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# ROLE OF THROMBIN IN CORONARY ARTERY BYPASS GRAFTING

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Academic Dissertation

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*To my family*

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# LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which are referred to in the text by their Roman numerals.

**I** Raivio P, Kuitunen A, Suojaranta-Ylinen R, Lassila R, Petäjä J. Thrombin generation during reperfusion after coronary artery bypass surgery associates with postoperative myocardial damage. *J Thromb Haemost* 2006; 4: 1523-9.

**II** Raivio P, Fernández JA, Kuitunen A, Griffin JH, Lassila R, Petäjä J. Activation of protein C and hemodynamic recovery after coronary artery bypass surgery. *J Thorac Cardiovasc Surg* 2007; 133: 44-51.

**III** Raivio P, Petäjä J, Kuitunen A, Lassila R. Thrombophilic variables do not increase the generation or procoagulant activity of thrombin during cardiopulmonary bypass. *Ann Thorac Surg*, in press.

**IV** Raivio P, Kuitunen A, Petäjä J, Ilveskero S, Lassila R. Monitoring high-dose heparinization during cardiopulmonary bypass – a comparison between prothrombinase-induced clotting time (PiCT) and two chromogenic anti-factor Xa activity assays. *Thromb Haemost*, in press.

# ABBREVIATIONS AND ACRONYMS

| | |
|---|---|
| ACT | activated clotting time |
| Anti-Xa | anti-factor Xa activity |
| APC | activated protein C |
| CABG | coronary artery bypass grafting |
| CAD | coronary artery disease |
| Ck-Mbm | mass of the Mb fraction of creatine kinase |
| CPB | cardiopulmonary bypass |
| EuroSCORE | European System for Cardiac Operative Risk Evaluation |
| F1+2 | prothrombin fragment F1+2 |
| FPA | fibrinopeptide A |
| GAG | glycosaminoglycan |
| GP | glycoprotein |
| GUSTO | Global Use of Strategies To Open occluded coronary arteries trial |
| HCII | heparin cofactor II |
| HMW | high molecular weight |
| ICU | intensive care unit |
| IL | interleukin |
| MCP-1 | monocyte chemotactic protein-1 |
| PAI-1 | plasminogen activator inhibitor-1 |
| PAR | protease-activated receptor |
| PDGF | platelet-derived growth factor |
| PiCT | prothrombinase-induced clotting time |
| Serpin | serine protease inhibitor |
| SFC | soluble fibrin monomer complexes |
| TAFI | thrombin-activatable fibrinolysis inhibitor |
| TAT | thrombin-antithrombin complex |
| TFPI | tissue factor pathway inhibitor |
| TnT | troponin T |
| t-PA | tissue-type plasminogen activator |
| vWF | von Willebrand factor |

# ABSTRACT


## BACKGROUND AND OBJECTIVES

Thrombin is a multifunctional protease, which has a central role in the development and progression of coronary atherosclerotic lesions and is a possible mediator of myocardial ischemia-reperfusion injury. Its generation and procoagulant activity are greatly upregulated during cardiopulmonary bypass (CPB). On the other hand, activated protein C (APC), a physiologic anticoagulant that is activated by thrombomodulin-bound thrombin, has been beneficial in various models of ischemia-reperfusion. Therefore our aim in this study was to test whether thrombin generation or protein C activation during coronary artery bypass grafting (CABG) associate with postoperative myocardial damage or hemodynamic changes. Because we postulated that thrombin generation during reperfusion would associate with clinically significant sequelae, we wanted to further investigate the regulation of thrombin during CABG. Specifically, we tested whether preoperative thrombophilic factors associate with increased CPB-related generation of thrombin or its procoagulant activity. Also, we measured the anticoagulant effects of heparin during CPB with a novel coagulation test, prothrombinase-induced clotting time (PiCT), and compared the performance of this test with the present standard of laboratory-based anticoagulation monitoring.

## METHODS

One hundred patients undergoing elective on-pump CABG were studied prospectively. Comprehensive thrombophilia screening was performed preoperatively to identify patients with thrombophilic variables. Activation of coagulation was assessed with serial measurements of markers of thrombin generation [prothrombin fragment F1+2 (F1+2)] and its procoagulant activity [soluble fibrin complexes (SFC)], a marker of fibrin degradation (D-dimer), and APC before, during, and after CABG. Anticoagulation during CPB was assessed with serial measurements of heparin effects with a novel coagulation test, PiCT and two chromogenic anti-factor Xa activity (anti-Xa) assays, which are the present standard of laboratory-based anticoagulation monitoring. Antithrombin and protein C activities were measured to evaluate their association with heparin activity. Clinical outcome was evaluated with measurements of hemodynamic parameters and cardiac biomarkers, mass of the Mb fraction of creatine kinase (Ck-Mbm) and troponin T (TnT).

## RESULTS

A progressive increase in markers of thrombin generation (F1+2), fibrinolysis (D-dimer), and fibrin formation (SFC) was observed during CPB, which was further distinctly propagated by reperfusion after myocardial ischemia, and continued to peak after the neutralization of heparin with protamine. APC levels increased only slightly during CPB before the release of the aortic clamp, but reperfusion and more significantly heparin neutralization caused a massive increase in APC levels. Protein C activation was clearly delayed in relation to both thrombin generation and fibrin formation.

We demonstrated a correlation between F1+2, a marker of thrombin generation, measured during reperfusion and postoperative levels of biochemical markers of myocardial necrosis. Multivariable logistic regression analyses identified thrombin generation during reperfusion to be independently associated with myocardial damage. Enhanced thrombin generation during reperfusion also associated with an adverse hemodynamic change, *i.e.* increased post-operative pulmonary vascular resistance. Thrombin and fibrin formation both preceded and dominated over protein C activation. When an early response of APC to thrombin generation was observed, i.e. at the end of ischemia and during early reperfusion, the postoperative hemodynamic profile was favorable. Conversely, high preoperative APC level and high peak APC level, which coincided with peak thrombin generation, associated with unfavorable postoperative hemodynamic performance. Despite the dynamic association of APC with postoperative hemodynamic recovery, there was no association between protein C activation and evidence of postoperative myocardial damage. A preoperative thrombophilic state did not associate with perioperative generation of thrombin or the procoagulant activity of thrombin in patients undergoing CABG.

We showed that there was poor agreement between PiCT and anti-Xa measurements and between two chromogenic anti-Xa assays in the setting of CPB. Basal antithrombin and protein C activity associated with PiCT and anti-Xa levels measured with one of the two anti-Xa assays. Also, in our cohort of patients with extensive heparinization, lower heparin levels (either PiCT or anti-Xa) during CPB associated with inferior thrombin control during late reperfusion after heparin neutralization and high heparin activities during CPB associated with fewer perioperative transfusions of blood products.

## CONCLUSIONS

In conclusion, we showed that thrombin generation during reperfusion after CABG associated with postoperative myocardial damage and pulmonary vascular resistance. Protein C activation during CPB was clearly delayed in relation to both thrombin generation and fibrin formation. Even though APC associated dynamically with postoperative hemodynamic performance, it did not associate with postoperative myocardial damage. Overall, our results suggest that hypercoagulation after CABG, especially during reperfusion, might be clinically important. Preoperative thrombophilic variables did not associate with perioperative thrombin generation or its procoagulant activity in patients undergoing CABG. Our results do not favor routine thrombophilia screening before CABG. There was poor agreement between PiCT and anti-Xa assays and between two chromogenic anti-Xa assays in monitoring heparin levels in the challenging setting of CPB. Further studies are needed to establish optimal laboratory-based methods for monitoring high heparin levels during CPB, but our results suggest that PiCT could be an alternative to the chromogenic anti-Xa assays. We also demonstrated that patients with high heparin levels during CPB received fewer transfusions than other patients did.

# INTRODUCTION

Open heart surgery became possible after the pioneering work of John Gibbon who performed the first successful clinical open heart surgical operation using CPB in 1953 (Stammers 1997). Thereafter many others perfected this technology which made the repair of complex cardiac lesions possible. To date, millions of patients have been treated successfully using CPB. Cardiac surgery with CPB remains fundamental in the treatment of cardiac diseases despite the advent of off pump-coronary artery bypass surgery and interventional cardiology.

While often irreplaceable, CPB has significant limitations. Apart from eliciting a systemic inflammatory reaction and profoundly disturbing hemostasis, CPB causes a significant increase in thrombin generation and its procoagulant activity. Thrombin is a multifunctional protease that has been shown to contribute to myocardial ischemia-reperfusion injury in animal studies. While thrombin is procoagulant, proinflammatory, and proapoptotic, the physiologic anticoagulant, APC, is anticoagulant, anti-inflammatory, and antiapoptotic. When levels of both thrombin and APC are propagated their biological net effects are incompletely understood. A central goal of this study was to achieve a better understanding of the significance of the complex interplay between thrombin and APC in the setting of CPB for CABG.

# REVIEW OF THE LITERATURE

## 1. Thrombin and its functions

"Can´t live without it; probably die from it" (Mann 2003b)

Thrombin is a multifunctional protease with functions extending from coagulation activation and inhibition to many aspects of cellular regulation (Figure 1). The central importance of thrombin is evident from studies of homogenous transgenic mice that are deficient in several components of the coagulation pathway essential to thrombin generation and its regulation. Transgenic mice deficient in tissue factor, factor VII, tissue factor pathway inhibitor, factor X, factor V, prothrombin, or protein C are incompatible with life (Mann 2003b). In humans, probably due to genetic heterogeneity that provides alternative pathways, deficiencies in coagulation components that are lethal in mice produce pathology that ranges from mild to severe (Mann 2003b). Even more importantly, unregulated and inappropriate production of thrombin promotes atherosclerosis and can lead to thrombosis, embolism, and, ultimately, death.

### 1.1. Role of thrombin in coagulation

Thrombin is a serine protease that has multiple coagulation-related natural substrates and cofactors (Table 1). Thrombin is the most potent known stimulator of platelet aggregation and degranulation, it is the final enzyme in the coagulation pathway leading to fibrin formation, and it has important anticoagulant functions. Depending on how thrombin activity is directed its net effect is either procoagulant or anticoagulant. The multiple functions of thrombin in hemostasis are directed by substrate-specificity largely controlled by cofactors (Lane *et al.* 2005). The thrombin molecule has an active site cleft and two exosites on its surface (anion-binding exosites ABEI and ABEII) that are crucial for substrate and cofactor recognition (Huntington 2005). The competitive binding of cofactors plays a crucial role in redirecting thrombin activity from procoagulant to anticoagulant in areas of intact endothelium. The cofactors fibrin and thrombomodulin compete for binding to exosite ABEI and the cofactors heparan sulfate (a glycosaminoglycan) and platelet glycoprotein Ibα compete for binding to exosite ABEII. In areas of damaged vessel procoagulant fibrin and platelets (glycoprotein Ibα) are abundant and will be favored in binding to thrombin while in areas of intact endothelium thrombomodulin and heparan sulfate are more abundant and will dominate exosite binding favoring the anticoagulant function of thrombin. Thrombomodulin also has a much higher affinity for thrombin than fibrin does. (Lane *et al.* 2005)

#### 1.1.1. Thrombin formation

The initiating event leading to thrombin generation is the complexing of tissue factor with factor VIIa, which is incapable of proteolytic activity unless it is bound to tissue factor (Figure 2). Tissue factor is constitutively expressed on subendothelial cells, such as vascular smooth muscle cells, but endothelial cells, monocytes, and platelets express tissue factor only after stimulation (Mackman *et al.* 2007, Steffel *et al.* 2006). Circulating microparticles originating from platelets, leukocytes, endothelial cells, or vascular smooth muscle cells also express tissue factor (Furie and Furie 2005, Steffel *et al.* 2006). Another form of circulating tissue factor,

