pro-TAFI, TAFI and TAFIi) concentrations by using a monoclonal antibody specific for human TAFI. Reagents were obtained from Haemochrom Diagnostica (Haemochrom Diagnostica GmbH, Essen).

(2) The ASSERACHROM TAFI ELISA is based on a similar principle as the Haemochrom assay, but two specially selected monoclonal antibodies are used, one as a capturing mAb and one as a detecting mAb. This renders the assay specific for pro-TAFI, with little or no reactivity towards TAFI or TAFIi. The reagents were from Stago (Diagnostica Stago, France). The assay is regarded as genotype independent (Verdú 2006).

(3) The Pefakit TAFI assay (Pentapharm Ltd., Basel) is a chromogenic assay for determination of TAFI enzyme activity concentration, reflecting the pro-TAFI concentration in plasma.

#### *Comparison of results*

The results of the Haemochrom as well as of the Pefakit assays are expressed as relative mass concentrations normalized to that of a plasma pool supplied with the kits. The Asserachrom results are expressed as mass concentrations calibrated against a preparation of freeze-dried TAFI of a known concentration.

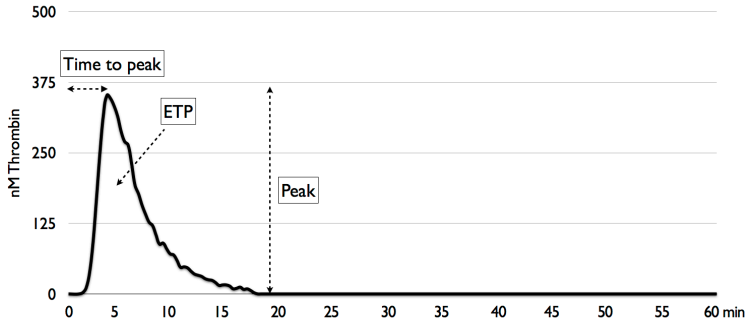
Before comparing results, we established a regression function between the Haemochrom method and the Asserachrom method, calibrated against the same plasma pool. The regression function was used to establish a conversion function between mass concentration and relative mass concentration. All Asserachrom results were then converted to relative mass concentration using this function.

### 3.2.3 Oh-index

The method has been described previously by He et al. (He, 2007). Briefly, in platelet-poor plasma, recombinant human tissue factor (final conc. 2.1 pmol/L), purified phospholipids (final conc. 2.5  $\mu\text{mol/L}$ ) and  $\text{CaCl}_2$  (final conc. 17.5 mmol/L) are used to activate coagulation. Fibrinolysis is initiated by recombinant tissue plasminogen activator (final conc. 135  $\mu\text{g/L}$ ). The optical density (OD) is recorded every 30 seconds until it has returned to baseline. The OD values are used to obtain time to start of detectable fibrin formation ( $t_1$ ), maximum rate of fibrin formation ( $h_1$ = maximum “ $\Delta\text{OD}/\text{time unit}$ ”), maximum rate of fibrin degradation ( $|h_2|$ = maximum “ $\Delta\text{OD}/\text{time unit}$ ”), and time required to reach  $h_1$  ( $t_2$ ),  $h_2$  ( $t_4$ ) and the maximum OD ( $t_3$ ). These quantities are used to estimate the Coagulation profile ( $C_p$ ) and Fibrinolysis profile ( $F_p$ ):

$$C_p = (t_1)^{-1} * \frac{|h_1|}{(t_2 - t_1)} \quad \text{and} \quad F_p = t_1 * \frac{|h_2|}{(t_4 - t_3)}$$

The ratio of  $C_p$  to  $F_p$  is called the “Overall Haemostasis Index” (Oh-index) and is suggested to reflect the balance between coagulation and fibrinolysis in the plasma sample (He 2007).



**Figure 5.** Calibrated Automated Thrombogram (CAT) with the measured variables “peak thrombin concentration”, “time to peak of thrombin concentration” and the area under the curve (AUC), which represents the “endogenous thrombin potential (ETP)”.

### 3.2.4 Calibrated Automated Thrombogram (CAT)

We used the method described by Hemker et al. (Hemker 2003) and commercialized by Thrombinoscope BV, (Maastricht, the Netherlands). Samples were run according to the manufacturer’s instructions. Briefly, coagulation is triggered in platelet-poor plasma by adding a reagent containing tissue factor (5 pmol/L) and phospholipids (4  $\mu\text{mol/L}$ ). A fluorogenic thrombin substrate is added and the fluorescence intensity measured. We report the peak thrombin concentration and its time to peak, and the area under the curve (AUC),

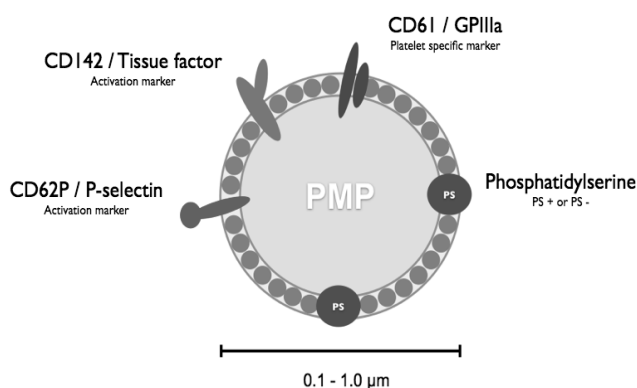
which represents the endogenous thrombin potential (ETP), i.e. the total amount of thrombin produced over time.

### 3.2.5 Microparticles

Previously frozen platelet-poor plasma was thawed and centrifuged at  $2000 \times g$  for 20 minutes at room temperature. The supernatant was then re-centrifuged, at  $13,000 \times g$  for 2 minutes at room temperature. 20  $\mu\text{L}$  of sample was incubated for 20 minutes in the dark with phalloidin-Alexa-660 (Invitrogen, Paisley, UK), lactadherin-FITC (Haematologic Technologies, Vermont, USA), and CD61-APC (Abcam, Cambridge, UK) plus CD62P-PE (BD, NJ, USA) or CD142-PE (BD, NJ, USA). Microparticles (MP) were measured by flow cytometry on a Beckman Gallios instrument (CA, USA). The MP gate was determined by using Megamix beads (BioCytex, Marseille, France), which is a mixture of beads with diameters of 0.5  $\mu\text{m}$ , 0.9  $\mu\text{m}$  and 3  $\mu\text{m}$ . The acquired settings were also controlled with fresh platelets to insure proper gating.

Platelet microparticles (PMP) were defined as particles of less than 1.0  $\mu\text{m}$  in size, negative to phalloidin (in order to exclude cell membrane fragments; see Mobarrez 2010) and positive to CD61 (GPIIIa).

PMP were sorted into particles expressing or lacking phosphatidylserine,  $\text{PS}^+$  or  $\text{PS}^-$ . In order to do so we used lactadherin instead of annexin V to improve the sensitivity of PS detection (Hou 2011; Perez Pujol 2007). Conjugate isotype-matched immunoglobulin (IgG1-FITC, IgG1-PE and IgG1-APC) with no reactivity against human antigens was used as a negative control to define the background noise of the cytometric analysis. Among the  $\text{PS}^+$  PMP and the  $\text{PS}^-$  PMP respectively the expression of CD142 (tissue factor) and the expression of CD62P (P-selectin) were measured.



**Figure 6.** A schematic figure of a platelet derived microparticle

The absolute number of MP was calculated by means of the following formula:  
(MPs counted x standard beads/L)/standard beads counted (FlowCount, Beckman Coulter).

### **3.2.6 Other laboratory methods**

Commercially available kits and calibrators were used to measure all the quantities below, and the measurement procedures were carried out according to the manufacturers' instructions;

#### **3.2.6.1 *Von Willebrand factor (VWF) antigen concentrations***

VWF antigen concentrations were measured in plasma using the LIATEST from Diagnostica Stago (Asnieres, France) and assayed using BCS equipment (Dade Behring, Marburg, Germany).

#### **3.2.6.2 *Prothrombin fragment (F1+2) concentrations***

Prothrombin fragment (F1+2) concentrations in plasma were measured by Enzygnost® F1+2 (monoclonal) (Dade Behring), using Multiscan Labsystem MCC/340 (Labsystem, Sweden).

#### **3.2.6.3 *PAI-1 antigen concentrations***

PAI-1 antigen concentrations in plasma were determined by an ELISA, TintElize® PAI-1 (Biopool AB, Umeå, Sweden), using Multiscan Labsystem MCC/340 (Labsystem, Sweden).

#### **3.2.6.4 *Fibrinogen and hsCRP plasma concentrations***

These were determined immunologically using reagents from Dade Behring, and nephelometrically assayed using BN Prospec (Dade Behring, Marburg, Germany).

#### **3.2.6.5 *IL-6 concentrations***

IL-6 concentrations in plasma were determined by using high-sensitivity Human IL-6 Quantikine kits, HS ELISAs (Novakemi AB, Stockholm, Sweden) using EVOLIS (Bio-Rad, France).

#### **3.2.6.6 *Routine analyses***

Concentrations of serum creatinine, plasma cholesterol, and plasma glucose were all measured by routine laboratory techniques at the clinical chemistry laboratory of Karolinska University Hospital.



## 4 STATISTICAL ANALYSES

A comprehensive database was created in the JMP package (versions JMP 5.1 and 7.0, SAS Institute, Cary, NC, USA). This software was also used for statistical analyses and creation of Figures, when appropriate. Innate statistical functions of Excel 2007 (Microsoft, Seattle WAS, USA) were also used, and many Figures were created by using SigmaPlot 12 (Systat Software Inc., San Jose, CA, USA).

A  $p$ -value  $<0.05$  was considered statistically significant for all tests.

All data were tested for normality with the Shapiro–Wilk's  $W$  test. Data were expressed as median values (range or interval) or mean values  $\pm$  SD, as appropriate.

Spearman's or Pearson's correlation coefficients were used, as appropriate, for estimation of correlations between variables.

Differences between the healthy controls and patients on each sampling occasion were evaluated by Student's independent  $t$ -test for different variances or by Wilcoxon's Rank Sum test (including Bonferroni correction; Paper IV), as appropriate.

*Paper I:* Differences between baseline and treatment values in the investigation of possible interactions between dalteparin treatment and measured parameters were evaluated by using Student's  $t$ -test for paired samples (ADAMTS13) and Wilcoxon's Signed Rank test (von Willebrand factor).