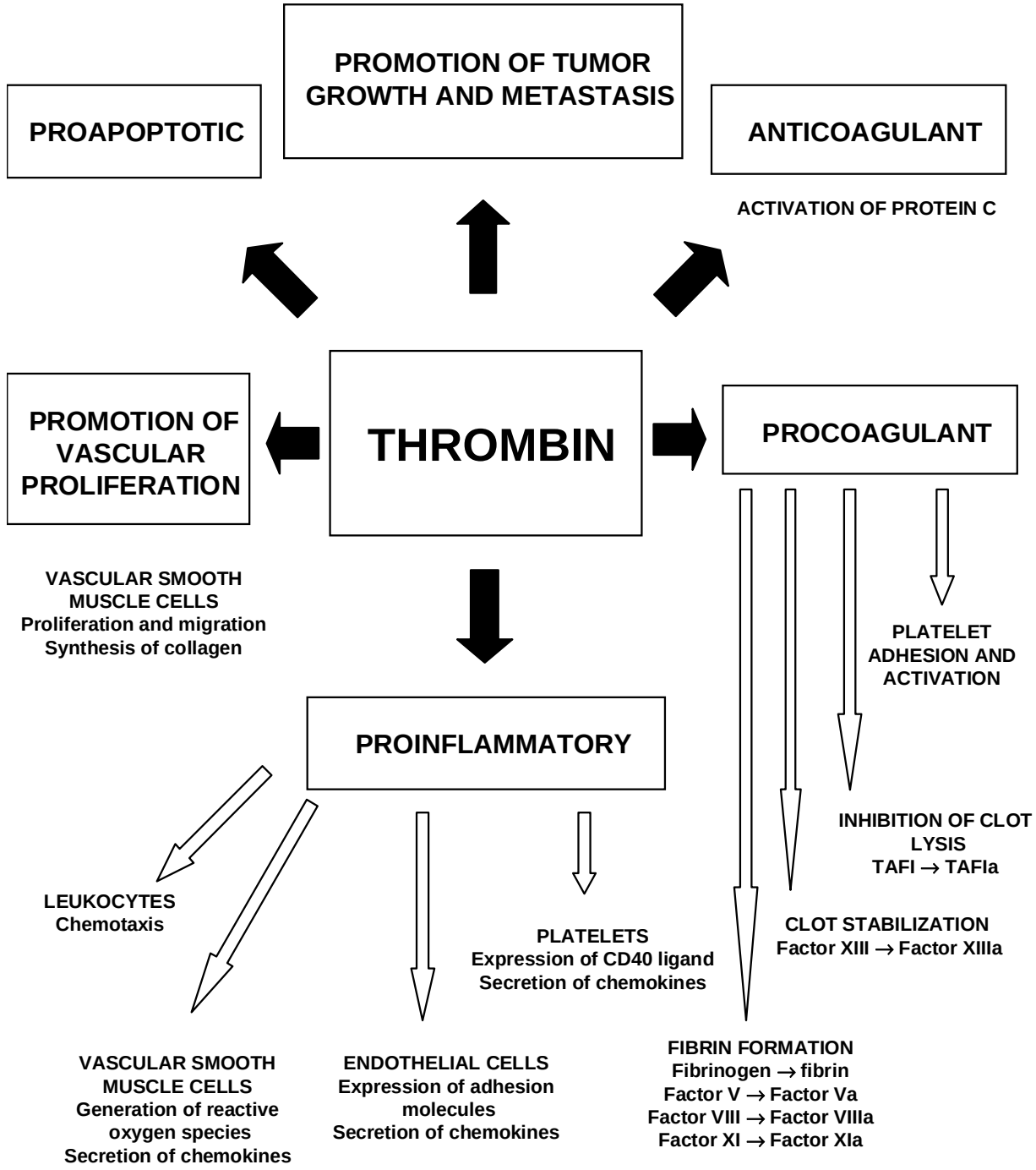


**Figure 1.** Functions of thrombin. See reviews by Croce and Libby 2007, Esmon 2005, Lane *et al*. 2005, Nierodzik and Karpatkin 2006, and text for further references.

| Substrate/ effector | Effect of cleavage/ binding by thrombin | Known cofactor | Fold activation |
|---|---|---|---|
| **Procoagulant or prohemostatic** | | | |
| Fibrinogen | Cleavage of fibrinopeptide A to form fibrin monomer (fibrin I) and cleavage of fibrinopeptide B generating fibrin II | $Na^+$ | 7-20 |
| Factor V | Activation to factor Va | None | - |
| Factor VIII | Activation to factor VIIIa | None | - |
| Factor XI | Activation to factor XIa | GpIbα | $5 \times 10^3$ |
| ADAMTS13 | Inactivation of ADAMTS13 (leads to reduced processing of vWF) | None | - |
| Factor XIII | Activation to factor XIIIa (cross-links fibrin) | Fibrin | 80 |
| TAFI | Activation of TAFI (inhibits fibrinolysis) | Thrombomodulin | $1.25 \times 10^3$ |
| PAR-1 | Platelet activation | GpIbα | 5-7 |
| PAR-4 | Platelet activation | None | - |
| GpV | Possibly facilitates cleavage of PARs | GpIbα | 6-10 |
| **Anticoagulant** | | | |
| Protein C | Activation to APC (inhibits factors Va and VIIIa) | Thrombomodulin | $1\text{-}10 \times 10^3$ |
| Antithrombin | Inhibition of thrombin | Heparin, GAGs | $1\text{-}20 \times 10^3$ |
| Heparin cofactor II | Inhibition of thrombin | Heparin, GAGs | $1\text{-}70 \times 10^3$ |

**Table 1.** Natural coagulation-related substrates/ effectors of thrombin, their cofactors, and magnitude of activation by the cofactor (Fold activation) as presented in reviews by Adams and Huntington (2006), Brass (2003), Huntington (2005), and Lane *et al*. (2005). ADAMTS13 = a disintegrin and metalloprotease with thrombospondin type 1 motif, APC =activated protein C, GAGs = glycosaminoglycans, Gp = (platelet) glycoprotein, PARs = protease-activated receptors, TAFI = thrombin-activatable fibrinolysis inhibitor, vWF = von Willebrand factor

soluble tissue factor, has been recently discovered. It is also procoagulant, but it is not bound to microparticles (Steffel *et al.* 2006). The relative contribution of each form of tissue factor, i.e. soluble, microparticle-bound, cellular and vessel wall-associated tissue factor, to the initiation and propagation of coagulation is still unclear (Steffel *et al.* 2006).

When vessel wall injury occurs tissue factor and factor VIIa complex on the negatively charged phospholipid membrane surfaces of tissue factor-presenting cells in the presence of calcium ions to form the extrinsic tenase complex (Mann *et al.* 2003) (Figure 2). The factor VIIa-tissue factor complex (extrinsic tenase complex) both activates factor X directly to produce factor Xa and it also activates factor IX to produce factor Xa by the intrinsic tenase complex. Factor Xa in turn complexes with factor Va to form the prothrombinase complex, which cleaves prothrombin into thrombin (Figure 2). When prothrombin is cleaved the active protease thrombin and F1+2 are generated. (Lane *et al.* 2005, Mann 2003a) A platform of negatively charged phospholipids, which are brought to the surface of cells such as platelets following their activation, and the presence of calcium ions are required for the proteolytic action of coagulation enzymes to occur with the exception of the procoagulant reactions catalyzed by thrombin (Gomez *et al.* 2005).

The thrombin-generating coagulation pathway was previously considered to include the extrinsic and contact, or intrinsic, pathways. Current knowledge of the process leading to thrombin formation has been compiled to form the “revised pathway of coagulation” (Mann 2003a) (Figure 2). Factors VIII and IX were included in the tissue-factor-initiated pathway of coagulation when Osterud and Rapaport (1977) showed that factor IX can be activated by the tissue factor - factor VIIa complex. Further understanding came from the knowledge that factor XI is activated directly by thrombin (Gailani and Broze 1991). The components of the former intrinsic pathway: factor XII, prekallikrein, and high-molecular-weight kininogen, do not appear to be fundamental for coagulation, but the significance of their contribution to hemostasis remains an open question (Mann 2003a).

Tissue factor-induced thrombin generation can be divided into two phases based on whole blood experiments, the initiation phase and the propagation phase. At first, during the initiation phase very small, nanomolar, amounts of thrombin are generated. The major bolus of thrombin (>96%) is produced secondarily during the propagation phase. During the initiation phase sub-picomolar amounts of factor Xa and factor IXa are formed. The very small amount of thrombin that is subsequently formed activates the extrinsic and intrinsic tenase complexes in an autocatalytic process leading to more formation of the catalyst (thrombin). In this process the intrinsic tenase complex is 50 times more efficient in activating factor X to Xa than the extrinsic tenase complex. The transition from slow to very rapid thrombin generation that occurs represents the transition from the initiation phase to the propagation phase. Clot formation in whole blood experiments occurs close to the end of the initiation phase, when only approximately 3-5% of the total amount of thrombin is formed. Therefore, the propagation phase is undetected by clot-based assays. (Mann *et al.* 2003, Mann 2003a)

### 1.1.2. Procoagulant functions of thrombin and the formation of a stable platelet plug

The principle procoagulant functions of thrombin are the activation of platelets mainly through proteolytic cleavage of protease-activated receptors (PARs) 1 and 4 and the cleavage of

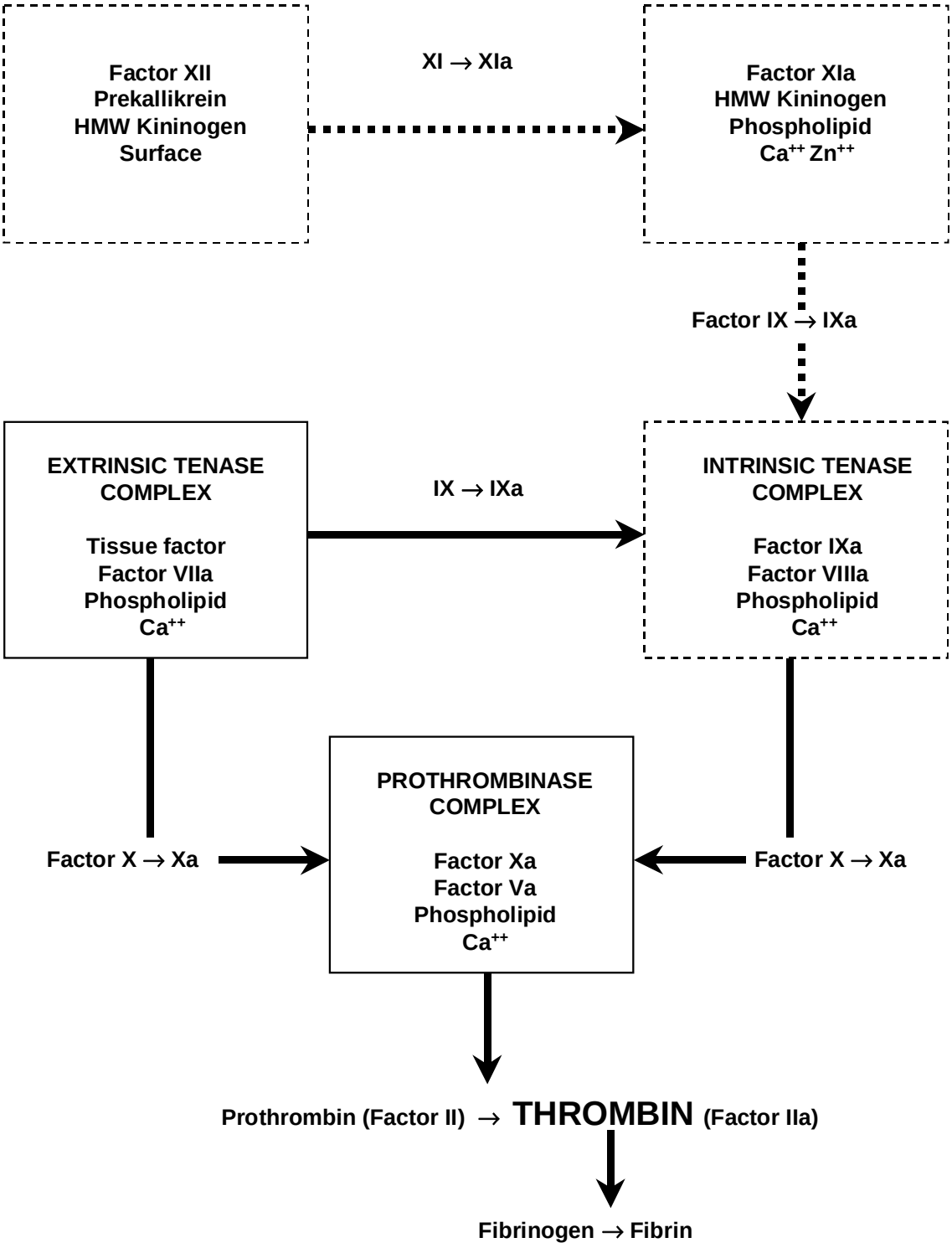


**Figure 2.** The revised pathway of coagulation. Components of the former intrinsic pathway of coagulation are shown with dashed lines. Modified from Mann *et al*. 2003.

fibrinogen to fibrin to produce the fibrin meshwork of platelet plugs (Brass 2003) (Figure 1). Thrombin converts fibrinogen to fibrin by first cleaving fibrinopeptide A (FPA) to generate fibrin monomer (fibrin I) which spontaneously polymerizes to protofibrils. Fibrinopeptide B is next cleaved from fibrin I to produce fibrin II protofibrils that undergo lateral aggregation to form the scaffold for the fibrin clot. Factor XIII, which is also activated by thrombin, in turn, stabilizes fibrin by covalent cross-linking. (Lane *et al.* 2005)

Injury to the vessel wall causes collagen, von Willebrand factor (vWF), and tissue factor to be exposed. This leads to a series of events that ultimately leads to the formation of a stable fibrin-anchored platelet plug. The formation of the platelet plug is initiated with tethering, rolling, and arrest of platelets on exposed collagen with vWF supporting platelet-collagen and platelet-platelet interactions to form a platelet monolayer. Local tissue factor-induced thrombin generation on activated platelets aids in the initiation of platelet plug formation. The extension of the platelet plug occurs when platelet agonists thrombin, adenosine diphosphate, and thromboxane $A_2$ further activate platelets and cause the recruitment of additional platelets which accumulate on the platelet monolayer to from a fibrin-anchored platelet plug in which platelet-platelet interactions are supported by the binding of fibrinogen, fibrin, and vWF to platelets. (Brass 2003) Contrary to classical models of thrombus formation in which the platelet plug first forms and is then stabilized by formation of a fibrin clot, intravital microscopy of the microcirculation of living mice has revealed that platelet plug and fibrin clot formation occur nearly simultaneously (Furie and Furie 2005).

### 1.1.3. Anticoagulant function of thrombin and physiological inhibition of thrombin

Thrombomodulin has a central role in directing thrombin activity from procoagulant to anticoagulant. It is an integral membrane protein that is expressed on the surface of endothelial cells and thrombin binds to it with high affinity. Once thrombin binds to thrombomodulin it is no longer capable of cleaving its procoagulant substrates. Thrombomodulin also acts as a cofactor of thrombin in the activation of the anticoagulant protein C pathway. Thrombomodulin-bound thrombin activates protein C, which is docked on its receptor, endothelial protein C receptor, on the endothelial surface. The proteolytic cleavage of protein C by thrombin is enhanced over 1000-fold by thrombomodulin alone and approximately 10 000-fold by thrombomodulin in the presence of a phospholipid membrane. APC binds to its cofactor, protein S, and proteolytically inactivates factors Va and VIIIa, which leads to down-regulation of thrombin generation. (Gomez *et al.* 2005, Huntington 2005, Lane *et al.* 2005)

The major physiologic anticoagulants are APC, tissue factor pathway inhibitor (TFPI), antithrombin, and heparin cofactor II (HCII). While APC is the anticoagulant product of proteolytic cleavage of protein C by thrombin and TFPI is the major inhibitor of the tissue factor-factor VIIa complex, antithrombin and HCII inhibit thrombin directly (Table 1). Antithrombin and HCII are serpins (serine protease inhibitors). Antithrombin is the main circulating inhibitor of coagulation proteases and physiologically HCII has at best only a secondary role in the inhibition of thrombin (Huntington 2005, Lane *et al.* 2005). In addition to thrombin, antithrombin inhibits coagulation factors Xa, IXa, XIa, and XIIa in a process termed “suicide substrate inhibition”, in which the interaction between the protease and antithrombin causes a conformational change in both molecules leading to geometric distortion of the active site of the protease (Kottke-Marchant and Duncan 2002). Glycosaminoglycans (heparin, heparan sulfate, and dermatan sulfate) accelerate the inhibition of thrombin by antithrombin up to 20 000-fold and the inhibition of thrombin by HCII up to 70 000-fold (Lane *et al.* 2005).

### 1.1.4. Fibrinolysis and thrombin

Plasmin is the major fibrinolytic protease responsible for the cleavage of fibrin. The zymogen, plasminogen, is converted to the active enzyme, plasmin, by tissue-type plasminogen activator (t-PA) or urokinase. Plasmin also transforms both t-PA and urokinase form single-chain to more active two-chain polypeptides. t-PA is synthesized and secreted primarily by endothelial cells and is the major intravascular activator of plasminogen. Fibrin acts as a cofactor for plasmin generation by increasing the efficiency of plasminogen cleavage by t-PA. Fragments known as D-dimers are released when cross-linked fibrin is degraded by plasmin. (Cesarman-Maus and Hajjar 2005)

There are three major physiological inhibitors of fibrinolysis: $\alpha_2$-antiplasmin, plasminogen activator inhibitor-1 (PAI-1), and thrombin-activatable fibrinolysis inhibitor (TAFI). $\alpha_2$-antiplasmin is a serpin that inhibits plasmin directly. PAI-1 is the most important and rapidly acting inhibitor of both t-PA and urokinase and its activity leads to reduced plasmin formation. TAFI is a carboxypeptidase that circulates in plasma as a zymogen. It is activated by thrombin and therefore acts as an intermediate between coagulation and fibrinolysis. TAFI efficiently inhibits fibrinolysis by removing carboxy-terminal lysine residues from partially degraded fibrin. These lysine residues are binding sites for both plasminogen and t-PA on fibrin and TAFI therefore abrogates the cofactor function of fibrin in plasmin formation. The endothelial cell receptor thrombomodulin stimulates the activation of TAFI by thrombin approximately 1250-fold. Activation of TAFI by thrombin down-regulates plasmin formation and stabilizes the fibrin clot. (Cesarman-Maus and Hajjar 2005, Mosnier and Bouma 2006) TAFI may also have important functions in the regulation of inflammation as it inactivates bradykinin and the anaphylatoxins C3a and C5a (Bouma and Mosnier 2006).

## 1.2. Thrombin and inflammation

Thrombin is an important link between coagulation and inflammation. The proinflammatory effects of thrombin are mediated through activation of endothelial cells, smooth muscle cells, and platelets as well as release of cellular mediators and its anti-inflammatory effects are mediated through activation of natural anticoagulant mechanisms. Interestingly many of the proinflammatory effects of thrombin have implications for the promotion of atherosclerosis (Croce and Libby 2007) (Figure 1). On the other hand, inflammation shifts the hemostatic balance toward coagulation by elevating platelet count and platelet reactivity, by downregulating natural anticoagulant mechanisms, by initiating and propagating thrombin generation, and by impairing fibrinolysis (Esmon 2005).

Thrombin activates endothelial cells by cleaving PARs-1 and -2 resulting in the expression of several leukocyte adhesion molecules (VCAM-1, ICAM-1, and E-selectin) and in the secretion of inflammatory chemokines [interleukin (IL)-6, IL-8, monocyte chemotactic protein-1 (MCP-1), platelet-derived growth factor (PDGF), and macrophage migration inhibitory factor (MIF)]. IL-6, on the other hand, is also prothrombotic by increasing the levels of circulating fibrinogen and PAI-1. (Croce and Libby 2007, Esmon 2005) Thrombin stimulates the proliferation and migration of vascular smooth muscle cells and the synthesis of collagen, generation of reactive oxygen species, and secretion of chemokines (IL-6 and MCP-1) by smooth muscle cells (Patterson *et al.* 2001). Thrombin-mediated activation of platelets also results in the secretion of chemokines (PDGF among others) and the expression of CD40 ligand which also induces

the secretion of chemokines and the expression of adhesion molecules by endothelial cells, smooth muscle cells, and macrophages (Croce and Libby 2007, Esmon 2005).

### 1.3. Thrombin and apoptosis

Moderate concentrations of thrombin protect neurons from toxic insults while high concentrations or prolonged exposure to thrombin induce apoptosis and cell death (Donovan and Cunningham 1998). A similar dual effect of thrombin has been observed on human tumor cells. Lower concentrations of thrombin (1-5 nmol/ L) enhanced tumor cell growth while high concentrations (5-10 nmol/ L) induced apoptotic cell death in cultures of several human tumor cell lines (Ahmad *et al.* 2000, Zain *et al.* 2000). High concentrations of thrombin have also been shown to have proapoptotic effects on cultured vascular smooth muscle cells (Rossignol *et al.* 2004). In addition to its apoptotic effects on nucleated cells, the platelet activator thrombin has also been shown to trigger apoptosis in platelets (Leytin *et al.* 2006). Interestingly, both the antiapoptotic and proapoptotic effects of thrombin in these experiments have been shown to be PAR-1-mediated.

While thrombin is proapoptotic in high concentrations, APC has been shown to have both *in vitro* antiapoptotic effects on hypoxic cultured brain endothelial cells and *in vivo* neuroprotective effects in murine stroke models (Cheng *et al.* 2003, Liu *et al.* 2004).

### 1.4. Thrombin in tumor angiogenesis, tumor growth, and metastasis

The association of venous thrombosis and cancer is well recognized and was first reported over 140 years ago by Armand Trousseau. However, more recent research has revealed an independent role for thrombin in inducing tumor cell adhesion to platelets, in stimulating tumor angiogenesis, in enhancing tumor cell growth, and in increasing tumor cell seeding and spontaneous metastasis. Thrombin induces the secretion of vascular growth factors and it has a significant stimulatory effect on angiogenesis. Platelets are required for the hematogenous dissemination of tumor cells as tumor cell-platelet aggregates are protected from natural-killer cell-mediated elimination. Thrombin increases tumor cell-platelet adhesion by activating platelets and by inducing the expression of fibronectin and vWF on the platelet surface. Thrombin-treated tumor cells also express glycoprotein IIb-IIIa that aids in their adhesion to platelets and endothelial cells. The development of experimental pulmonary metastases is enhanced up to over 150-fold by thrombin treatment of injected tumor cells and 5-fold by overexpression of the thrombin receptor PAR-1 by tumor cells. (Nierodzik and Karpatkin 2006) Also, the thrombin inhibitor hirudin inhibits tumor growth and metastasis in mice with spontaneously metastasizing breast tumor (Hu *et al.* 2004).

Of particular interest are clinical observations that support the role of thrombin in cancer progression. Improved survival in patients with small-cell lung cancer has been reported with oral anticoagulant, heparin, and low molecular weight heparin treatment (Nierodzik and Karpatkin 2006). Moreover, treatment of a first episode of venous thromboembolism with warfarin for 6 months *versus* 6 weeks associated with a reduction in the incidence of urogenital cancers in the ensuing 6 years of follow-up (Schulman and Lindmarker 2000). Finally, a recent analysis of the Second Northwick Park Heart Study revealed an increase in digestive tract cancer incidence and mortality during eleven years of follow-up of subjects who had evidence of persistent activation of coagulation at baseline defined as two consecutive yearly measurements of F1+2 and FPA in the highest quartile (Miller *et al.* 2004).

# 2. Thrombin, coagulation, and atherosclerosis

## 2.1. Experimental evidence

Many of the cellular responses elicited by thrombin can promote the development of proliferative vascular lesions. Thrombin has powerful proinflammatory effects on endothelial cells, smooth muscle cells, and platelets including the secretion of many chemokines and the PAR-1-mediated induction of proliferation and migration of vascular smooth muscle cells and synthesis of collagen by vascular smooth muscle cells (see 1.2. Thrombin and inflammation above) (Croce and Libby 2007). Thrombin also induces the proliferation of endothelial cells (Herbert *et al.* 1994) and the proliferation and migration of fibroblasts (Chen and Buchanan 1975).