*Paper II:* Comparison between the healthy controls and patients was carried out by using a one-sided Student's  $t$ -test, since our hypothesis was based on the assumption that patients are characterized by increased TAFI antigen and activity concentrations. The significance of the differences between the ELISA methods were estimated by using paired Student's  $t$ -tests.

Method imprecision was calculated by utilizing duplicate measurements:

$$sd = \sqrt{\frac{\sum_{i=1}^N d_i^2}{2 \times N}}$$

where  $d_i$  is the difference between observations and  $N$  is the number of samples.

*Paper III:* Differences between patients at admission and S2, and admission and S3 were evaluated by Student's  $t$ -test for paired samples or Wilcoxon's Signed Rank, as appropriate. The associations between TIMI risk scores and clinical findings were assessed by contingency analysis.

*Paper IV:* Differences over time in patients were first evaluated with an overall test, repeated measures ANOVA, and since there were highly significant differences over time the individual sampling results (S1-S2, S1-S3 and S2-S3) were then compared by using Wilcoxon's Signed Rank test.

## 5 RESULTS AND DISCUSSION

### 5.1 Paper I

We studied the concentrations of both VWF antigen and ADAMTS13 antigen and ADAMTS13 activity concentrations in two different diabetic populations. To our knowledge this had not been performed previously. Group one consisted of twenty-three patients with type 1 diabetes mellitus without macrovascular complications (DM). Group two consisted of 65 patients with mainly type 2 diabetes in combination with peripheral artery occlusive disease (DM+PAOD) and chronic foot ulcers. The latter group also participated in a prospective, randomized trial to investigate the effect of long-term treatment with dalteparin (5000 U subcutaneously once daily;  $n = 33$ ) or placebo (physiological saline;  $n = 32$ ) on ulcer healing (Kalani 2003; Kalani 2007).

Dalteparin has previously been shown to increase plasma levels of VWF (Oldgren 2005), and therefore we used baseline and treatment values to investigate possible interactions between dalteparin treatment and ADAMTS13. For comparison we used 23 and 59 age- and gender-matched healthy individuals as controls to the DM and DM+PAOD patients, respectively. For the DM+PAOD patients baseline values were used to compare ADAMTS13 and VWF concentrations with those in healthy controls. For demographics see Table 4.

We found differences between genders, with higher ADAMTS13 activity concentration in plasma from women compared with men among the controls, while the concentration of ADAMTS13 antigen did not differ significantly. In the DM+PAOD group, the female patients had significantly higher concentrations of both ADAMTS13 activity and antigen compared with male patients. In DM patients, no differences in ADAMTS13 between genders were observed, but the sample size was relatively small. No gender differences were observed as regards VWF antigen in any of the groups investigated.

In the healthy controls, there was a weak but statistically significant negative correlation between age and ADAMTS13 antigen concentration ( $r = -0.29$ ,  $p < 0.05$ ) and a positive correlation between age and VWF concentration ( $r = 0.37$ ,  $p < 0.01$ ), whereas there was no significant relationship between age and ADAMTS13 activity concentration.

No statistically significant relationships were observed between age and ADAMTS13 activity or antigen concentrations, or VWF antigen concentration in any of the studied patient groups.

**Table 4. Characteristics of patients and healthy controls.**

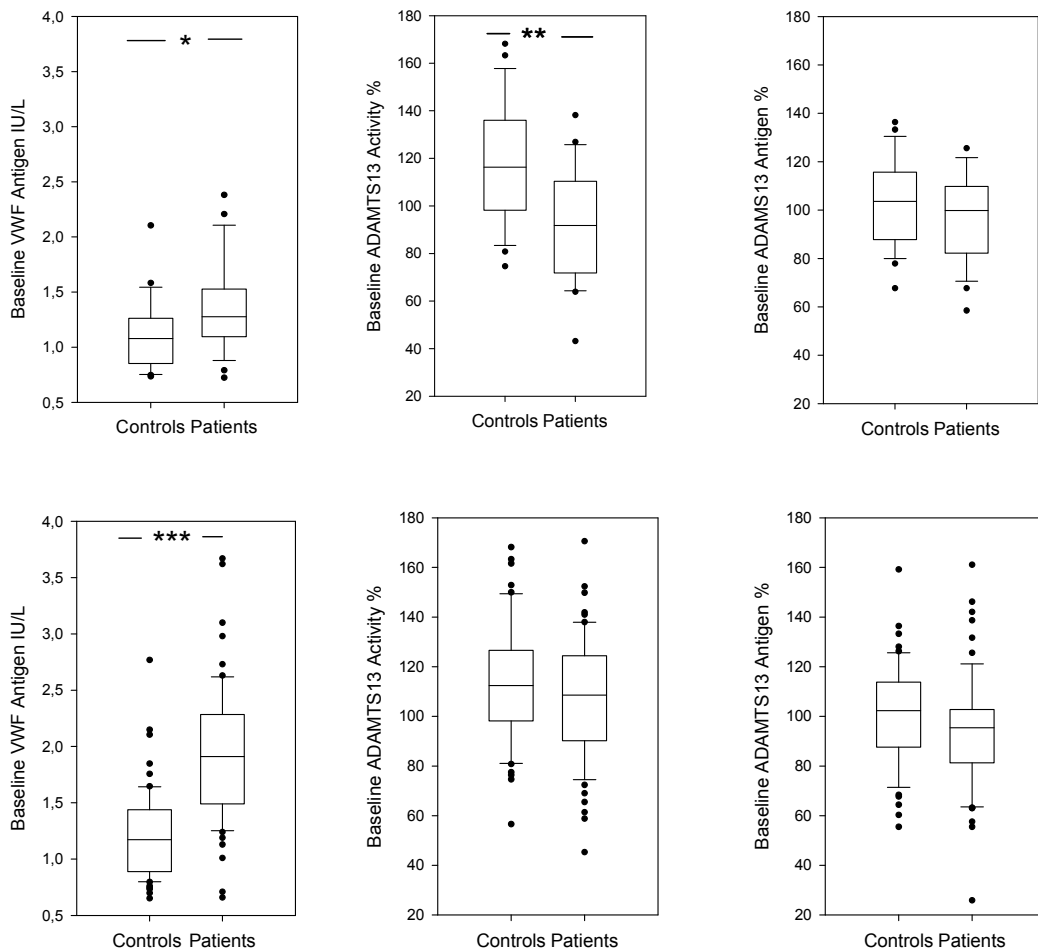
	<i>DM(+)</i> PAOD <i>n</i> =65	<i>Healthy</i> <i>controls</i> <i>n</i> =59	<i>DM</i> <i>n</i> =23	<i>Healthy</i> <i>controls</i> <i>n</i> =23
Age (years)	74 (38-90)	67 (42-85)	60 (39-69)	60 (42-69)
Gender (% male/female)	74/26	64/36	61/39	61/39
Diabetes type (%)	type 2 (84) type 1(16)		type 1 (100)	
Diabetes duration (years)	19.5 (1-66)		38.5 (1.5-61)	
HbA1c (%)	6.7 (5.0-11.0)		6.4 (4.7-8.8)	
hsCRP (g/L)	5.3*** (0.3-118)	0.7 (0.1-17.3)	3.5*** (1.0-19)	0.6 (0.1-4.8)
ASA n (%)	65(100)	0	1 (4)	0
ACEi n (%)	16 (25)	0	7 (30)	0
Statins n (%)	8 (12)	0	9 (39)	0

DM(+)*PAOD*: Diabetes and peripheral artery occlusive disease.

DM: Diabetes without peripheral artery occlusive disease.

Values for age, diabetes duration, HbA1c and hsCRP are expressed as median and interval (min-max values). \*\*\*  $p < 0.001$  compared to controls.

In DM patients, VWF antigen concentrations in plasma were slightly but significantly higher and ADAMTS13 activity concentration significantly lower than in the controls ( $p < 0.05$  for VWF,  $p < 0.01$  for ADAMTS13 activity), whereas there was no significant difference in ADAMTS13 antigen concentrations between the two groups (Fig. 7A). The ratio between VWF and ADAMTS13 antigen (VWF:ADAMTS13) concentration was significantly higher in DM patients compared with the healthy controls ( $p < 0.01$ ) due to higher VWF antigen concentrations in the patients. There were no significant differences in ADAMTS13 antigen or activity concentrations between DM+PAOD patients and their healthy controls in spite of significantly higher VWF antigen concentrations in patients compared with controls ( $p < 0.001$ ; Fig. 7B). The VWF:ADAMTS13 antigen ratio was significantly higher also in the DM+PAOD patients ( $p < 0.001$ ), and as in the DM patient group, this was due to elevated VWF antigen concentrations.



**Figure 7 A:** Upper panel DM patients and their healthy controls

**Figure 7 B:** Lower panel DM+PAOD and their healthy controls

The box plots show median, upper and lower quartile with extension to points still within 1.5 IQR from the quartiles. \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$

Following treatment with dalteparin, VWF antigen concentrations increased significantly from  $1.9 \pm 0.7$  kIU/L at baseline to  $2.3 \pm 1.0$  kIU/L at the end of treatment ( $p < 0.001$ ), whereas VWF antigen concentrations remained unchanged in patients receiving placebo treatment. However, ADAMTS13 activity and antigen concentrations did not change significantly during either treatment period.

There were no statistically significant correlations between VWF concentrations vs. ADAMTS13 activity or antigen concentrations in any of the patient groups or in the controls. As expected, there were significant correlations between ADAMTS13 activity

and antigen concentrations in the healthy controls ( $r = 0.42$ ,  $p < 0.001$ ,  $n = 59$ ) as well as in the DM ( $r = 0.68$ ,  $p < 0.001$ ,  $n = 23$ ) and DM+PAOD groups ( $r = 0.74$ ,  $p < 0.001$ ,  $n = 65$ ).

### Discussion

Our main finding was that patients with type 1 diabetes mellitus had significantly lower concentration of ADAMTS13 activity in plasma than healthy individuals, suggesting a link between disturbed function of ADAMTS13 and diabetic microangiopathy. In patients with diabetes mellitus and PAOD the elevation in VWF antigen concentrations was pronounced, whereas ADAMTS13 antigen and activity concentrations were similar to those observed in healthy controls. Patients with diabetes mellitus and PAOD were relatively severely diseased, some with infections and inflammation secondary to foot ulcers, as a plausible cause for VWF antigen elevation together with normal function of ADAMTS13. We observed gender differences, with higher ADAMTS13 activity and antigen concentrations in women and indications of a slightly decreased concentration of ADAMTS13 antigen with age. The increase in VWF antigen concentrations during treatment with dalteparin did not seem to be due to changes in ADAMTS13 activity or antigen concentrations.

## 5.2 Paper II

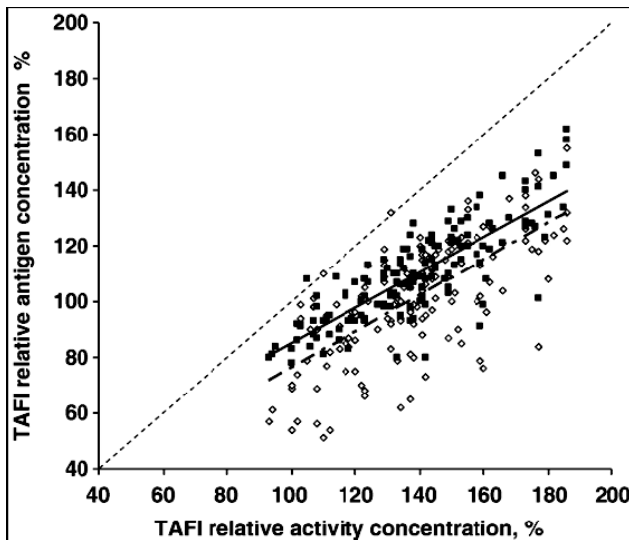
In order to identify an appropriate method to be used in studies of the thrombin activatable fibrinolysis inhibitor (TAFI) in ACS, we compared three assays based on different measurement principles of TAFI (Table 5). TAFI was measured as relative activity by using a functional method (Pefakit) and as antigen concentration by using two different immunological methods (Haemochrom and Asserachrom), in 36 patients suffering from ACS. The patients were sampled on four occasions: acutely, i.e. at admission to the coronary care unit (sample 1), 24 h after admission (sample 2), in convalescence at three (sample T) and at six months (sample 3) after admission. We also used plasma from 14 healthy controls sampled once.

**Table 5. Measurement principles.**

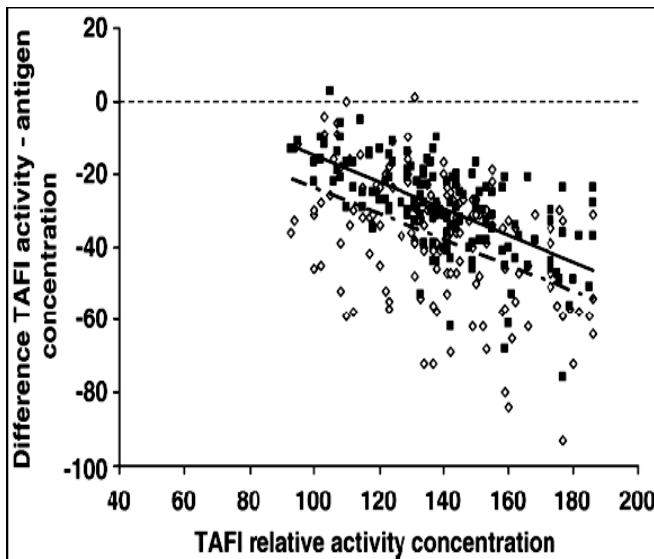
<i>Method</i>	<i>Principle</i>	<i>Measured variables</i>
<b>Pefakit</b>	<i>Activity assay.</i> Native pro-TAFI in a plasma sample is activated and a chromogenic substrate is selectively degraded by the produced TAFI.	Pro-TAFI
<b>Asserachrom</b>	<i>Immunological assay.</i> Two specially selected monoclonal antibodies render this assay specific for pro-TAFI.	Pro-TAFI
<b>Haemochrom</b>	<i>Immunological assay.</i> One monoclonal antibody specific for human TAFI.	Pro-TAFI, TAFI and TAFIi

Pro-TAFI is the proenzyme for the active form of TAFI, whereas TAFIi stands for the inactivated form.

The results of the Pefakit assay as well as those of the Haemochrom assay were expressed in relative mass concentrations, while results of the Asserachrom assay were expressed in mass concentrations calibrated against a preparation of freeze-dried TAFI of a known concentration. Before comparing results, data from Asserachrom measurements were transferred to and expressed in relative mass concentration using a conversion function, established as described in detail in the Methods section. For comparisons between methods we used all data from patients and controls ( $n = 150$ ). Statistically significant correlations ( $p < 0.01$ ,  $n = 150$ ) between the immunoassays and the functional assay (i.e. Pefakit) were observed ( $r^2 = 0.67$  and  $0.47$  for Asserachrom and Haemochrom, respectively (Fig. 8)). Of the three assays, the Pefakit values were higher within the measurement interval than those of the immunoassays, of which the Asserachrom assay gave higher concentrations than the Haemochrom assay (Fig. 9). There was a highly significant difference between the results obtained with the Asserachrom and Haemochrom methods ( $p < 0.001$ ,  $n = 150$ ), and the correlation between the results of the immunochemical methods was highly significant but not very strong ( $r^2 = 0.51$ ,  $n = 150$ ).



**Figure 8.** Relationships between relative TAFI activity concentrations and relative TAFI antigen concentrations measured with Asserachrom and Haemochrom assays, respectively. The solid line (and filled symbols) show TAFI antigen concentrations according to the Asserachrom assay and the dashed line below it (and open symbols) show TAFI antigen concentrations according to the Haemochrom assay



**Figure 9.** A bias plot showing the difference between the two immunochemical methods and the functional method (Pefakit). Difference between relative TAFI antigen concentrations measured by the Asserachrom assay (solid line and filled symbols) and the Haemochrom assay (dashed line and open symbols), respectively and relative TAFI activity concentrations (Pefakit).

Compared with healthy controls the patients had significantly higher pro-TAFI antigen concentrations measured by the Asserachrom kit both acutely (sample 1,  $p < 0.05$ ) and in convalescence (samples T,  $p < 0.01$  and 3,  $p < 0.05$ ). In addition, TAFI activity concentration, measured by the Pefakit assay, were significantly higher in patients during convalescence vs. healthy controls (one-sided unpaired *t*-test in samples T and 3,  $p < 0.05$ ). The difference in TAFI activity concentrations in samples from the acute phase and from the controls did not reach statistical significance. In contrast, the total TAFI antigen concentration measured by the Haemochrom kit showed no significant difference between patients and healthy controls on any occasion.

### **Discussion**

Our results confirm that measured concentrations of TAFI are strongly dependent on the method used and that there may be a need for reconsideration of previous study results regarding TAFI (Willemse 2006). We observed low or moderate correlations between all three assays, indicating heterogeneity of the results between individuals, although the relatively large number of observations rendered the coefficients statistically different. In a bias plot (Fig. 9) it is obvious that the differences between activity and antigen concentrations (both Asserachrom and Haemochrom) increase with increasing activity concentrations. The Haemochrom method, said to measure all three forms of TAFI, gave lower concentrations than the Asserachrom method, which is specific for pro-TAFI, which in turn indicated lower concentrations than the functional method, measuring TAFI activity.

The Asserachrom method is regarded as genotype independent while the Haemochrom assay is not (Gils 2003). The presence of a common polymorphism (Thr325Ile) has been shown to cause a reduced response to some antibodies and can thus be the cause of the lower TAFI concentrations measured by the Haemochrom method. We found that the Pefakit kinetic assay principle, with high precision and easy performance, speaks in favour of the chromogenic assay based on determination of TAFI activity concentration, corresponding to the concentration of pro-TAFI. In addition we found elevated concentrations of pro-TAFI, here measured with both the Asserachrom and Pefakit methods, in patients suffering from ACS. Due to the small sample size this has to be re-evaluated in further studies with a chosen reference method where the Pefakit and Asserachrom assays seem to be appropriate candidates.

### 5.3 Paper III

In order to study coagulation and fibrinolysis in ACS we used a recently developed assay, the so-called Oh-index. This is a global method which provides simultaneous measurements of fibrin formation and fibrinolysis (fibrin degradation) expressed as the Coagulation and Fibrinolysis Profile (abbreviated as Cp and Fp), respectively. We also investigated thrombin generation *in vitro* using the commercially available calibrated automated thrombogram (CAT), and assessed thrombin generation *in vivo* by measuring concentrations of F1+2 in plasma. In order to characterize the patients further we also assessed markers of inflammation and endothelial function.

**Table 6. Treatment at the three different sampling occasions.**

	<i>Sample 1 (S1)</i>	<i>Sample 2 (S2)</i>	<i>Sample 3 (S3)</i>
Sample occasion	Acute, at admission	Within 24h after S1 but before CAI	Six months after the acute event
Aspirin (%)	Yes (100)	Yes (100)	Yes (88)
Clopidogrel (%)	No	Yes (95)	Yes (8)
LMH (%)	No	Yes (100)	Yes (1)*
GPIIb/IIIa receptor inhibitor (%)	No	Yes (8)	No
Warfarin (%)	No	No	Yes (9)*

\* not included in Figure 10, see below.

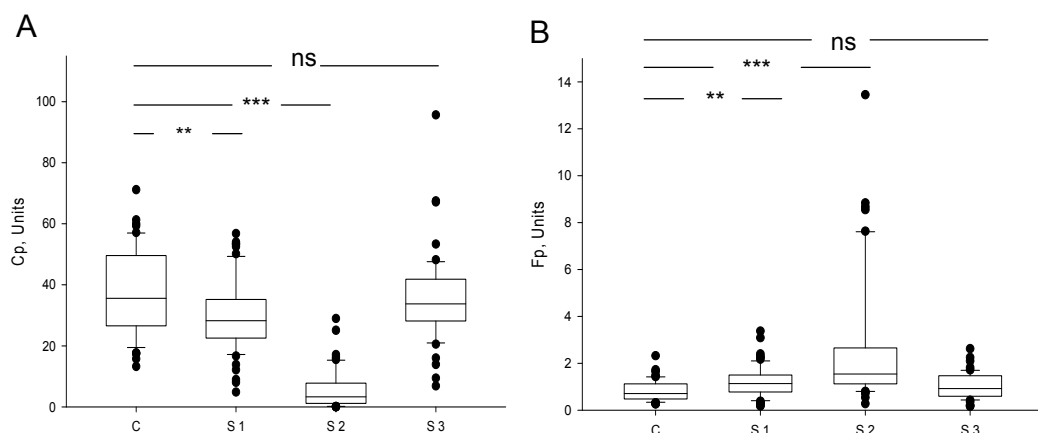


Eighty-seven ACS patients were sampled on three occasions (see Table 6). For comparison, 61 gender- and aged matched healthy controls were sampled once. Due to limited access to samples, the Oh-index was measured in 65 patients and 55 controls and CAT was used in 49 patients and 53 controls. The patients were partitioned according to a modified TIMI score (for details see Methods section). In all 87 patients, 56 % (n = 49) belonged to the high risk group (i.e. score  $\geq 4$  points), and in the 65 patients in whom the Oh-index was estimated the corresponding figure was 62 % (n = 40).