In addition, several lines of experimental evidence suggest that thrombin has an important role in the development of vascular disease by promoting the formation of neointimal hyperplasia and atherosclerotic lesions. In rat and baboon models thrombin receptor PAR-1 expression is upregulated in vascular smooth muscle cells of the media and neointima in response to vascular injury (Wilcox *et al.* 1994). Mice deficient in PAR-1 (PAR-1 -/-) displayed a trend toward less neointimal formation after mechanical carotid artery injury than wild type mice did (Cheung *et al.* 1999). Also, in a rat model of restenosis after carotid artery balloon angioplasty a synthetic PAR-1 antagonist significantly reduced neointimal thickness (Andrade-Gordon *et al.* 2001) and an antibody against PAR-1 reduced neointimal smooth muscle accumulation and expression of PAR-1 messenger RNA (Takada *et al.* 1998). In contrast to human platelets, PAR-1 is not expressed in rodent and rabbit platelets (Cheung *et al.* 1999) and in mice thrombin activates platelets by cleaving PAR-4 with PAR-3 serving to facilitate this cleavage (Brass 2003). Because PAR-1 does not mediate thrombin-induced platelet activation in rodents or rabbits these experimental results suggest that non-platelet effects of thrombin have a role in the development of neointimal hyperplasia in response to vascular injury. Contrary to these findings the local application of an antisense PAR-1 oligonucleotide in a rabbit model of vascular injury inhibited PAR-1 expression, but did not decrease neointimal hyperplasia even though the direct thrombin inhibitor hirudin did. This suggests that thrombin plays a role in neointima formation in response to vascular injury, but the effects of thrombin are not necessarily PAR-1 mediated in rabbits (Herbert *et al.* 1997). Also, in rabbit models of catheter balloon angioplasty of atherosclerotic arterial lesions, recombinant hirudin reduced both angiographic and histopathological restenosis more efficiently than heparin (Sarembock *et al.* 1991) and another thrombin inhibitor, hirulog-like peptide, reduced both neointimal hyperplasia and tissue factor expression of the neointima (Chen *et al.* 2003).

Important evidence that supports a role for thrombin in the development of atherosclerosis has come from experiments in which transgenic mice with mutations that promote thrombin generation were crossbred with atherosclerosis-prone apolipoprotein E-deficient mice. Mice with combined heterozygous TFPI deficiency and apolipoprotein E deficiency developed more aortic atherosclerosis than wild-type mice cross-bred with apolipoprotein E-deficient mice did (Westrick *et al.* 2001). Similarly, mice homozygous for factor V Leiden with APC resistance and deficient in apolipoprotein E developed more atherosclerosis than wild-type mice cross-bred with apolipoprotein E-deficient mice did (Eitzman *et al.* 2005). Mice heterozygous for factor V Leiden had an intermediate extent of atherosclerosis suggesting that even low-grade thrombin generation promotes atherosclerosis (Eitzman *et al.* 2005).

## 2.2. Histopathological evidence

The most common mechanism leading to coronary thrombosis in acute coronary syndromes is the rupture of the protective fibrous cap of an atherosclerotic plaque. Plaque rupture provokes thrombosis through thrombin generation initiated by the exposure of tissue factor, which is abundant in atherosclerotic plaques, and through platelet activation. (Libby and Theroux 2005) However, autopsy studies have shown that recurrent episodes of subclinical plaque disruption and minor thrombosis without arterial occlusion contribute also to the progression of stable coronary artery disease (CAD) (Burke *et al.* 2001, Mann and Davies 1999). In these studies multiple healed plaque rupture sites with layering causing stenosis have been identified in the majority of coronary lesions of subjects who died of sudden coronary death.

Other histopathological studies of human chronic atherosclerotic lesions also suggest that thrombin plays a role in the development of these lesions. While the thrombin receptor PAR-1 is expressed almost solely in the endothelial layer in normal human arteries it is expressed widely in the neointima of human atherosclerotic lesions (Nelken *et al.* 1992) and thrombin activity has been detected in the neointima of these lesions (Stoop *et al.* 2000).

## 2.3. Epidemiological evidence linking coagulation and coronary atherosclerosis

The strongest epidemiological evidence linking coagulation markers with the risk of CAD has been obtained from large-scale long-term prospective population-based studies and meta-analyses of published studies. Of various coagulation-related markers studied, fibrinogen shows the strongest association with CAD risk in prospective studies (Fibrinogen Studies Collaboration 2005). The association between CAD risk and fibrinogen is only slightly weaker than that between CAD and classical risk factors such as smoking, blood pressure, and cholesterol and fibrinogen may add to their predictive value (Lowe 2005). Level of D-dimer, a marker of fibrin turnover and a surrogate measure of thrombin activity (Lassila *et al.* 1993), also associates with the future risk of cardiovascular events and mortality from CAD in prospective studies and may add to the predictive value of conventional risk factors (Danesh *et al.* 2001, Smith *et al.* 2005). Other coagulation markers that have been shown to associate with increased risk of cardiovascular events in prospective studies include t-PA antigen (Lowe *et al.* 2004, Smith *et al.* 2005), PAI-1 (Smith *et al.* 2005), vWF (Rumley *et al.* 1999, Whincup *et al.* 2002), factor VIII (Rumley *et al.* 1999), and both low and high antithrombin levels (Meade *et al.* 1991).

F1+2, a commonly used marker of thrombin generation, has not been shown to be a risk marker for CAD in prospective population based studies of healthy subjects (Cooper *et al.* 2000, Smith *et al.* 2005). However, epidemiological evidence directly linking thrombin generation and CAD has come from studies evaluating the association between CAD and gene polymorphisms, which lead to increased thrombin generation. A recent meta-analysis of mostly cross-sectional studies showed that factor V polymorphism G1691A (factor V Leiden) (60 studies evaluated) and the G20210A polymorphism of the prothrombin gene (40 studies evaluated) associate moderately but significantly with the risk of CAD (Ye *et al.* 2006).

# 3. Thrombin and ischemia-reperfusion

## 3.1. Experimental evidence

Inhibition of thrombin has been shown to have beneficial effects in several experimental models of myocardial ischemia-reperfusion injury. Thrombin increased cell death of cultured cardiomyocytes subjected to simulated ischemia-reperfusion dose-dependently and this effect was reversed by the direct thrombin inhibitor lepirudin (Mirabet *et al.* 2005). In a rabbit model of myocardial ischemia-reperfusion, direct thrombin inhibition with hirudin reduced myocardial infarction size and polymorphonuclear leukocyte infiltration in the endothelium and subendothelium of reperfused myocardium (Erlich *et al.* 2000). In a porcine model of CPB, thrombin inhibition with hirudin, added to standard heparin anticoagulation, improved immediate hemodynamic recovery after ischemia-reperfusion and reduced cardiomyocyte apoptosis (Jormalainen *et al.* 2004, Jormalainen *et al.* 2007). Snow *et al.* (1991) showed that APC, the physiologic anticoagulant that is activated by thrombin, also attenuated myocardial ischemia-reperfusion in a porcine model. Infusion of a monoclonal antibody that prevents protein C activation led to slower and incomplete recovery of left ventricular function after left anterior descending coronary artery occlusion, while infusion of APC led to almost immediate recovery of left ventricular function.

Substances that inhibit thrombin or thrombin generation have attenuated ischemia-reperfusion injury also in other animal models of ischemia-reperfusion. In experimental models of intestinal ischemia-reperfusion, antithrombin reduced neutrophil rolling and adhesion during reperfusion (Ostrovsky *et al.* 1997) and antithrombin and APC reduced intestinal histological injury and dysfunction and inhibited systemic inflammation (Schoots *et al.* 2004). In rat models of liver ischemia-reperfusion, pretreatment with either a selective factor Xa inhibitor or APC reduced neutrophil sequestration (Yamaguchi *et al.* 2000) and treatment with TFPI reduced liver necrosis and tissue factor expression (Yoshimura *et al.* 1999). TFPI also protected from ischemia-reperfusion injury of the spinal cord (Koudsi *et al.* 1996).

APC has exerted protective effects also in models of ischemia-reperfusion of the brain (Cheng *et al.* 2003, Shibata *et al.* 2001), spinal cord (Hirose *et al.* 2000), and kidney (Mizutani *et al.* 2000). The protective effects of APC in ischemia-reperfusion injury seem to be distinct from its anticoagulant effects and are either anti-inflammatory (Hirose *et al.* 2000, Mizutani *et al.* 2000, Shibata *et al.* 2001) or directly anti-apoptotic as is the case after ischemia-reperfusion of the brain (Cheng *et al.* 2003).

## 3.2. Thrombin and outcomes of acute coronary syndromes and interventions for cardiac disease

A marker of thrombin generation (F1+2) has associated with clinical outcomes in acute coronary syndromes. In a substudy of the Global Use of Strategies To Open occluded coronary arteries (GUSTO)-1 trial comparing thrombolytic treatment strategies for ST-elevation myocardial infarct, levels of F1+2 at baseline and 12 hours after thrombolysis associated with the risk of death or reinfarction (Granger *et al.* 1998). Ardissino *et al.* (2003) found a U-shaped association between F1+2 and the risk of myocardial reinfarction or cardiac death as both low and high levels of F1+2 associated with higher risk in a substudy of a trial comparing recombinant hirudin to heparin in the treatment of acute coronary syndrome (GUSTO IIb). A similar U-shaped association between baseline F1+2 and the risk of death,

myocardial infarction, or refractory angina was observed in a substudy of the Thrombin Inhibition in Myocardial Ischemia (TRIM) study assessing the efficacy of heparin *vs*. inogatran, a direct thrombin inhibitor, in unstable CAD (Oldgren *et al.* 2001). Patients with increasing vs. decreasing thrombin markers [F1+2 and thrombin-antithrombin complexes (TAT)] despite anticoagulation also had a higher event rate (Oldgren *et al.* 2001).

Low levels of thrombin are thought to generate markedly increased levels of the endogenous circulating anticoagulant, APC, exerting, therefore, a net anticoagulant effect (Griffin 1995). This "thrombin paradox" has been characterized in a primate model in which systemic infusions of low doses of thrombin were antithrombotic and increased APC levels (Hanson et *al*. 1993). Even though it can be postulated that the findings of a U-shaped association between markers of thrombin generation and outcomes after acute coronary syndromes might be evidence supporting the "thrombin paradox", this hypothesis has not been tested clinically.

Thrombin markers have been shown to associate also with clinical outcomes after percutaneous coronary intervention and cardiac surgery. In a series of patients undergoing percutaneous transluminal coronary angioplasty and serial FPA measurements, patients with late angiographic restenosis had higher FPA levels measured soon after the procedure than those without restenosis (Salvioni *et al.* 1998). After cardiac surgery with CPB, levels of F1+2 correlated with measures of postoperative organ function including left ventricular stroke work index, $Pa0_2$/ fraction of inspired oxygen ratio, and serum creatinine (Dixon *et al.* 2005) and after off-pump CABG, F1+2 and D-dimer levels associated with early postoperative cognitive decline (Lo *et al.* 2005).

## 4. Coagulation and CPB

CPB disturbs hemostasis profoundly. The hemostatic changes induced by CPB are associated with the exposure of blood to the non-biological surfaces of the oxygenator, reservoir, and tubing of the extracorporeal circuit, with marked hemodilution caused by CPB, with surgical trauma, and with the high doses of unfractionated heparin that are administered (Bevan 1999). CPB-induced hemostatic changes include a significant decrease in platelet counts, a platelet function defect related to release of platelet alpha-granule contents and down-regulation of surface receptors for vWF and fibrinogen (Bevan 1999), increase in circulating platelet-derived procoagulant microparticles (Abrams *et al.* 1990), consumption and dilution of coagulation factors, and increased thrombin generation and fibrinolysis (Bevan 1999). The following review will focus on thrombin generation and fibrinolysis during CPB.

### 4.1. Thrombin generation during CPB

Thrombin generation and activity during CPB has been well characterized with measurements of markers of thrombin generation (F1+2), inhibition of free thrombin by antithrombin (TAT), and the fibrinogen-cleaving activity of thrombin (FPA) (Boisclair *et al.* 1993a, Boisclair *et al.* 1993b, Brister *et al.* 1993, Davies *et al.* 1980, Hunt *et al.* 1998, Knudsen *et al.* 1996, Slaughter *et al.* 1994, Tanaka *et al.* 1989). These studies have demonstrated that CPB causes a progressive increase in thrombin generation. However, reperfusion after cardiac ischemia has been shown to induce a distinct more rapid increase in thrombin generation during CPB (Knudsen *et al.* 1996, Petäjä *et al.* 1999). Indeed, in a computer model of the vascular system

that accounted for marker clearance, hemodilution, and blood loss, Chandler and Velan (2003) showed that instead of steady continuous increase in thrombin generation, CPB results in the generation of bursts of non-hemostatic thrombin and soluble fibrin especially soon after the initiation of CPB and during early reperfusion after cardiac ischemia. Studies which have demonstrated transcoronary gradients of FPA levels during early reperfusion (Petäjä *et al.* 1999) and of F1+2 levels after cardiac ischemia (Kalweit *et al.* 2005) suggest that the generation of thrombin across the coronary circulation during ischemia and reperfusion contributes to but does not fully explain the reperfusion-related increase of systemic levels of thrombin markers. Rewarming during CPB causes systemic vasodilation which occurs partly after the release of the aortic clamp. Speculatively, vasodilation might cause hypoperfused areas of the circulation to be reperfused and increase systemic thrombin generation. Also, a possible contribution of the pulmonary circulation to the reperfusion-associated generation of thrombin is unknown.