### ***Coagulation and Fibrinolysis Profiles***

The Coagulation profile (Cp) was significantly lower in patients at admission compared with that in healthy controls (Fig. 10A), and it decreased considerably during treatment with LMH ( $p < 0.001$ , S1 vs. S2). However, in the stable phase (S3) there was no significant difference between patients and healthy controls (Fig. 10A).

As can be seen in Figure 10B the Fibrinolysis profile (Fp) was higher in patients compared with healthy controls at admission (S1), increased profoundly and significantly during treatment with LMH ( $p < 0.001$  S1 vs. S2) and then normalized in the stable phase (S3).



**Figure 10A and B.** Coagulation profile (Cp; panel A), Fibrinolysis profile (Fp; panel B) in healthy controls (C), and in patients with ACS at three sampling occasions (S1-S3); see Table 6. The box plot shows median, upper and lower quartile with extensions to the 10th and 90th percentiles. \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .

### ***Thrombin Generation as measured by CAT and F1+2***

Below are shown peak thrombin concentrations and their times to peak as well as the areas under the curve representing the endogenous thrombin potential (ETP), i.e. the total amount of thrombin produced over time, see Fig. 5.

Patients had significantly higher values of “peak thrombin” than controls, both at admission (S1) and in S3, whereas there was no significant difference in “time to peak” or ETP between the groups on these two occasions (Table 7). As expected, during LMH

treatment (S2) ETP decreased ( $p<0.001$ ), “time to peak” was prolonged and “peak thrombin” reduced.

There were no significant differences between patients and healthy controls in the plasma concentrations of prothrombin fragment F1+2 at admission (S1) or six months after the acute phase (S3), data not shown. Also, F1+2 concentrations decreased as expected from S1 to S2 during LMH treatment ( $p<0.001$ ).

**Table 7. Thrombin generation in plasma**

	<i>Controls</i>	<i>S1</i>	<i>S2</i>	<i>S3</i>
Time to peak (min)	6.1 (4.3-8.2)	6.7 (4.6-9.9)	12.9*** (0.0-46.6)	6.1 (4.8-8.8)
Peak thrombin (nM)	261 (178-364)	283* (181-373)	73*** (0.0-339)	298** (212-354)
ETP (nM/min)	1683 (1192-2559)	1852 (1235-2724)	949*** (0.0-2283)	1799 (1221-2634)

Data expressed as median and interval (min-max). \* $p<0.05$ , \*\* $p<0.01$ , \*\*\* $p<0.001$ , patients compared to controls.

### ***TAFI and PAI-1***

TAFI activity concentrations were significantly elevated in patients compared with healthy controls both at admission (S1) and after six months (S3; Table 8), but with a slight decrease in between (S2).

At admission (S1) there were no significant differences in PAI-1 antigen concentrations in plasma between patients and healthy controls (Table 8). However, in S2, PAI-1 concentrations increased significantly ( $p<0.001$ ; S1 vs. S2) and they remained elevated in the stable phase ( $p<0.001$ ; S1 vs. S3).

### ***Inflammatory Variables and von Willebrand Factor***

Plasma concentrations of IL-6, hsCRP and fibrinogen were all elevated in patients compared with healthy controls at all time points (Table 8). Furthermore, these parameters had a dynamic course, with a significant increase in concentrations from S1 to S2 ( $p<0.001$  for all three) and thereafter significant decreases in IL-6 and hsCRP concentrations ( $p<0.05$  for IL-6,  $p<0.001$  for hsCRP).

Concentrations of VWF antigen in patients were significantly elevated at all time points compared with the healthy controls. However, there were no differences in ADAMTS13 activity or antigen concentrations between controls and patients at any time point (data not shown).

**Table 8. Plasma concentrations of markers of inflammation, fibrinolysis and coagulation in patients and healthy controls.**

	<i>Controls</i>	<i>S1</i>	<i>S2</i>	<i>S3</i>
IL-6 (µg/L)	0.9 (0.3-3.8)	1.9*** (0.3-18.2)	3.0*** (0.5-64.7)	1.4*** (0.4-16.7)
CRP (mg/L)	0.6 (0.1-17.3)	2.0*** (0.1-39.3)	3.1*** (0.1-39.8)	1.6*** (0.1-23.5)
Fibrinogen (g/L)	2.7 (2.0-4.4)	3.1*** (1.9-7.3)	3.3*** (2.2-7.3)	3.5*** (2.3-6.8)
PAI-1 antigen (µg/L)	14.7 (5.8-49.4)	14.3 (5.6-188.5)	22.6 *** (5.8-193.9)	22.7 *** (6.2-116.0)
TAFI (%)	124 (69-173)	134 * (83-186)	127 (90-186)	134** (87-180)
VWF antigen (kIU/L)	1.2 (0.7-2.8)	1.5*** (0.7- 4.0)	1.7*** (0.7-3.8)	1.5*** (0.7-3.6)

Data expressed as median and interval (min-max). \*p<0.05, \*\*\*p<0.001, patients compared to controls.

### ***TIMI Risk Score***

In all 87 patients, 56 % (n = 49) belonged to the high-risk group, and in the 65 patients in whom the Oh-index was estimated the corresponding figure was 62 % (n=40).

Patients with a high TIMI risk score were more frequently readmitted to hospital for cardiovascular reasons compared with those with a low TIMI score, at the six-month follow-up. In addition, patients with a high TIMI risk score more often had pathological echocardiograms during the index hospitalization (data not shown).

Patients with a high TIMI risk score had an increased Cp and increased concentrations of F1+2 at admission compared with patients with a low TIMI risk score, and for F1+2 this difference remained in the stable phase. For Fp there was no significant difference between groups.

### ***Correlations***

Regarding coagulation, there was a significant correlation between Cp and F1+2 (r = 0.43, p<0.001) in patients at admission, whereas there was no significant correlation between Cp and ETP at any time. At S2, i.e. during treatment with LMH, there was a

highly significant correlation between Cp and “peak thrombin” ( $r = 0.91$ ,  $p < 0.001$ ), but this relationship was not seen on the other two sampling occasions or in the controls.

Regarding fibrinolysis, there was a significant inverse correlation between Fp and both TAFI activity concentration and PAI-1 antigen concentrations at admission (S1;  $r = -0.32$ ,  $p < 0.05$  for TAFI,  $r = -0.35$ ,  $p < 0.01$  for PAI-1) and in the stable phase (S3;  $r = -0.34$ ,  $p < 0.05$  for TAFI,  $r = -0.51$ ,  $p < 0.001$  for PAI-1). In the controls this relationship was seen only between Fp and PAI-1 antigen concentrations ( $r = -0.34$ ,  $p < 0.05$ ).

### ***Discussion***

Our global method concerning coagulation and fibrinolysis did not indicate that patients with ACS have increased fibrin formation capacity, either in the acute or in the stable phase. This observation was made in spite of the fact that the majority of patients included in the present study had an intermediate to high risk as assessed by the TIMI risk score (i.e. score  $\geq 4$  points) in combination with an ongoing inflammatory reaction and endothelial activation. Furthermore, we did not document any increased thrombin generation *in vivo* as assessed by a single marker (F1+2). However, the detected difference in thrombin generation measured by CAT persisted 6 months after admission, suggesting that patients might have a persistent disposition to react with pronounced thrombin formation.

The increased fibrinolysis profile observed in the acute phase could be interpreted as a response to increased coagulation in order to achieve a balance in haemostasis. However, this increase in fibrinolytic capacity was somewhat unexpected, as the patients had more cardiovascular risk factors such as smoking, diabetes and hypertension, all of which have been reported to be connected with elevated concentrations of PAI-1 and thus impaired fibrinolysis. Our patients had significantly elevated concentrations of PAI-1 and we also reproduced our findings in Study II showing that TAFI activity concentration was significantly elevated in ACS patients. Taken together, this may indicate that “global fibrinolysis” is importantly influenced by factors other than PAI-1 and TAFI.

Despite repeated measurements we found no significant differences between patients and healthy controls in ADAMTS13 antigen or activity concentrations. Furthermore, there were no significant correlations between VWF antigen vs. ADAMTS13 activity or antigen concentrations, respectively. Thus, we find no support for the idea that elevated concentrations of VWF among ACS patients are a result of reduced function of ADAMTS13.

According to the Cp and Fp data shown in Figures 10A & B, there was relatively great inter-individual variability. If outliers differ with respect to prognosis it will be of interest in future prospective studies (see General discussion for further comments).

## 5.4 Paper IV

In this study we measured the plasma concentration of platelet derived microparticles (PMP) during and after ACS. Fifty-one patients with ACS and 61 gender- and aged matched healthy controls were investigated. Patients were sampled at admission, within 24 hours but before coronary angiography, and 6 months later. Controls were sampled once. PMP were measured by flow cytometry and defined as particles sized 0.1 -1.0  $\mu\text{m}$ , negative to phalloidin (labels cell-fragments), and positive to CD61 (GPIIIa). Thus all data below allude to CD61<sup>+</sup> PMP. Among those the presence of phosphatidylserine (PS), CD62P (P-selectin) or CD142 (Tissue factor) were also measured and PMP were sorted into subpopulations expressing or lacking these markers respectively.

### ***PS<sup>+</sup> PMP and PS<sup>-</sup> PMP***

Patients had significantly higher numbers of both PS<sup>+</sup> and PS<sup>-</sup> PMP at admission, and they decreased significantly ( $p<0.001$ , for both) from S1 to S2, i.e. following initiation of clopidogrel and LMH treatment, and then decreased further from S2 to S3, i.e. 6 months later ( $p<0.001$ , for both). Compared to healthy controls, patients had significantly higher numbers of both PS<sup>+</sup> and PS<sup>-</sup> PMP at admission (S1;  $p<0.001$  for both PS<sup>+</sup> and PS<sup>-</sup>) as well as during treatment (S2;  $p<0.001$  for PS<sup>+</sup>,  $p<0.01$  for PS<sup>-</sup>). In S3 however the numbers of PS<sup>+</sup> and PS<sup>-</sup> PMP were similar to healthy controls ( $p>0.05$ ; Table 9).

The ratio between PS<sup>+</sup>PMP and the total number of PMP increased slightly but non-significantly in patients from S1 into S2 and S3 resulting in a significantly higher ratio in patients at S2 ( $p<0.05$ ) and S3 ( $p<0.05$ ) compared to healthy controls. This reflects that a comparatively greater part of PMP in patients express PS at these two time-points.

### ***PS<sup>+</sup> CD62P<sup>+</sup> and PS<sup>-</sup> CD62P<sup>+</sup> PMP (Figure 11)***

Patients had elevated numbers of both entities of CD62P<sup>+</sup> PMP at admission, with a decrease following addition of clopidogrel and LMH (S1 to S2,  $p<0.001$ , for both) and then an even further decrease from S2 to S3 ( $p<0.01$  for PS<sup>+</sup> and  $p<0.001$  for PS<sup>-</sup>) (Fig. 11). However, in comparison to healthy controls the numbers of PS<sup>+</sup> CD62P<sup>+</sup> PMP and PS<sup>-</sup> CD62P<sup>+</sup> PMP were higher in patients at all three sampling occasions, ( $p<0.001$  for both variables at all occasions) (Table 9).

### ***PS<sup>+</sup> CD142<sup>+</sup> and PS<sup>-</sup> CD142<sup>+</sup> PMP (Figure 12)***

Also for both entities of CD142<sup>+</sup> PMP, patients had the highest numbers at admission (S1) with a significant decrease from S1 to S2 ( $p<0.001$ , for both) and a further decrease from S2 to S3 ( $p<0.001$ , for both) (Fig. 12). Consistent with CD62P<sup>+</sup> PMP, the number of both PS<sup>+</sup> CD142<sup>+</sup> PMP as well as the number of PS<sup>-</sup> CD142<sup>+</sup> PMP in patients remained significantly higher compared to the healthy controls at all three sampling occasions ( $p<0.001$  for both parameters at all occasions) (Table 9).

**Table 9. Numbers ( $\times 10^6$  /L) of PMP in patients with ACS and in healthy controls.**

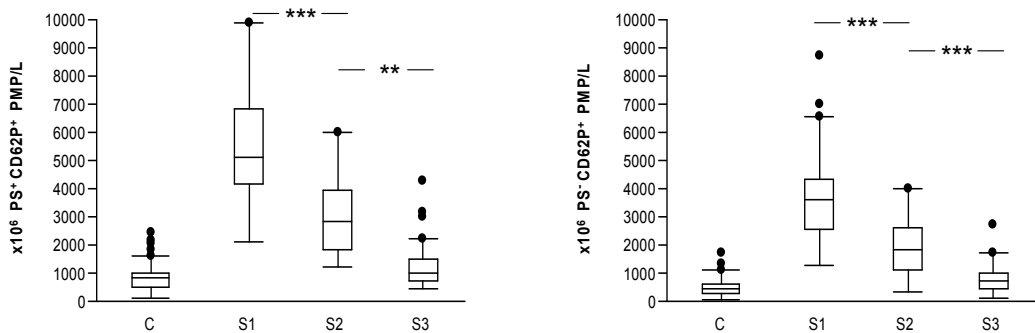
	<i>Controls</i>	<i>S1</i>	<i>S2</i>	<i>S3</i>
PS <sup>+</sup> PMP	9837 (6947-14939)	23799*** (12488-48381)	14115*** (6071-29828)	10933 (6349-18272)
PS <sup>-</sup> PMP	6063 (1426-8942)	11558*** (5280-29996)	6850** (2194-15063)	5293 (656-9456)
PS <sup>+</sup> CD62P <sup>+</sup> PMP	826 (113-2472)	5133*** (2095-9874)	2837*** (1232-5993)	1001*** (436-4298)
PS <sup>-</sup> CD62P <sup>+</sup> PMP	434 (28-1695)	3598*** (1292-8707)	1859*** (325-3973)	745*** (98-2700)
PS <sup>+</sup> CD142 <sup>+</sup> PMP	26 (9-65)	138*** (64-201)	99*** (38-179)	60*** (28-96)
PS <sup>-</sup> CD142 <sup>+</sup> PMP	16 (2-31)	98*** (39-209)	56*** (10-119)	22*** (3-81)

Data expressed as median and interval (min-max). \*\*p<0.01, \*\*\*p<0.001, patients compared to controls.

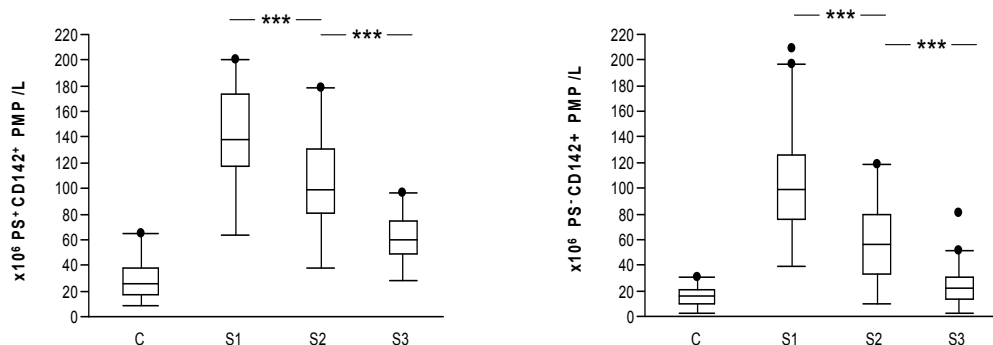
### Correlations

There were no correlations between platelet counts in venous blood and any of the PMP entities measured for either controls or patients.

Univariate correlation analyses could not reveal any significant relationships between levels of PS<sup>+</sup> PMP, CD142<sup>+</sup> PS<sup>+</sup> PMP or CD62P<sup>+</sup> PS<sup>+</sup> PMP and any of the inflammatory markers measured (i.e. hsCRP, IL-6, fibrinogen, or VWF).



**Figure 11.** Concentrations of PS<sup>+</sup> CD62P<sup>+</sup> PMP (left) and PS<sup>-</sup> CD62P<sup>+</sup> PMP (right) respectively in controls and patients at three sampling occasions S1, S2 and S3. The box plots show median, upper and lower quartile with extension to points still within 1.5 IQR from the quartiles. \*\* p<0.05, \*\*\* p<0.001.



**Figure 12.** Concentrations of PS<sup>+</sup> CD142<sup>+</sup> PMP (left) and PS<sup>-</sup> CD142<sup>+</sup> PMP (right) respectively in controls and patients at three sampling occasions S1, S2 and S3. Box plots as in Fig. 11. \*\*\* p<0.001.

## Discussion

In the present study we found that the numbers of all PMP subpopulations measured were highly increased in patients with ACS at admission to hospital. PMP concentrations followed a dynamic course, with a significant reduction of about 30-50% in all types of PMP, and this reduction followed the start of clopidogrel and LMH treatment (Fig. 11-12, Table 9). Six months after the acute event the numbers of PMP exposing CD62P and CD142 were still significantly higher than in healthy controls, and this was true for both PS<sup>+</sup> and PS<sup>-</sup> PMP. Of note, almost all patients were treated with aspirin during the study. Given that aspirin treatment in CAD reduces PMP (Bulut 2011) the difference in PMP numbers between patients and controls are likely to be underestimated.

The course of PS<sup>-</sup> PMP exposing the activation marker CD62P indicate that PMP formed during platelet activation may not necessarily expose significant amounts of PS. It is likely, however, that a part of the circulating PMP does not reflect platelet activation but originates directly from megakaryocytes in the bone marrow. This has been shown to include circulating PMP exposing PS but probably not activation markers like CD62P (Flaumenhaft 2009, see Fig. 3). The dynamics of both PS<sup>+</sup> and PS<sup>-</sup> PMP expressing CD62P observed in the present study fits well with the idea that PMP measurements in plasma reflect the degree of platelet activation in vivo.

The largest difference between patients and healthy controls at admission was observed for the PMP subpopulations that exposed CD62P (median value about six times higher for PS<sup>+</sup> and eight times for PS<sup>-</sup>) and CD142 (about five times higher for PS<sup>+</sup> and six times for PS<sup>-</sup>). The PS<sup>+</sup> and the PS<sup>-</sup> PMP populations not grouped for CD62P or CD142 were only

around two times higher in ACS patients (Table 9). Of note, the absolute numbers of CD142<sup>+</sup> PMP were comparatively low (less than 1 %; see Table 9), but the temporal pattern in ACS patients and the differences to healthy controls were highly significant and paralleled with CD62P<sup>+</sup> PMP (Figure 12, Table 9). Our interpretation is that CD142<sup>+</sup> PMP may be yet another useful marker of platelet activation. Unfortunately we performed no tissue factor activity (CD142) assay so we can not estimate its functional importance.

The effect of treatment with clopidogrel and LMH on PMP has not been investigated previously in the setting of ACS. As our study is observational we can not conclude that the significant fall in PMP concentration from S1 to S2 is due to antithrombotic treatment alone. However, some previous studies in patients with heart failure or diabetes suggest that treatment with thienopyridines reduces PMP numbers (Nomura 2004; Serebruany 2003). The effect of LMH, which like clopidogrel treatment was started after S1, is hard to appreciate but it is possible that this treatment through inhibition of thrombin may to some degree reduce PMP formation.

We used lactadherin instead of annexin V to detect PS exposure. Lactadherin is a compound which recently has been shown to be more sensitive than annexin V to detect PS (Hou 2011). About 2/3 of PMP were PS<sup>+</sup> in the healthy controls, and a similar PMP profile was observed in the ACS patients.

In summary, we find that the number of PMP is highly and significantly increased in the acute phase of ACS and then rapidly decreases following additional treatment with clopidogrel and parenteral anticoagulation. Especially subpopulations of PMP expressing CD62P or CD142 are strongly increased, and they continue to be elevated also 6 months after the acute event reflecting persistent platelet activation.



## 6 GENERAL DISCUSSION

In an acute ischaemic situation deviations in haemostasis may precede the disease or reflect the ongoing pathological process. It may further be assumed that when a thrombotic event is triggered, individuals react in different ways; some individuals are more prone to develop thrombosis than others. In some individuals even a superficial intimal erosion could be enough to trigger a hypercoagulative response (Falk 1995). Furthermore, reactions to therapeutics show great inter-individual variability as exemplified by “treatment resistance” towards antiplatelet therapy (Ahmad 2011). Thus, some individuals are hyporesponders suffering thrombotic events in spite of treatment while others are more prone to bleeding. Indeed, strategies involving extended potent antithrombotic treatment among low-risk individuals result in no clear benefit, but instead an increased risk of bleeding complications (Bhatt 2006). Tools to describe an individual’s haemostatic balance, and help to sort out “bleeders” and “clotters” in a given situation would optimize handling in clinical practice.