### 4.2. Fibrinolysis during CPB

Several studies have demonstrated activation of the fibrinolytic system during CPB with elevated levels of t-PA, fibrin degradation products, and plasmin-antiplasmin complexes (Chandler *et al.* 1995, Hunt *et al.* 1998, Stibbe *et al.* 1984, Tanaka *et al.* 1989, Teufelsbauer *et al.* 1992, Whitten *et al.* 1999). Mainly increased levels of t-PA rather than urokinase cause enhanced blood fibrinolytic activity during CPB (Stibbe *et al.* 1984). Elevated t-PA levels return to baseline soon postoperatively while PAI-1 levels which diminish during CPB increase significantly above baseline values on the first and second post-operative days suggesting a shift toward hypercoagulability in the fibrinolytic balance (Chandler *et al.* 1995, Hunt *et al.* 1998, Mannucci *et al.* 1995).

Follow-up data of patients who underwent CABG with CBP have shown that markers of coagulation (F1+2 and TAT) and fibrinolysis (D-dimer) are elevated up to 30 and 60 days after CABG, respectively (Mannucci *et al.* 1995, Parolari *et al.* 2003).

### 4.3. Mechanisms of activation of coagulation during CPB

Although tempting as an explanation for coagulation activation during CPB, the intrinsic or contact activation pathway of coagulation is no longer thought to contribute significantly to coagulation in vivo (Bevan 1999, Mann 2003a). Studies performed in patients during CPB have suggested that the tissue factor/ factor VIIa pathway, previously known as the extrinsic coagulation pathway, is the initiator of also CPB-related activation of coagulation rather than the contact activation pathway. Boisclair *et al.* (1993a) showed that there was no association between factor XIIa levels and thrombin generation measured with F1+2 during CPB and that factor IX activation did not occur before near maximal F1+2 increase. Factor X activation occurred ahead of factor IX activation also suggesting that contact activation could not be responsible for the observed thrombin generation (Philippou *et al.* 1995). Burman *et al.* (1994) observed significant thrombin generation demonstrated by F1+2 and TAT equalling that generally observed during CPB in a 12-year old girl with severe factor XII deficiency who underwent cardiac surgery. Therefore, a factor XII-dependent pathway (contact activation) could not have been directly responsible for the increased thrombin generation.

Soluble plasma tissue factor levels in the systemic circulation increase during cardiac surgery with CPB (Hattori *et al.* 2005, Philippou *et al.* 2000). Tissue factor requires monocytes,

platelets, or microparticles to provide a phospholipid surface for activating factor VII (Edmunds and Colman 2006). Monocytes, which can be stimulated to express tissue factor (Furie and Furie 2005), have been implicated in the initiation of thrombin generation during CPB. In a model of prolonged simulated extracorporeal circulation, circulating monocytes were induced to express tissue factor after two hours of extracorporeal circulation and tissue factor expression was highest in cells recovered from the surface of the circuit and the oxygenator (Kappelmayer *et al.* 1993). Also during clinical CPB, Chung and coworkers (Chung *et al.* 1996) have demonstrated increased tissue factor expression of circulating monocytes, but other investigators have shown either no increase in circulating monocyte tissue factor expression during CPB (Barstad *et al.* 1996, Parratt and Hunt 1998) or delayed tissue factor expression after CPB (Ernofsson *et al.* 1997). However, both circulating monocytes and more distinctly monocytes retrieved from the oxygenator of the extracorporeal circuit exhibited an increase in monocyte procoagulant activity (Barstad *et al.* 1996, Parratt and Hunt 1998). One possible explanation is the direct activation of factor X by circulating monocytes, which is mediated by the monocyte surface receptor CD 11b without evidence of tissue factor expression (Parratt and Hunt 1998). However, since cellular upregulation of tissue factor expression is slow compared to the rapid increase in thrombin generation observed during CPB (Philippou *et al.* 2000), other mechanisms must be involved.

Procoagulant microparticles are another possible platform for activation of the tissue factor/ factor VIIa pathway and therefore a potentially important source of thrombin generation during CPB. CPB increases the amount of circulating platelet-derived microparticles (Abrams *et al.* 1990), which stimulate thrombin formation *in vitro* (Nieuwland *et al.* 1997).

The surgical wound is an important source of tissue factor-mediated thrombin generation during CPB. Thrombin generation in pericardial blood during CABG is fulminant compared to systemic blood (Chung *et al.* 1996, Tabuchi *et al.* 1993, Weerwind *et al.* 2003) with over 30-fold concentrations of F1+2 and up to 50-fold concentrations of TAT in the pericardial blood in comparison to systemic blood reported (Sturk-Maquelin *et al.* 2003). Also fibrinolytic activity (levels of t-PA and fibrin and fibrinogen degradation products) is higher in blood oozing into the pericardial cavity during the operation than in systemic blood (Tabuchi *et al.* 1993). Furthermore, monocyte tissue factor expression is higher in pericardial blood than in systemic blood (Chung *et al.* 1996) and plasma levels of soluble tissue factor (Philippou *et al.* 2000, Sturk-Maquelin *et al.* 2003) and factor VIIa are significantly more pronounced in pericardial cavity blood than systemic blood (Chung *et al.* 1996, Philippou *et al.* 1999). During cardiac surgery pericardial blood also contains procoagulant platelet-derived microparticles at nearly ten times the concentration found in systemic blood and in contrast to systemic blood pericardial blood contains also microparticles derived from other cellular sources such as erythrocytes, monocytes, and granulocytes (Nieuwland *et al.* 1997). Even though microparticle-bound tissue factor obtained from pericardial blood during CPB stimulates thrombin formation (Sturk-Maquelin *et al.* 2003), recent studies have shown that microparticle-free soluble plasma tissue factor from pericardial blood is capable of activating factors VII and X in the presence of either wound monocytes or activated monocytes (Hattori *et al.* 2005). Furthermore, in this setting, monocytes are a more efficient platform for factor VII activation than microparticles or platelets (Hattori *et al.* 2005, Khan *et al.* 2006).

### 4.4. Effect of eliminating cardiotomy suction

Blood collecting into the surgical field (the mediastinum and pericardial and thoracic cavities) during cardiac surgery has routinely been suctioned during heparinization (cardiotomy suction) and returned into the extracorporeal circuit in most cardiac surgical centers. Since blood in the surgical wound has been shown to be procoagulant, studies have been performed to determine the effect of eliminating cardiotomy suction on activation of coagulation and blood loss. Tabuchi *et al.* (1993) retained suctioned mediastinal blood during CABG until the distal anastomoses were sutured and observed increases in systemic levels of TAT, fibrinogen degradation products, and fibrin degradation products after the suctioned blood was returned into the extracorporeal circuit. Thereafter, several small prospective randomized trials of CABG patients have addressed the same issue and all suggest that the elimination of cardiotomy suction may be beneficial in attenuating the activation of coagulation during CPB. De Haan and coworkers (de Haan *et al.* 1995) randomized patients into two groups: suctioned blood was either reinfused at the end of the operation or retained. A subgroup was analyzed for hemostatic parameters. Levels of TAT, t-PA, and fibrin degradation increased significantly more in the retransfusion group and the increased concentrations of these markers were higher than expected by the mere infusion of the suctioned blood. Also postoperative blood loss was higher in the retransfusion group. In a similar more recent study, discarding of suctioned blood prevented increased thrombin generation (F1+2 and TAT) during CPB and greatly attenuated thrombin generation after CPB (De Somer *et al.* 2002). Another randomized study showed that elimination of cardiotomy suction blunted the elevation of F1+2 levels after CPB (Aldea *et al.* 2002). When aspirated blood is processed with a cell saver, the aspirated plasma is discarded and the resulting packed red blood cells are reinfused (Albes *et al.* 2003). This approach also reduces CPB-induced thrombin generation (Albes *et al.* 2003).

### 4.5. Heparin-coated circuits for CPB

Extracorporeal circuits have been coated with heparin in an attempt to increase their biocompatibility. Several studies have shown that heparin-coated circuits reduce the inflammatory response associated with CPB as evidenced by reduced complement and granulocyte activation (Despotis *et al.* 1999). The effect of heparin coating of the extracorporeal circuit on activation of coagulation has been less consistent. Most studies have not demonstrated an effect on levels of markers of activation of coagulation (Boonstra *et al.* 1994, Gorman *et al.* 1996, Muehrcke *et al.* 1996, Ovrum *et al.* 1995, Wagner *et al.* 1994, te Velthuis *et al.* 1997) while others have shown reduced thrombin formation (Aldea *et al.* 2002, Gu *et al.* 1991). Maintaining reduced systemic levels of heparin when heparin-coated circuits are used has been advocated, because some investigators have observed reduced blood loss with this strategy while, again, others have shown no benefit (Despotis *et al.* 1999). When using closed heparin-coated circuits, Aldea and coworkers found similar levels of markers of thrombin generation in patients who were treated with full and reduced heparin doses (Aldea *et al.* 1998). However, reports of increases in markers of coagulation activation with this approach (Kuitunen *et al.* 1997, Ovrum *et al.* 1996) have led to recommendations that systemic heparin doses should not be reduced (Despotis *et al.* 1999, Edmunds 1994).

### 4.6. Antifibrinolytic agents and CPB

Hyperfibrinolysis has been implicated as an important contributor to the CPB-related coagulopathy that can result in increased perioperative hemorrhage. Gram and coworkers

(Gram *et al.* 1990) showed that increased fibrinolytic activity after heparin neutralization associated with increased post-CPB hemorrhage. Several studies have shown the efficacy of administering the antifibrinolytic agents aprotinin, tranexamic acid, and ε-aminocaproic acid in preventing blood loss during CPB (Fremes *et al.* 1994, Laupacis and Fergusson 1997). All of these agents inhibit fibrinolysis effectively, but only the serpin, aprotinin, neutralizes plasmin activity directly as evidenced by reduced levels of plasmin-antiplasmin complexes during CPB, while both tranexamic acid and ε-aminocaproic acid inhibit fibrinolysis by displacing plasminogen from the fibrin surface and increase the levels of plasmin-antiplasmin complexes (Eberle *et al.* 1998, Kuitunen *et al.* 2005). Interestingly, aprotinin also reduces thrombin generation during CPB (Dietrich *et al.* 1995, Feindt *et al.* 1994, Kuitunen *et al.* 2005, Marx *et al.* 1991) and inhibits thrombin-mediated platelet activation (Poullis *et al.* 2000).

Safety concerns were raised after reports of increased rates of myocardial infarction and saphenous vein graft thrombosis with aprotinin use (Alderman *et al.* 1998, Cosgrove *et al.* 1992). Yet, meta-analyses of randomized trials have not shown aprotinin treatment to associate with an increased risk of mortality, myocardial infarction (Levi *et al.* 1999, Sedrakyan *et al.* 2004), or renal failure (Sedrakyan *et al.* 2004). On the contrary these studies found aprotinin treatment to associate with a decreased risk of perioperative mortality (Levi *et al.* 1999) and stroke (Sedrakyan *et al.* 2004). However, recent non-randomized observational studies utilizing propensity score matching have found aprotinin treatment to be associated with renal dysfunction (Karkouti *et al.* 2006, Mangano *et al.* 2006) and late mortality (Mangano *et al.* 2007).

### 4.7. Off-pump coronary artery bypass surgery and activation of coagulation

Studies examining patients undergoing off-pump CABG surgery have demonstrated that, in contrast to on-pump CABG, markers of thrombin generation and fibrin degradation are not increased immediately postoperatively after off-pump CABG, but 24 hours after surgery they increase to levels comparable with levels observed during on-pump CABG (Casati *et al.* 2001, Lo *et al.* 2004, Mariani *et al.* 1999). Furthermore, the increases in F1+2 and D-dimer levels persist at least until the fourth postoperative day (Lo *et al.* 2004). There are also reports on increased vWf concentrations after off-pump CABG surgery (Casati *et al.* 2001, Lo *et al.* 2004). A hypercoagulable state is therefore present also after off-pump CABG surgery (Kurlansky 2003).