Several studies have revealed elevated concentrations of single haemostatic markers in ACS indicating a prothrombotic state. However, few markers have been evaluated with respect to diagnostic sensitivity and specificity, and when done most markers have not performed satisfactorily (van der Putten 2006). There do not seem to be simple correlations between the concentrations of a single factor and overall haemostatic function. For instance, TF, which is a central factor for coagulation, might be elevated in CAD, but one of its natural inhibitors – TFPI – has been shown to be elevated in the same condition (Soejima 1999). This might in the end lead to balanced haemostasis. Since haemostatic factors could be altered by many conditions as well as by ongoing treatments, the use of single markers as diagnostic tools is problematic (van der Putten 2006).

When discussing haemostasis in an atherosclerotic disease like ACS, it is inevitable that inflammation is included, since there is close interplay between these two systems. The vascular endothelium is the first and most important “target” to be affected by the atherosclerotic process. When LDL-cholesterol accumulates in the intima it is oxidized to a highly atherogenic and proinflammatory form, “oxidized LDL”, which activates the endothelium to express molecules that bind inflammatory cells which also enter the vessel wall. The inflammatory process intensifies during the development of an atherogenic plaque and a number of different cytokines are produced (Hansson 2005). In patients with ACS there are findings of elevated concentrations of inflammatory markers such as cytokines (e.g. IL-6 and IL-1) and acute-phase reactants (e.g. CRP and fibrinogen) (Hansson 2009). Elevated plasma levels of these inflammatory markers are linked to worse prognosis in ACS patients (Lindahl 2000; Koenig 2003). IL-6, which may stimulate production of a wide spectrum of acute-phase reactants including CRP and fibrinogen, has also been shown to stimulate production of PAI-1 in the liver and in adipose tissue (Rega

2005). Among other effects, some cytokines may influence the haemostatic activity of the endothelium. Cytokines may thus induce TF expression, inhibit NO production, cause platelet activation, and attenuate the anti-thrombotic properties of the endothelium through reduction of thrombomodulin production and protein C activation (Ajjan 2006). Accordingly, in many patients a primary proinflammatory state may lead to a secondary prothrombotic state.

## **6.1 ADAMTS13**

Diabetes is a well-known cause of endothelial dysfunction and a major risk factor of CAD, and therefore particularly suited for a study of ADAMTS13 and VWF, and their interrelationship. We found that VWF concentrations were significantly elevated in the patients with mainly type 2 DM and established macrovascular disease proven by peripheral artery occlusive disease (DM+PAOD, Study I), whereas ADAMTS13 antigen and activity concentrations did not differ significantly from those of healthy controls. Therefore, it is likely that the elevated VWF concentrations in this condition are not related to ADAMTS13 dysfunction. This is in line with previous results indicating that elevated VWF concentrations in patients with peripheral vascular disease are related to widespread endothelial cell damage (Lip 1997). It is thought that the VWF stored in Weibel-Palade bodies of endothelial cells and released on demand, consists of very large multimers, whereas the VWF that is secreted constitutively consists mostly of smaller multimers (Sadler 1998). It is possible that DM+PAOD patients have disturbed endothelial function with increased secretion of smaller VWF fragments that are equally recognized as larger multimers when measured by an ELISA.

In patients with DM without macrovascular complications (type 1 diabetes patients only), we found that ADAMTS13 activity concentration was reduced compared with that in healthy controls. Type 1 diabetes patients are prone to microvascular complications to which reduced ADAMTS13 activity possibly could contribute. This would then be a phenomenon similar to but less severe than TTP, a thrombotic microangiopathy in which ADAMTS13 is proven to be severely reduced. In this latter group of DM patients ADAMTS13 antigen concentrations were similar to those in the controls, indicating that the patients have a reduced ability to activate ADAMTS13 in spite of normal antigen concentrations. This is in agreement to what is observed in the microvascular thrombotic disease TTP, where patients with the acquired form can present normal ADAMTS13 antigen levels as measured by ELISA even though ADAMTS13 activity is decreased as a result of inhibitory antibodies (Tsai 2006). Accordingly, the use of different assays for ADAMTS13 may provide different results and this also emphasizes the need for standardized measurements in order to obtain results that are comparable between studies.

The ACS patients in Study III had significantly higher concentrations of VWF antigen compared with healthy controls, both acutely and in the stable phase of the disease. As regards ADAMTS13 antigen as well as activity concentrations there were no significant

differences between groups at any time point. In addition, we found no significant correlations between VWF antigen and ADAMTS13 activity or antigen concentrations. In a large case-control study Crawely et al. reported results similar to ours. They found no significant differences in plasma concentrations of ADAMTS13 antigen between controls and patients sampled between 3 and 9 months after an AMI, and furthermore VWF and ADAMTS13 concentrations were not associated (Crawely 2008). However, Crawely et al. found that ADAMTS13 antigen concentrations showed a negative association with the risk of AMI after adjustment for other risk factors of CHD. Furthermore, Miura et al. found that low ADAMTS13 antigen concentrations were associated with recurrent cardiovascular events in patients with CAD followed for a maximum of three years (Miura 2010). Experimental studies have shown that ADAMTS13 attenuates thrombus formation both *in vitro* and *in vivo* (Moriguchi-Goto 2009). Thus, one interpretation could be that once thrombus formation is triggered, e.g. by plaque erosion or rupture, then the function of ADAMTS13 is important in the regulation of the action of VWF on platelet adhesion and aggregation. Reduced function of ADAMTS13 may thus have negative consequences on thrombus growth, which to some degree may influence prognosis.

Regarding the possible role of ADAMTS13 in platelet responsiveness, some interesting observations have been made. ACS patients undergoing percutaneous coronary intervention (PCI) and having low platelet responsiveness to dual treatment with clopidogrel and aspirin, have been reported to have significantly lower ADAMTS13 *activity* than patients with normal antiplatelet responsiveness (Marcucci 2008). In the same study no significant differences in ADAMTS13 *antigen* concentrations between low- and normal “responders” were observed. Even though our data indicate that ADAMTS13 concentrations are not altered during an acute thrombotic event at a group level, this does not contradict the reasoning above concerning ADAMTS13 as pathophysiologically important in coronary thrombus formation. Individuals with ADAMTS13 in the lower range might thus have a tendency to form a larger and/or firmer thrombus. However, there are also arguments against this idea. Patients with “ADAMTS13 model disease” TTP do not become symptomatic until ADAMTS13 concentrations reach very low levels (<5–10%). Thus, there seems to be a great excess of the enzyme in plasma, raising the question of whether relatively small variations in plasma concentrations are clinically important.

One approach in assessment of the VWF–ADAMTS13 interrelationship has been to look at the ratio between their plasma concentrations. This VWF:ADAMTS13 ratio has been regarded as a potential risk marker by some investigators. Thus, Kaikita et al. found a positive association between the VWF:ADAMTS13 ratio and subsequent in-hospital cardiovascular events after AMI (Kaikita 2006). Interestingly, the patients in both Studies I and III did not respond with increased concentrations of either ADAMTS antigen or activity concentrations in spite of increased concentrations of VWF antigen. In Study I the VWF:ADAMTS13 ratio was significantly elevated in both groups of patients with diabetes mellitus compared with their controls. We also found that the ACS patients in Study III had significantly higher VWF:ADAMTS13 ratios than their controls both in the acute phase as

well as six months after the index event. However, since our study numbers were small, with few cardiovascular events, there was not enough statistical power to carry out outcome analyses.

## 6.2 TAFI

We found elevated concentrations of TAFI activity (corresponding to elevated concentrations of pro-TAFI) in ACS patients compared with healthy controls, both acutely and six months after the event. Previous data on TAFI and CAD are controversial. Santamaria et al. used a spectrophotometric endpoint activity assay and found that high TAFI activity concentrations were associated with an increased risk of acute CAD (Santamaria 2004). In contrast, Meltzer et al. performed a retrospective case-control study and found that *low* TAFI activity concentrations (measured by Pefakit) were associated with an increased risk (Meltzer 2009c). Of note, these patients were sampled on average 2.6 years after their MIs, while our patients were sampled acutely and after six months, and Santamaria et al. sampled within six months from the acute event. An explanation for these divergent findings could, however, be that TAFI not only inhibits fibrinolysis but also is involved in the regulation of inflammation (Myles 2003). Low TAFI concentrations could thus promote inflammation and thereby have long-term proatherosclerotic and prothrombotic effects. Such mechanisms may be revealed when sampling is carried out in a stable phase of CAD, as Meltzer et al. did in their study (Meltzer 2009c). In the acute phase, however high TAFI activity might contribute to attenuation of fibrinolysis and decreased thrombus degradation and thus increase the risk of total vascular occlusion. This is partly supported by the results of experimental studies showing that thrombus-adherent monocytes could depress fibrinolysis through TF-mediated improvement of TAFI activation (Semeraro 2009). ACS is associated with enhanced inflammation, and TAFI concentrations increase during inflammation (Skeppholm 2009), which may be yet another mechanism of importance in the acute phase of CAD.

Further support for the role of TAFI in acute thrombotic disease comes from the promising yet early studies of TAFI inhibitors as a treatment alternative (Willemse 2009). Conceptually they are based on the idea of a benefit in reducing elevated TAFI concentrations in line with our findings in Studies II and III.

## 6.3 Coagulation and Fibrinolysis

In Study III we found clear-cut signs of enhanced inflammation in the ACS patients. Thus, plasma concentrations of the inflammatory markers CRP (high-sensitivity analysis), IL-6 and fibrinogen were all elevated, as were those of PAI-1, an important anti-fibrinolytic variable also influenced by inflammation. These findings persisted six months after the index event. Somewhat unexpectedly, our hypothesis that the global method (Oh-index) would indicate a hypercoagulable state among patients compared with healthy controls was not confirmed. Moreover, we found no signs of increased thrombin generation *in vivo*,

which would have been detected by increased plasma concentrations of F1+2. Likewise, we found no influence on the endogenous thrombin potential (ETP, i.e. total amount of thrombin generated in plasma upon addition of TF). The only signs of increased coagulation observed were significantly higher values of “peak thrombin concentrations” assessed by way of the Calibrated Automated Thrombogram (CAT).

Also unexpected was the finding of increased fibrinolytic capacity (Oh-index assay) in ACS patients at admission. It is not clear how to interpret this finding. One explanation could be that it in some way reflects a triggered fibrinolytic system which acts upon the influence of increased coagulation and thrombin production.