## 5. Natural anticoagulants and heparin anticoagulation during CPB

### 5.1. Natural anticoagulants

The role of the natural anticoagulants APC, TFPI, antithrombin, and HCII during CPB is incompletely understood. During CPB plasma APC levels increase rapidly during reperfusion after cardiac ischemia (Petäjä *et al.* 1999). Interestingly, patients with higher increases in coronary sinus blood APC levels during reperfusion tended to have a more favorable postoperative hemodynamic profile after CABG (Petäjä *et al.* 1999). Also, protein C activation during reperfusion after CABG had an inverse correlation with neutrophil sequestration in the human myocardium and with L-selectin expression of circulating neutrophils, implicating APC as a possible regulator of myocardial ischemia-reperfusion injury (Petäjä *et al.* 2001).

Heparin causes the release of TFPI from endothelial cells (Sandset *et al.* 1988). There is significant variability in the TFPI response to heparinization in patients undergoing CPB and evidently some patients do not respond to heparin with an increase in TFPI levels (Adams *et al.* 2002). On average, CPB with heparin anticoagulation causes a very significant increase in both total and free TFPI levels (Donahue *et al.* 2006, Fischer *et al.* 2004, Kojima *et al.* 2001) with over 10-fold increases in the levels of free TFPI reported (Donahue *et al.* 2006, Fischer *et al.* 2004). When heparin is neutralized with protamine after CPB TFPI levels decrease but remain higher than preoperatively (Donahue *et al.* 2006, Sun *et al.* 2000). Donahue and coworkers (Donahue *et al.* 2006) showed that TFPI undergoes proteolytic degradation during CPB and this degraded form of TFPI remains circulating in plasma after heparin neutralization, which may result in a decrease in endothelium-associated TFPI after CPB.

Effective heparin anticoagulation requires sufficient levels of plasma antithrombin. Antithrombin levels decrease during CPB as a result of hemodilution and consumption of antithrombin (Cullmann *et al.* 1980, Hashimoto *et al.* 1994, Ranucci *et al.* 2004), which might lead to ineffective anticoagulation. Furthermore, preoperative heparin treatment decreases antithrombin levels in cardiac surgical patients (Dietrich *et al.* 1991), but reduced systemic heparinization during CPB with the use of heparin-coated circuits associates with better preserved antithrombin levels during CPB (Ranucci *et al.* 2002).

HCII levels decrease immediately after the initiation of CPB as a result of hemodilution (Cardigan *et al.* 1996, Chan *et al.* 1997, Turner-Gomes *et al.* 1994). The role of HCII is thought to be less important clinically (Hirsh and Raschke 2004). However, experimental CPB has been performed in pigs with dermatan sulfate anticoagulation, which is based on the potentiating effect of dermatan sulfate on the inhibition of thrombin by HCII (Brister and Buchanan 1995).

## 5.2. Heparin anticoagulation

### 5.2.1. Mode of action of unfractionated heparin

Heparin is a heterogeneous mixture of branched glycosaminoglycans with molecular weights ranging from 3000 to 30 000. Heparin binds to antithrombin and the heparin/antithrombin complex inactivates the procoagulant serine proteases, factors IIa (thrombin), Xa, IXa, XIa, and XIIa. The anticoagulant effect of heparin is mainly achieved by antithrombin-dependent inactivation of thrombin and factor Xa. Heparin also activates HCII, which inhibits thrombin. The HCII-mediated anticoagulant effect of heparin requires higher heparin concentrations than the antithrombin-mediated one and might therefore play a role during CPB (Hirsh and Raschke 2004). Heparin also has a third anticoagulant effect, which is antithrombin- and HCII-independent. It is mediated by heparin binding to factor IXa, requires very high heparin concentrations, and results in inhibition of factor X activation. In all, unfractionated heparin has a relative anti-factor IIa (thrombin)/ anti-factor Xa activity ratio of 1:1. (Hirsh and Raschke 2004) Also, heparin-releasable TFPI is thought to contribute significantly to the anticoagulant effects of heparins (Sandset *et al.* 1988). The release of TFPI is more efficient after unfractionated heparin administration than after low molecular weight heparin administration (Brodin *et al.* 2004). In addition, heparin has been shown to enhance the anticoagulant activity of APC by enhancing the proteolytic inactivation of factor V by APC (Petäjä *et al.* 1997).

### 5.2.2. Monitoring of heparin anticoagulation for CPB

Anti-Xa measurements are considered the golden standard of laboratory-based monitoring of heparin anticoagulation (Hirsh and Raschke 2004) even though various studies have reported clinically significant variability in the results of different anti-Xa assays (Kitchen et al. 1999, Kitchen et al. 2000, Kovacs et al. 1999). As laboratory-based assays can not be used to guide heparin dosage during CPB, activated clotting time (ACT) has become a widely accepted point-of-care test for monitoring heparin during CPB (Despotis et al. 1999). However, the effect of ACT-based protocols guiding heparin and protamine dosage vs. fixed dosage protocols on transfusion requirements and post-operative blood loss in various studies has been inconsistent (Despotis et al. 1999). There are inherent problems related to ACT-based monitoring of heparin during CPB. ACT may be prolonged by factors other than heparin, such as hemodilution, hypothermia, quantitative and qualitative platelet abnormalities, and drugs such as aprotinin, which prolongs celite-based ACT but not kaolin-activated ACT (Despotis et al. 1999). Indeed, ACT levels reflect plasma heparin levels poorly (Culliford et al. 1981, Despotis et al. 1994). Whole blood heparin concentration measurements during CPB with an on-site automated protamine sulfate titration assay have been reported to correlate well with laboratory-based anti-Xa measurements in one study (Despotis et al. 1994). However, others have reported poor agreement between these measurements (Gruenwald et al. 2000, Hardy et al. 1996).

### 5.2.3. Heparin dosage during CPB

Early heparin dosage protocols were empiric, but Bull and coworkers (Bull et al. 1975b) showed that they resulted in significant variability in dose response and duration of heparin effects. Bull et al. (1975a) introduced ACT-guided heparin dosage and suggested that ACT levels should be maintained above 300 seconds. Determining the minimum safe ACT for CPB has proved difficult. Young et al. (1978) suggested 400 seconds as the minimum safe ACT based on the appearance of fibrin monomer in plasma of monkeys with ACT values below 400 seconds during CPB. In subsequent studies evaluating coagulation during CPB ACT has usually been maintained at levels greater than either 400 or 480 seconds (Boisclair et al. 1993b, Brister et al. 1993, Chandler and Velan 2003, Davies et al. 1980, Hunt et al. 1998, Knudsen et al. 1996, Petäjä et al. 1999, Slaughter et al. 1994). There is both indirect and direct evidence suggesting that maintaining even higher heparin concentrations during CPB may be beneficial. Plasma FPA correlated inversely with heparin levels during CPB (Gravlee et al. 1990, Hashimoto et al. 1994). High-dose heparin anticoagulation for patients undergoing aortic surgery under deep hypothermic circulatory arrest resulted in reduced levels of TAT and D-dimer (Okita et al. 1997). Also, in randomized settings higher heparin doses during CPB associated with reduced F1+2 and D-dimer levels (Koster et al. 2002), reduced FPA and D-dimer levels and reduced consumption of coagulation factors (Despotis et al. 1996), and fewer transfusions (Despotis et al. 1995).

# AIMS OF THE PRESENT STUDY

In order to further elucidate the significance of thrombin generation and its functions in patients undergoing CABG the aims of this study were

1. to test whether post-ischemic activation of coagulation during reperfusion after CABG associates with postoperative myocardial damage or hemodynamic compromise.

2. to measure protein C activation during CABG in relation to thrombin generation and to the procoagulant activity of thrombin and to investigate the associations of protein C activation with postoperative hemodynamic recovery and myocardial damage.

3. to investigate the effect of preoperative thrombophilic variables on perioperative thrombin generation, the procoagulant activity of thrombin, and fibrin turnover.

4. to compare the performance of a novel coagulation test, PiCT, and two chromogenic anti-Xa assays in measuring heparin effects in the setting of high-dose heparinization during CPB.

# MATERIAL AND METHODS

## 1. Study setting and patient population (I-IV)

This study was a prospective, single center study performed in the Department of Cardiothoracic Surgery, Helsinki University Central Hospital, Helsinki, Finland between October 2002 and June 2004. Institutional ethical committee approval was obtained. Anesthesia, CPB, transfusions, fluid therapy, and postoperative medication followed a strict clinical protocol.

The study population consisted of 100 consecutive patients who were scheduled for primary, elective on-pump CABG. Written informed consent was obtained. Apart from patients with exclusion criteria patients were excluded only if it was necessary for logistical reasons related to patient recruitment and blood sampling (*i.e.* weekends, holidays *etc*.). Exclusion criteria were as follows: concomitant valve or other cardiac surgery; use of warfarin, unfractionated or low-molecular weight heparin, or aspirin less than five days prior to surgery; renal failure (serum creatinine >120 µmol/ L before the preoperative angiogram); abnormal preoperative international normalized ratio; anemia (preoperative hemoglobin <130 g/ L for males and <120 g/ L for females); and thrombocytopenia (preoperative platelet count <150 x $10^9$/ L). All patients were interviewed preoperatively and medication, history of smoking, family history of venous and arterial thrombosis, history of diabetes mellitus, stroke, transient ischemic attack, myocardial infarction, percutaneous coronary intervention, peripheral arterial disease, deep venous thrombosis, pulmonary embolism, other major concomitant diseases, New York Heart Association functional class, and left ventricular ejection fraction were recorded. Operative risk was evaluated according to the European System for Cardiac Operative Risk Evaluation (EuroSCORE) (Nilsson *et al.* 2004), which takes into account several patient-related, cardiac, and operation-related preoperative risk factors including age, sex, the presence of pulmonary, neurologic, and renal comorbidity, the presence of extracardiac arterial disease, previous cardiac surgery, active endocarditis, unstable angina, left ventricle dysfunction, recent myocardial infarct, pulmonary hypertension, urgency, and the type of surgery performed. The demographic and clinical data are summarized in **I**, Table 1.

## 2. Clinical study protocol (I-IV)

### 2.1. Anesthesia

The patients were premedicated with oral lorazepam (0.04-0.06 mg/kg) two hours prior to the induction of anesthesia. The patients´ regular oral cardiovascular medications were given at the same time as the premedication. Anesthesia was induced with sufentanil (1-2 µg/kg), propofol (1-2 mg/kg), and pancuronium (0.1 mg/kg) and maintained with sufentanil (0.5-1.0 µg/kg/h) and isoflurane (inspiratory concentration of at least 0.2 volume %). After CPB isoflurane was replaced with a propofol infusion (4-10 mg/kg/h). Propofol (1-4 mg/kg/h) was used for postoperative sedation in the cardiac surgical intensive care unit (ICU). Sedation was continued until the patient´s core temperature had reached 36 º C. Sufentanil (0.1 µg/kg/h) or oxycodone (0.05 mg/kg intravenously or 0.1 mg/kg intramuscularly) was used for postoperative analgesia in the ICU.

### 2.2. Anticoagulation protocol

The patients received an initial intravenous dose of 400 IU/kg of porcine mucosal heparin (Heparin Leo®, LEO Pharma, Ballerup, Denmark) and 5000 IU of heparin was added to the priming solution of the CPB circuit. Heparinization was monitored with kaolin-ACT measurements (Automated Coagulation Timer II, Medtronic, Minneapolis, MN, USA) every 20 minutes during CPB and additional heparin doses of 5000 IU were administered, as needed, to maintain an ACT above 600 s. After CPB heparinization was neutralized with 1 mg of protamine sulfate per 100 IU of the initial dose of heparin. In case of increased postoperative blood loss (continuous chest tube output > 3 mL/ kg/ 30 minutes) in the ICU, ACT was measured and if it was prolonged more than 50 seconds compared with the preoperative value an additional 25 mg dose of protamine sulfate was administered. When no increased postoperative blood loss was present, low molecular weight heparin treatment with dalteparin was started six hours postoperatively and continued twice daily thereafter (2500 IU subcutaneously twice daily for patients who weighed under 60 kg and 5000 IU subcutaneously twice daily for patients who weighed over 60 kg).

### 2.3. CPB

CPB was instituted with a non-coated circuit, a membrane oxygenator (Trillium®Affinity®, Medtronic), and a roller pump (Stöckert SIII, Stöckert Instrumente GmbH, Munich, Germany), and non-pulsatile pump flow of 2.4 L/min/$m^2$ was maintained. The CPB circuit was primed with 2000 mL of Ringer´s acetate and 100 mL of mannitol. Antifibrinolytic agents were not administered. Hematocrit was maintained above 0.20 during CPB. Blood suctioned from the operative field was returned to the systemic circulation through a filtered cardiotomy reservoir throughout the operation until protamine administration. The patients were allowed to cool passively and were rewarmed to a core temperature of 36 °C before weaning from CPB. After CPB the content of the extracorporeal circuit was collected into non-anticoagulated blood bags and returned to the patient.

### 2.4. Surgery

After median sternotomy the left internal mammary artery (LIMA), right internal mammary artery (RIMA), saphenous vein, or radial artery grafts were harvested. The ascending aorta and the right atrial appendage were cannulated and CPB was initiated. Intermittent antegrade cold (+10-12ºC) blood cardioplegia was used for myocardial protection. The aorta was cross-clamped during suturing of all anastomoses. LIMA was anastomosed to the left anterior descending coronary artery in all cases except one. Four patients received also a RIMA graft. Additional aortocoronary anastomoses were performed using saphenous vein or radial artery grafts. Intraoperative volume flow measurements of all grafts were performed with a transit time flow meter (Medi-Stim Butterfly Flowmeter®, Medi-Stim AS, Oslo, Norway) to ascertain immediate graft patency. The operative data are summarized in **I**, Table 2.

### 2.5. Blood component transfusion triggers

The cut-off hemoglobin value for postoperative packed red blood cell transfusions was 80 g/L throughout the study. In case of increased postoperative bleeding (continuous chest tube output > 3 mL/kg/30 minutes) platelet count, APTT, and INR were measured. If bleeding continued and APTT was prolonged more than 1.5 -fold compared to the preoperative value,

or INR was >1.8, fresh frozen plasma was transfused (15 mL/kg) and platelet counts below 100 x $10^9$/L indicated a transfusion of one unit of platelets /10 kg of body weight.

### 2.6. Hemodynamic management and fluid therapy

When the postoperative cardiac index was under 2.0 L/min/$m^2$, pulmonary capillary wedge pressure was first adjusted to 12-15 mmHg by optimizing preload with an infusion of Ringer´s acetate, 6% hydroxyethyl starch, or 4% human albumin. If the cardiac index remained below 2.0 L/min/$m^2$, epinephrine (0.02-0.2 µg/kg/minute) was infused. If the cardiac index still remained below 2.0 L/min/$m^2$, a milrinone infusion (0.5 µg/kg/minute) was added. When the mean arterial pressure was under 70 mmHg, preload was first optimized (see above), and when necessary, norepinephrine (0.01-0.1 µg/kg/minute) was infused.

### 2.7. Postoperative medication

100 mg of aspirin was administered orally on the morning of the first postoperative day and once daily thereafter. Non-steroidal anti-inflammatory drugs, dypyridamole, clopidogrel, warfarin, aprotinin, or tranexamic acid were not allowed during the study.

## 3. Outcome measures

### 3.1. Clinical outcome measures

Reoperations, neurological complications, acute renal failure, respiratory complications, and infections were registered prospectively. Two observers, who were blinded to the Ck-Mbm and TnT values of the patients, analyzed the preoperative and postoperative electrocardiograms of all patients. New pathological Q waves were identified according to the criteria of a recent consensus document (Anonymous 2000). Blood component transfusions during the perioperative period and chest tube drainage up to 16 h postoperatively were recorded.

### 3.2. Hemodynamic measurements (I-II)

A radial artery cannula and a pulmonary artery catheter were inserted and heart rate, arterial pressure, central venous pressure, pulmonary artery pressure, and pulmonary capillary wedge pressure were measured. Cardiac output measurements were performed with thermodilution. Mean arterial pressure, mean pulmonary artery pressure, stroke volume, stroke volume index, cardiac index, systemic vascular resistance index, and pulmonary vascular resistance index were calculated with standard formulae at five time points: preoperatively (coinciding with time point A for blood sampling, see below and Figure 3), after termination of CPB (time point E), on arrival in the ICU, six hours after protamine (time point F), and on the first postoperative day (time point G).

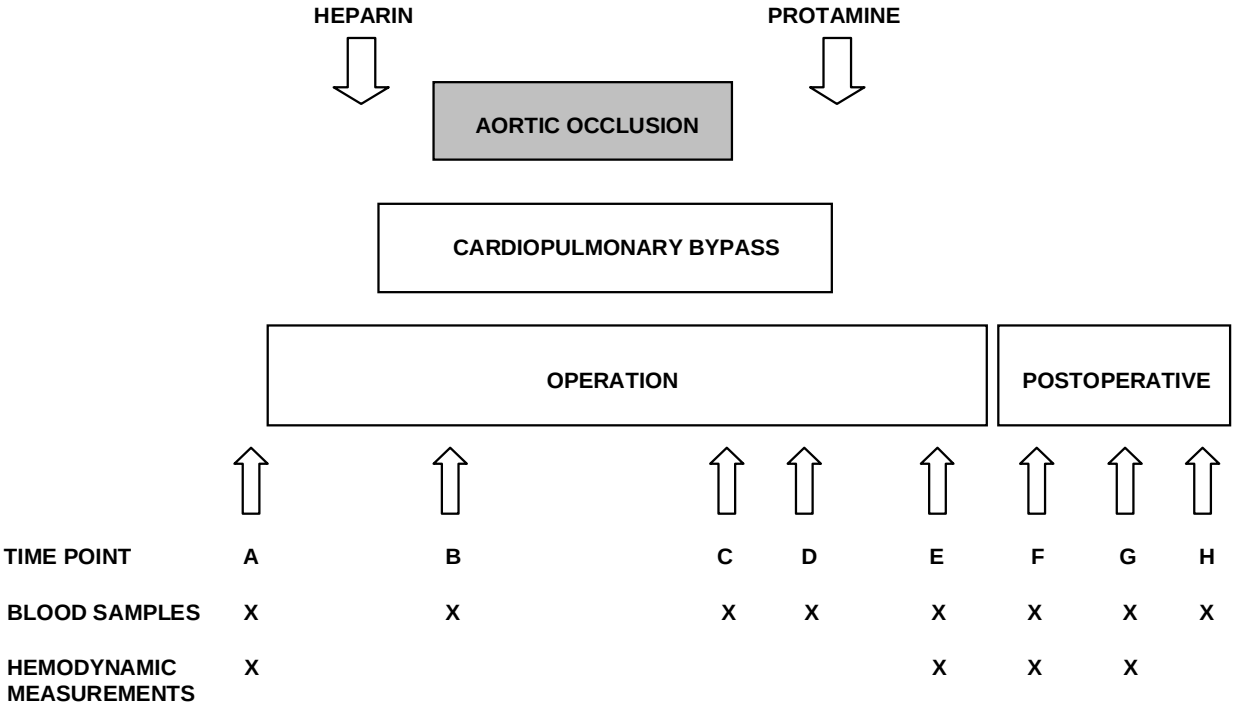


**Figure 3.** Blood sampling protocol. The time points measured were preoperatively (A), 15 minutes of CPB (B), immediately before the release of the aortic clamp (C), 15 minutes after the release of the aortic clamp (D), 30 minutes (E) and 6 hours after protamine administration (F), and on the first (G) and fifth (H) postoperative days. Blood sampling and hemodynamic measurements were performed as shown. The time points are not in scale.

## 4. Blood sampling and laboratory analyses

### 4.1. Preanalytical testing

The handling of blood samples was performed bedside next to the operating theater to avoid delays in the processing of the samples. To ascertain the effect of various blood sampling techniques and to test the stability of the samples, a pilot study of altogether 10 patients was performed. There were no significant differences between F1+2 values in samples collected through either radial arterial lines of two different lengths or directly from the CPB circuit (Table 2). The samples also remained stable for up to two hours on ice (Table 2). Based on the pilot study samples A through G (see "Blood sampling" below) were collected through a radial artery line and sample H was collected either through an atraumatic venipuncture or through a radial artery line. We also performed testing of the plasma samples (n=12) to rule out significant platelet contamination as the centrifugation speed of our local centrifuge was somewhat low (1500 g). Under these conditions platelet counts in plasma remained on average below 10 x $10^9$/ l, which is acceptable but might not rule out minor platelet contamination.

| | **Preoperative** | | | **40 min of CPB** |
|---|---|---|---|---|
| | On ice 20 min | On ice 2 h | RT 2 h | On ice 20 min |
| Arterial line (30 cm) | 0.90 (0.55) | 0.97 (0.53) | 1.15 (0.57) | - |
| Arterial line (180 cm) | 0.93 (0.49) | - | - | 2.47 (1.40) |
| Extracorporeal circuit | - | - | - | 2.43 (1.36) |

**Table 2.** The effect of the blood sampling technique on F1+2 measurements [nmol/ L, mean (SD), n=5]. Samples were taken from arterial lines of two different lengths or directly from the extracorporeal circuit and were incubated on ice or in room temperature (RT) for up to 2 h.

### 4.2. Blood sampling

Blood samples were collected at eight time points (A-H): preoperatively (A), at 15 minutes of CPB (B), immediately before the release of the aortic clamp (C), 15 minutes after the release of the aortic clamp (D), 30 minutes (E) and six hours (F) after protamine administration, and on the first (G) and fifth (H) postoperative days (Figure 3). Samples A through G were collected through a radial artery catheter and sample H was collected either through an atraumatic venipuncture or through a radial artery catheter. The radial artery line was flushed with only physiological saline without heparin. The first 5 or 10 mL of each sample were discarded depending on the length of the arterial line. For measurements of F1+2, SFC, D-dimer, anti-Xa, and PiCT, nine volumes of blood were collected into vacuum test tubes with one volume of 3.8% sodiumcitrate (Venoject®, Terumo Europe N.V., Leuven, Belgium) (**I-IV**). For measurement of APC, a citrate benzamidine anticoagulant mixture was used according to Gruber and Griffin (1992) (**II**). The samples were cooled on ice and centrifuged (1500 g/ 10 minutes) at +4°C. Plasma was separated and stored at -80°C. For other coagulation measurements, nine volumes of blood were collected into vacuum test tubes with one volume of either 3.8% or 3.2% sodiumcitrate and for measurement of homocysteine three volumes of blood were collected on five volumes of K3-EDTA on ice (**III**). These samples were centrifuged (2000 g/ 10 minutes) at +20°C before separation of plasma (**III**).

### 4.3. Laboratory analyses

F1+2 was analyzed with an enzyme-linked immunoassay (Enzygnost F1+2micro®, Dade Behring, Marburg, Germany) (**I-IV**). SFC and D-dimer were analyzed with immunoturbidimetric assays (STA-Liatest®FM, Diagnostica Stago, Asnieres, France and Tina-quant D-Dimer®, Roche Diagnostics, Mannheim, Germany) (**I-IV**). APC and protein C were analyzed with an enzyme capture assay, as previously described (Gruber and Griffin 1992) (**II**). The levels of the cardiac biomarkers, Ck-Mbm and TnT, were determined with electrochemiluminescence immunoassays (Elecsys Ck-MbSTAT® and Elecsys TroponinTSTAT®, Roche Diagnostics GmbH, Mannheim, Germany) (**I-II, IV**).

The preoperative thrombophilia screen included the following analyses (**III**): Antithrombin and protein C activities were determined with chromogenic assays [Berichrom®Antithrombin III (A), Berichrom®Protein C, Dade Behring, Marburg, Germany] and expressed as percentage after calibration against reference plasma (HemosIL Calibration Plasma, Instrumentation

Laboratory, Lexington, MA, USA and Standard Human Plasma, Dade Behring, respectively) (**III-IV**). Free protein S was determined with an automated latex ligand immunoassay (Instrumentation Laboratory, Lexington, MA, USA). Factor VIII was analyzed with a coagulometric method (Dade Behring, Marburg, Germany). APC resistance was determined with a functional activated partial thromboplastin time (APTT)-based test with predilution of patient plasma with factor V-depleted plasma (Coatest®APC Resistance V, Chromogenix, Lexington, MA, USA). Factor V Leiden (FVR506Q) and the prothrombin G20210A gene mutations were identified with cyclic minisequencing. Cardiolipin IgG antibodies were determined with an enzyme immunoassay (Pharmacia Deutschland, Freiburg, Germany). Lupus anticoagulant was screened with a simplified dilute Russell´s Viper Venom Time test and confirmed with a high phospholipid-containing coagulation assay (DVVtest®, DVVconfirm®, American Diagnostica, Stamford, CT, USA). Homocysteine was determined with a fluorescence polarization immunoassay (AxSYM®Homocysteine, Axis-Shield, Dundee, UK).