Our result of an increased fibrinolytic capacity in ACS may at a first glance be in contrast to the results of studies indicating that a reduced fibrinolytic capacity is a risk factor of myocardial infarction (Meltzer 2009b) and other thrombotic manifestations on the arterial side (Guimarães 2009). However, as reasoned above, enhanced fibrinolysis as a response to thrombosis is to be expected, considering what regulates fibrinolysis. It is thus possible that the fibrinolytic capacity measured systemically in ACS, for example, could be higher than in a resting healthy person, in agreement with what we found in Study III. Nevertheless, subjects with relatively less fibrinolytic responsiveness may in the long-term be at risk of atherothrombotic complications, as shown in several studies. Indeed, a reduced fibrinolytic capacity as measured by the Oh-index method, and a prolonged clot lysis time, have recently been found to be associated with myocardial infarction (Leander; submitted 2011).

Concentrations of PAI-1 were elevated in ACS patients, as expected and in concordance with the results of previous studies (Hamsten 1985), and we found a significant inverse relationship between fibrinolytic capacity (Oh-index assay) and PAI-1 antigen concentrations, both at admission and after six months. This relationship was also evident in the group of healthy controls. An association between PAI-1 and CAD is, however, not evident in most studies when adjusting for other risk factors (Meltzer 2009a). This indicates that PAI-1 may be part of a complex risk profile rather than a single determinant of risk.

As discussed above, thrombin generation as measured by the Calibrated Automated Thrombogram (CAT) was used in Study III. The variable “peak thrombin concentration”, i.e. the maximal thrombin concentration reached when plasma coagulation is triggered by the addition of TF and phospholipids, was the only variable that indicated “hypercoagulation” in the ACS patients. Our CAT data are in agreement with the results of a recent study by Smid et al. (Smid 2011). They investigated 135 patients with ST-elevation myocardial infarction, and used the same CAT assay as we used, with the exception that they also used a lower concentration of TF (1 pmol/L) in addition to the commonly used 5 pmol/L concentration. Smid et al. also found that “peak thrombin concentration” (in their article, “peak height”) was significantly higher in patients compared with healthy controls, both in the acute and in the convalescent phase and for both TF concentrations, whereas as regards ETP there was a significant difference between these groups, but only when the low TF concentration (1 pmol/L) was used. It could be

argued that patients have a lower threshold for activation than healthy controls. This difference would be extinguished if the trigger concentration was sufficient to exceed the threshold for the healthy as well.

During a 12-month follow-up period Smid et al. found no association between ETP and recurring events. Very recently it was shown that thrombin generation as assessed by CAT was an independent predictor of ischaemic stroke but not CAD, and that the variable “peak thrombin concentration” was more strongly associated with risk than ETP (Carcaillon 2011). Taken together, data from our study, Smid et al, and Carcaillon et al show that peak thrombin concentration seem to be a more informative variable than ETP in terms of detection of thrombotic conditions, and perhaps prediction of vascular events (Smid 2011; Carcaillon 2011).

Smid et al also evaluated different coagulation factors as regards being determinants for CAT parameters and found TFPI – one of the natural inhibitors of TF – to be a negative determinant for both ETP and “peak thrombin concentration” (Smid 2011). These results illustrate some of the difficulties with so-called global methods. Since they are based on activation of plasma *in vitro* they can provide interesting information but they cannot claim to illustrate the haemostatic balance *in vivo*. For example, the amount of TFPI in a plasma sample may result in a disproportionate effect. This could be even more pronounced in the acute phase of an ACS event when patients may have consumed coagulations factors and have elevated concentrations of anticoagulants in their plasma. This reasoning could also be applied to the Oh-index analysis that we used.

For both the CAT method and the Oh-index the results might also be different with the use of platelet-rich-plasma, but then it would probably not be a suitable method in clinical practice due to methodological complexity.

### **6.3.1 Results related to clinical risk score**

We found that ACS patients with a high TIMI risk score ( $\geq 4$ ) had an increased fibrin formation capacity (Oh-index assay) and elevated F1+2 at admission compared with patients with a low TIMI risk score. Lee et al. (Lee 2005) also found elevated concentrations of haemostatic and inflammatory markers in ACS patients with high TIMI scores compared with those with a low score. Elevated troponin at admission indicates increased risk and is one of the factors included in the TIMI score (Antman 2000). Oldgren et al. (Oldgren 2004) found that troponin-positive ACS patients had higher plasma concentrations of F1+2 compared with troponin-negative patients. In the same study, the troponin-negative patients who, after 6 hours of intravenous treatment with a direct thrombin inhibitor (inogatran), showed unchanged or increased concentrations of F1+2 compared with pre-treatment levels, had an increased risk of recurrent events.

Our data in Study III are in agreement with those of Smid et al., who found that F1+2 plasma concentrations in STEMI patients were in the normal interval in the acute phase and remained unchanged at six months of follow-up, but nevertheless, patients with F1+2 concentrations above the median had an increased risk of recurrent ischaemic events (Smid 2011).

This illustrates two general problems. First, many coagulation factors can be produced locally at the site of a coronary thrombus, but plasma concentrations of the factor(s) measured in the peripheral circulation may not be increased. Secondly, although the increased risk for patients with, for example, a high TIMI risk score is partly due to increased thrombin formation as indicated by F1+2 concentrations, for instance, this is difficult to interpret in the individual patient without firmly established cut-off levels.

### **6.3.2 Treatment aspects**

During treatment with LMH the ACS patients in our study showed a significant decrease in fibrin formation capacity as well as in ETP and “peak thrombin concentration”. This is in line with previous results showing that CAT assay as well as OHP (the method that precede Oh-index) is sensitive to anticoagulant agents (Smid 2011; Brodin 2009; Antovic 2002). In contrast, the fibrin degradation capacity increased profoundly during treatment with LMH in comparison with admission values. This latter effect may be expected due to the formation of a looser fibrin network structure when thrombin formation is inhibited (He 2010) and a porous fibrin network will favour fibrinolysis. Interestingly, and for unknown reasons, TAFI activity was slightly but significantly lower at S2 during treatment with LMH in comparison with admission (S1) and convalescent (S3) concentrations.

Regarding ADAMTS13, there were no significant differences in antigen or activity concentrations in samples taken during treatment with LMH compared with concentrations at admission.

It should be emphasized that the patients in our ACS study (Studies II–IV) were relatively well treated with statins, angiotensinogen-converting enzyme inhibitors, angiotensin receptor antagonists and beta-adrenoreceptor antagonists. All these agents have been reported to modify the fibrinolytic system and – at least statins – attenuate inflammation (Ajjan 2006; Hansson 2005). In addition, statins and angiotensinogen-converting enzyme inhibitors have been shown to inhibit various aspects of coagulation and platelet function (Koh 2007; Ekholm 2002, Tehrani 2010; Mobarrez 2011). Also, treatment with aspirin has been shown to result in thicker fibrin fibrils, thereby forming a looser fibrin network more available for fibrinolysis (He 2009).

## **6.4 Platelet microparticles (PMP)**

It is known since long that platelet activation is evident in ACS, both acute and in a more stable phase after the acute event (Ault 1999). Activated platelets may increase the thrombin generation in plasma by 5 to 6 times (Merlini 2002), both by providing negatively charged surfaces and by the release of procoagulant factors. Activated platelets are not only closely involved in the coagulation cascade, e.g. by releasing factor V, but do also provide anti-fibrinolytic enzymes like TAFI and PAI-1. The substantial platelet contribution to PAI-1 plasma concentrations was illustrated in a study on stable CAD patients in whom an

elective PCI procedure caused a significant increase in circulating PAI-1. This was observed in patients not pretreated with clopidogrel whereas this was not seen in the pretreated patients (Katsaros 2008).

Due to the central role of platelets in haemostasis and thrombosis a lot of platelet function studies have been performed in CAD. Several methods for determination of platelet reactivity have been evaluated, but none proven to have a very good predictability with regard to cardiovascular complications or bleeding events (Breet 2010). Platelet microparticles (PMP) are shed from activated platelets and may possess procoagulant and proinflammatory properties by the expression of e.g. PS, CD62P (P-selectin) and CD142 (tissue factor). Measurements of PMP might therefore mirror platelet activation *in vivo*, but they may also by themselves have biological activities of importance for the pathophysiology CAD.

With a flow-cytometric assay we found that ACS patients had significantly higher concentrations of PMP in the acute phase compared to healthy controls, with decreasing values into the stable phase where PMP numbers became comparable to controls. Previous results indicate that circulating PMP subpopulations exposing activity markers better detect platelet activation than total numbers of PMP (van der Zee 2006). As discussed above we found that the largest difference in PMP numbers between patients and healthy controls was for PMP subpopulations exposing CD62P and CD142 and they were still significantly elevated six months after the acute event. In a small study on patients with AMI undergoing primary PCI, Gawaz et al showed increased generation of PMP following the procedure (Gawaz 1996). Interestingly, this increase was paralleled by a transient decrease in expression of various surface activation markers in a single platelet flow cytometric assay. Thus it seems more straightforward to measure and interpret flow cytometric data of PMP numbers than flow cytometric data on single platelet reactivity as regards platelet function *in vivo*. Our PMP data are in line with previous studies showing elevated concentration of PMP in ACS-patients acutely (Katopodis 1997) as well as in the stable phase after a myocardial infarction (Michelsen 2008). However, studies on the course of PMP formation during and after ACS have not been performed previously. The dynamics of the different PMP subpopulations observed in our study fits well with the idea that PMP measurements in plasma reflect the degree of platelet activation *in vivo*.

Although the increase in CD142<sup>+</sup> PMP numbers was up to 6-fold in Study IV, the absolute numbers were comparatively low (less than 1 %). The origins of CD142 molecules exposed on PMP are uncertain but they may be transferred from activated leukocytes but also originate from platelets (Rauch 2000; Schwartz 2006). Also the functional importance of CD142 exposed on PMP is controversial. However, CD142 (tissue factor) activity in plasma in patients with ACS was recently reported to carry prognostic information, and circulating microparticles were shown to be the major source of tissue factor activity (Steppich 2009).



We found that about 1/3 of PMP did not expose PS, a finding which was made in both healthy controls and in ACS patients. Others have recently reported that the majority of PMP in healthy individuals are PS<sup>-</sup> (Connor 2010), but this finding was made with annexin V, and thus the presence of PS may be underestimated (see above). Clearly more studies need to be done with respect to PS exposure, and the previous definition of PMP which includes PS exposure as a prerequisite has to be reconsidered. This is emphasized by our finding that ACS patients both in the acute and in the stable phase also have significantly elevated concentrations of PS<sup>-</sup> PMP expressing activity markers compared to healthy controls (Study IV).

As mentioned above, the effects of commonly used antithrombotic treatments on PMP have not been well studied. We found a significant decrease in all subpopulations of PMP investigated following treatment with clopidogrel and LMH. The decrease could be due to the treatment but also partly be due to a decline in disease activity in combination with an expected short half-life of PMP (in animal models reported to be as short as 10-30 minutes (Burnier 2009)). Randomized controlled studies are needed to in detail define the effects of various antithrombotic treatments on PMP. It may be, however, that PMP measurements could be used as tools in the monitoring of antiplatelet treatment.

Regarding prognostic information of PMP measurements there are hardly any studies published. In a preliminary report from the GRACE registry the risk of death or a new myocardial infarction 6 month after the acute event was significantly increased among patients with microparticles of non specified origin in the top tertile (Mallat 2004). Given that PMP in plasma reflect platelet function *in vivo*, and together with the many possible biological effects of microparticles (Morel 2006), it is clear that properly designed studies on PMP and prognosis would be worthwhile doing.

## 6.5 General remarks

There is perhaps a need for a redefined view of thrombotic disorders. Thrombus formation may be regarded as something that occurs beyond the normal balance between haemostasis and bleeding. From that point of view different pathophysiological pathways/mechanisms could be of interest when measuring activation markers as well as targets of antithrombotic treatment. Based on the fact that FXII deficiency does not cause bleeding problems, the factor has been regarded as not important for haemostasis *in vivo*. However, Gailani and Renné et al. have shown in different animal models that FXII deficiency is protective against arterial thrombosis, with no increased bleeding (Gailani 2007). This finding raises an important and interesting question regarding the contact activation pathway. It might not have a role in normal haemostasis but instead in the building of a stable thrombus in a pathological condition. For example, the contact pathway is described to be important for the generation of sufficient amounts of TAFI to achieve prolonged fibrinolysis (Heylen 2011). Furthermore, there are indications that the contact pathway of coagulation could be activated *in vivo* on the surface of platelets, a reaction that

may be enhanced through platelets expressing PS (Johne 2006). Also, the extracellular matrix protein laminin, which could be exposed to the circulation as a result of injury, has been shown to both activate FXII and as well as to stimulate platelets to expose PS (White-Adams 2010). Based on these observations it is reasonable to believe that PMP expressing PS and other activation markers localized around a thrombus might be activators of FXII, which in turn further contributes to the maintenance of sufficient thrombin generation and a stable clot (Gailani 2007).

TAFI, ADAMTS13 and FXII work alongside the tissue factor-induced pathway of the coagulation cascade. Hence, agents with effects on these enzymes could represent effective complementary antithrombotic treatment in ACS without excessive bleeding complications. There are interesting results showing that TAFI inhibition increases the efficiency of t-PA-mediated thrombolysis in animal studies, and a small pilot study in humans indicated that inhibition of TAFI enhanced endogenous lysis of pulmonary emboli during ongoing treatment with dalteparin (Willemse 2009). The experimental data reported by Moriguchi-Goto et al. showing that ADAMTS13 could attenuate thrombus growth, alluded to above (Moriguchi-Goto 2009), also indicate a plausible treatment strategy. We found in our ACS study (Study III) that the concentrations of ADAMTS13 antigen and activity concentrations were unchanged during treatment with LMH. This is of interest when considering ADAMTS13 as a future complementary therapeutic target.

## 7 CONCLUSION

The start point for this thesis was a clinician's need for tools to detect patients at high risk of thrombosis or bleeding. Haemostasis is complex, influenced by environmental as well as genetic factors. Analytical procedures are often rather complicated and influenced by preanalytical and experimental conditions which may further complicate the interpretation of data. Therefore precaution is called for when using both single haemostatic and more global methods in the risk evaluation of patients. In the light of these reflections our results indicate the following.

- ◆ Reduced function of ADAMTS13 might play a role in microthrombotic complications in patients with diabetes mellitus type 1, while elevated concentrations of the VWF in patients with diabetes mellitus and peripheral artery occlusive disease as well as in ACS-patients, is likely to be related to the release from a diseased vascular endothelium. The elevated ratio of VWF:ADAMTS13 found in these patients might however indicate a lack of ability to compensate for elevated VWF by increasing ADAMTS13 formation. This could contribute to the development of atherothrombotic complications.
- ◆ The measured concentrations of TAFI are strongly dependent on the method used. TAFI activity is elevated in ACS patients both in the acute phase and several months after the acute event. This could contribute to local attenuation of fibrinolysis with subsequent decreased thrombus degradation and thus an increased risk of thrombus progression and vascular occlusion.
- ◆ Both TAFI and ADAMTS13 are factors that influence haemostasis "outside" the tissue factor induced pathway of coagulation. They are therefore interesting molecules and/or drug targets in the development of future therapeutic agents. Such strategies should have the potential to attenuate thrombosis without increasing bleeding.
- ◆ In the acute phase of ACS, patients have an increased potential to generate thrombin as measured by the calibrated automated thrombogram, but not as measured by F1+2 concentrations in plasma, or by measurements of the fibrin formation capacity. Several months after the acute event, thrombin generation as measured by CAT is still elevated, but hypercoagulability is not detected by the F1+2 assay or by the Oh-index assay.
- ◆ The endogenous fibrinolysis inhibitor PAI-1, as well as TAFI (see above), is elevated during and after ACS reflecting impaired fibrinolysis. This is not detected with the Oh-index method.

- ◆ Our flow cytometry assay turned out to be easily performed and it can measure PMP in frozen-thawed samples. Data on PMP numbers obtained with this assay reflect the course of platelet activation during an ACS, and the assay seems to be sensitive to antithrombotic treatment. The use of frozen samples is an advantage as it makes it possible to analyze large patient populations.
- ◆ To conclude, this thesis has not provided sufficient information or data to support the clinical use of markers or methods to assess coagulation or fibrinolysis in the individual patient. However, our PMP data are in agreement with a dominating role of platelet function in the pathophysiology of acute coronary syndromes. As platelets influence coagulation partly through the formation of microparticles, flow cytometric assessments of PMP may provide important information over and above what could be shown with the other markers studied in the present thesis.

## 8 FUTURE PERSPECTIVES

- ♦ Evaluate our flow cytometric assay of PMP in larger trials, and to what extent it can be used for prognostic purposes and risk assessment of patients with CAD
- ♦ Study and characterize in more detail the effects of various antithrombotic treatments on PMP.
- ♦ The pathways that are more selectively involved in thrombosis than in normal haemostasis are interesting to explore since therapeutic interference with such “pathways” has the potential to reduce thrombus related damage without causing bleeding complications.

## 9 SVENSK SAMMANFATTNING

Hjärt-kärlsjukdom är, och förväntas under en längre tid vara, den ledande dödsorsaken i västvärlden. Ett s.k. akut koronart syndrom (AKS) innebär ett allvarligt tillstånd som innefattar både hjärtinfarkt och svår kärlkramp. Den utlösande orsaken är oftast en bristning i en åderförkalkad del av ett av hjärtats kranskärl. Denna bristning stimulerar till blodproppsbildning vilket kan medföra att kranskäret täpps igen och blodflödet i kranskäret avstannar. En del av hjärtat drabbas då av syrebrist och hjärtinfarkt utvecklas. Blodproppshämmande läkemedel har god effekt både akut och på längre sikt om man drabbas av AKS. Både risken för ny hjärtinfarkt och död minskar. Baksidan av behandlingen är att den också kan leda till ökad blödning, vilket i sig är ett risktillstånd. Kroppens system för blodproppsbildning är mycket komplext och innefattar både blodlevringssystemet samt blodplättarna (trombocyterna). Blodlevringen leder till bildningen av ett nätverk av s.k. fibrintrådar i vilket blodplättarna klumpar ihop sig till en propp. Blodet har även en förmåga att lösa upp skadliga blodproppar, det s.k. fibrinolytiska systemet. Genom att kunna mäta blodproppsbildning och blödningsrisk hos den enskilda patienten med ett blodprov skulle man kunna ”skraddarsy” blodpropps-hämmande behandling bättre än vad som idag är fallet.

I syfte att bättre förstå mekanismerna bakom blodproppsbildning (hemostasen) har vi undersökt olika metoder som skulle kunna vara till hjälp att förstå och bestämma proppbildningsrisken hos patienten. Vi har undersökt ett antal patienter med AKS med blodprov akut vid inkomst till sjukhuset, efter ett dygn då patienterna fått intensiv propphämmande behandling samt efter 6 månader. Vi har också undersökt friska personer som jämförelse.

Som förväntat hade patienterna förhöjda nivåer i blodet av ämnen som speglar åderförkalkning och inflammation. Med hjälp av en metod där man stimulerar blodproppsbildningen i ett provrör fann vi en kraftigare reaktion i blodprover från patienterna jämfört med de friska personerna. Vi använde även ytterligare en metod som vi själva utvecklat där man både stimulerar blodproppsbildning i ett provrör men samtidigt också undersöker blodets förmåga att lösa upp blodproppen (fibrinolysen). Denna metod visade emellertid inte att patienterna hade någon generell tendens till att bilda blodpropp och inte heller en nedsatt förmåga att lösa upp blodproppen.

Vi undersökte också särskilt blodplättarnas funktion i samband med blodproppsbildningen med en av oss utvecklad metod där man mäter små membranpartiklar (s.k. mikropartiklar) som avknoppas från kraftigt retade blodplättar. Vi fann att patienterna hade kraftigt förhöjda nivåer av dessa partiklar i blodet både i det akuta skedet och sex månader efter insjuknandet. Vi fann också att mikropartiklar kan bära särskilda molekyler som kan ha betydelse för proppbildning och inflammation, något som i sig lokalt kan påverka

proppbildningen men också ha ”fjärreffekter” i andra delar av cirkulationen än de områden som drabbas av själva blodproppen

Sammantaget finner vi stöd för blodplättarnas centrala roll för blodproppsbildning vid hjärtinfarkt. Det finns en möjlighet att blodprovsanalys av mikropartiklar från blodplättar skulle kunna användas till att bedöma patientens risk för blodpropp samt dennes svar på blodförtunnande behandling. Detta måste dock först utvärderas i större studier. Våra resultat avseende övriga analyser för blodproppsbildning och blodproppsnedbrytning ger inte stöd för att använda dessa metoder i värdering av den enskilde patienten.





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