Heparin levels were measured with PiCT (Pefakit®PiCT®, Pentapharm, Basel, Switzerland) (Calatzis *et al*. 2000) (detailed description in **IV**) and with two chromogenic anti-Xa assays [Berichrom®Heparin, Dade Behring, Marburg, Germany (anti-Xa A) (**I, IV**) and STA®-Rotachrom®Heparin, Diagnostica Stago, Asnieres, France (anti-Xa B) (**IV**)] according to the manufacturers´ instructions (detailed description in **IV**). For anti-Xa A dextran sulfate (0.02 g/L) was added to the sample to dissociate heparin from plasma proteins and antithrombin (1 IU/mL) was added in excess. For anti-Xa B neither dextran sulfate nor antithrombin were added.

F1+2, D-dimer, and APC were measured from all patients at all time points (see blood sampling above). SFC were measured from all patients at time point A and from patients 1-80 at time points B-F. Ck-Mbm and TnT were measured from all patients at time point G (18 hours postoperatively). The measurements included in the preoperative thrombophilia screen were measured from all patients at time point A. Additionally, antithrombin was measured at time point D from patients 1-80. PiCT was measured from all patients at time points A, B, D, and E. Anti-Xa assay A was measured from patients 1-80 at time points A-F and anti-Xa assay B was measured from all patients at time points A-F.

## 5. Statistical analysis

Based on a power calculation (**III**) it was estimated that a population of 100 patients was needed to yield the minimum number of patients with thrombophilia needed to demonstrate a 30 % increase in maximal F1+2 levels during CPB at 80 % power and p=0.05.

The data were analyzed with the SPSS for Windows 11.5.1 software (SPSS Inc., Chicago, Illinois, USA) and the NCSS 2000 software (NCSS, Kaysville, Utah, USA). Analysis of normality of the distribution of continuous variables was performed with the Kolmogorov-Smirnov test (**II-IV**). For clarity data in figures are expressed as mean and standard deviation or standard error of the mean (**I-IV**). Other data are presented as mean and standard deviation or standard error of the mean or as median and interquartile range (IQR), as appropriate (**III**). For univariate analysis of the association between variables Pearson´s (**I**) or Pearson´s and Spearman´s rank correlation coefficients were calculated, as appropriate (**II-IV**). Differences between repeated measurements were analyzed with repeated measures analysis of variance (ANOVA) and post hoc comparisons were made with Fisher's LSD Multiple-Comparison Test

(**I-IV**). Variables with skewed distributions were natural logarithmically transformed before these analyses (**II-IV**). Student´s t-test or Mann-Whitney U test was used for comparisons between two groups, as appropriate (**III-IV**), and associations between two dichotomous variables were tested with Chi-squared test (**III)** or Fisher´s exact test because of small expected cell counts (**IV**). Bland-Altman plots were used to examine the agreement between PiCT and anti-Xa assays (**IV**). Multivariable logistic regression analysis with block entry of variables was used to identify variables associated with postoperative myocardial damage and thrombin generation (**I**) and multivariable logistic regression analysis with a forward stepwise method was used to identify variables associated with protein C activation (**II**). Two-tailed p-values <0.05 were considered significant.

# RESULTS

### 1. Kinetics of thrombin generation, fibrin formation and turnover, and protein C activation during CPB

A progressive increase in markers of thrombin generation (F1+2), fibrinolysis (D-dimer), and fibrin formation (SFC) was observed during CPB (**I**, Figure 1 and **II,** Figure 1, panels A and B). This increase was further distinctly propagated by reperfusion after myocardial ischemia and continued to peak after the neutralization of heparin with protamine. APC levels increased only slightly during CPB before the release of the aortic clamp, but reperfusion and more significantly heparin neutralization caused a massive increase in APC levels, which peaked after heparin neutralization (**II**, Figure 1, panel A). Importantly, protein C activation was clearly delayed in relation to both thrombin generation and fibrin formation (**II**, Figure 1, panels A and B). Throughout the study thrombin generation dominated over protein C activation (**II**, Figure 1, panel C).

### 2. Thrombin generation and APC *vs.* myocardial damage

To test the hypothesis that thrombin generation during reperfusion associates with postoperative myocardial damage, correlations between levels of F1+2 and postoperative cardiac biomarkers were calculated. F1+2 levels during reperfusion at 30 minutes and 6 hours after protamine administration correlated with levels of Ck-Mbm ($r=0.30$, $p=0.003$, and $r=0.40$, $p<0.001$, respectively) and TnT ($r=0.24$, $p=0.014$, and $r=0.44$, $p<0.001$, respectively) measured on the first postoperative day (**I**). Patients with evidence of myocardial damage on the first postoperative day (highest deciles or quintiles of Ck-Mbm and TnT) had higher levels of F1+2 immediately before reperfusion (time point C) and during early (time point D) and later reperfusion (time points E-F) (**I**, Figure 2). Also patients with new pathological Q-waves had higher F1+2 than others during reperfusion (time points E-F) (**I**). Multivariable logistic regression analysis identified F1+2 during reperfusion to independently associate with postoperative myocardial damage (odds ratios for F1+2 were 2.5-4.4, 95% confidence intervals 1.04-15.7 when the highest deciles of Ck-Mbm and TnT were the dependent variable) (**I**). APC levels did not associate with markers of postoperative myocardial damage (**II**).

### 3. Association of thrombin generation and APC with hemodynamic performance

Because thrombin generation was shown to associate with postoperative myocardial damage, we separately analyzed the association of thrombin generation and APC with postoperative hemodynamic performance. Thrombin generation associated with unfavorable postoperative hemodynamics, as patients with F1+2 levels in the highest decile during reperfusion (time point E) had a higher postoperative pulmonary vascular resistance index than others (**I**, Figure 3). APC levels associated dynamically with postoperative hemodynamic performance. High preoperative APC associated with an unfavorable postoperative hemodynamic profile. Patients with preoperative APC/protein C ratio in the highest decile had a lower postoperative cardiac index than others (**II**, Figure 2). At the end of myocardial ischemia (time point C), and during early reperfusion (time point D), protein C activation associated with an opposite, favorable hemodynamic response. Patients with APC in the highest quintile before the release of the aortic clamp (time point C) had higher a cardiac index postoperatively than others (**II**, Figure

3) and those with APC/protein C in the highest quintile during early reperfusion (time point D) had a lower systemic vascular resistance index postoperatively (**II**). After heparin neutralization with protamine (time point E) APC associated again with an inferior hemodynamic profile. Those with APC level in the highest decile after heparin neutralization had a lower stroke volume index postoperatively (**II**, Figure 4) and those with APC/protein C in the highest decile or quintile after heparin neutralization had a higher postoperative pulmonary vascular resistance index than others (**II**).

### 4. Impact of thrombophilic variables on the generation and procoagulant activity of thrombin during CPB (III)

Since several thrombophilic factors have been shown to associate with basal thrombin generation, we hypothesized that patients with thrombophilic variables (see **III** for definition) would have enhanced CPB-related activation of coagulation. Even though patients with thrombophilic variables had slightly higher preoperative F1+2 than others [median (interquartile range) 0.55 (0.34) vs. 0.45 (0.21) nmol/L, p=0.009] they did not have higher F1+2, D-dimer, or SFC levels during CPB or postoperatively than patients without thrombophilic variables (**III**, Figure 1, panels A-C). Also, the patients with more than one thrombophilic factor did not have higher F1+2, D-dimer, or SFC levels than others did (**III**, Figure 2, panel A). Similar analyses were made separately for each thrombophilic variable tested. In these analyses none of the thrombophilic variables associated with higher perioperative levels of F1+2, D-dimer, or SFC (**III**, Figure 2, panels B and C).

### 5. Heparin levels during CPB

Mean ACT during CPB was constantly well above 600 s with the high total heparin doses administered (mean 575 IU/kg, SD 83 IU/kg) (**IV**, Figure 1). Heparin activity measured with PiCT [mean (SD)] ranged from 5.0 to 5.2 IU/mL (0.7-0.9 IU/mL) during CPB (**IV**, Figure 1, panel I). Anti-Xa heparin levels were slightly higher when measured with antithrombin supplementation to the sample (anti-Xa A) than under patient-specific plasma antithrombin levels (anti-Xa B) [4.7-5.0 (0.9-1.6) vs. 4.5-4.9 (0.9) IU/mL] (**IV**, Figure 1, panel II). Heparin levels during CPB were therefore high in the present study. In study **I** we reported mean anti-Xa levels during CPB to range from 9.0 to 9.5 IU/mL, which are 4.3-4.5 IU/mL higher levels than reported in study **IV**. This discrepancy is explained by the previous erroneous use of a reference curve with enoxaparin instead of unfractionated heparin.

### 6. Agreement between PiCT and anti-Xa

Because PiCT is a novel coagulation test that is sensitive for heparin and has potential advantages in monitoring heparin levels during CPB, its performance in this setting was compared with chromogenic anti-Xa assays, the present standard of monitoring heparin treatment. There was poor agreement between PiCT and anti-Xa and between the two anti-Xa assays. Heparin levels measured with PiCT levels correlated only modestly with the corresponding levels measured with anti-Xa (correlation coefficients ranging from r=0.32 to r=0.65) (**IV**, Table 1). Also, the limits of agreement in Bland-Altman plots (mean difference +- 2 SD) (Bland and Altman 1986) were unacceptably broad. The limits of agreement between PiCT and anti-Xa were between –1.0 to 2.2 IU/mL and –2.4 to 2.4 IU/mL and the limits of

agreement between the two anti-Xa assays were between –1.8 to 1.8 IU/mL and –2.5 to 3.1 IU/mL (**IV**, Figure 2).

### 7. Association of antithrombin and protein C with heparin levels

Preoperative antithrombin level correlated weakly with PiCT during CPB but not with anti-Xa (Table 2.). Antithrombin measured close to the end of CPB (time point D) correlated with simultaneously measured PiCT and anti-Xa. Preoperative protein C levels correlated with PiCT measured during CPB and weakly with anti-Xa levels measured at 15 min of CPB (time point B). (**IV**, Table 2).

Patients with low preoperative antithrombin [either in the lowest decile (≤66%) or subnormal (<84%)] had lower anti-Xa A and PiCT levels, respectively during CPB than others ($p<0.001$ and $p=0.028$, respectively) (**IV**, Figure 3, panels I and II). Also, patients with low preoperative protein C activity [in the lowest decile (≤76%)] had lower PiCT levels and anti-Xa B levels (**IV**, Figure 4, panels I and II) but not anti-Xa A levels than others.

### 8. Impact of heparinization on activation of coagulation and myocardial damage

There was an inverse correlation between the cumulative heparin dose (heparin dose/ minutes of CPB) and the peak value of F1+2 measured 30 minutes after protamine administration ($r=-0.39$, $p<0.001$) (**I**). Weaker inverse correlations were observed between the cumulative heparin dose and F1+2 measured immediately before the release of the aortic clamp and at two other time points during reperfusion. However, in multivariable logistic regression analysis, the cumulative heparin dose did not associate with thrombin generation (**I**). Anti-Xa A levels during CPB (time points C and D) correlated inversely with subsequent F1+2 levels after CPB ($r=-0.34$, $p=0.002$ and $r=-0.30$, $p=0.007$, respectively) (**IV**). Also, patients with low anti-Xa A or PiCT (in the lowest decile) had higher subsequent F1+2 after CPB than others (**IV**). Heparin activities (anti-Xa A, anti-Xa B, or PiCT) did not associate with levels of D-dimer or SFC (**IV**).

Interestingly, there was an inverse correlation between the cumulative heparin dose and postoperative Ck-Mbm ($r=-0.37$, $p<0.001$) and TnT ($r=-0.31$, $p=0.002$) (**I**). However, PiCT or anti-Xa heparin levels did not associate with Ck-Mbm or TnT (**IV**).

### 9. Association of anti-Xa and PiCT with transfusion requirements (IV)

Patients with high heparin levels during CPB (highest deciles of anti-Xa A, anti-Xa B, and PiCT) required fewer transfusions of packed red blood cells than others [mean (SD) 0.9 (1.0) vs. 2.5 (2.4) units, $p=0.014$, 1.0 (1.2) vs. 2.5 (2.3) units, $p=0.034$; and 0.6 (0.9) vs. 2.6 (2.3) units, $p=0.014$; respectively]. Conversely, patients with low PiCT during CPB (in the lowest decile) received transfusions of fresh frozen plasma more often than others (30% vs. 3%, $p=0.013$) and patients with either low PiCT or low anti-Xa B during CPB (in the lowest decile) received transfusions of platelets more often than others (both 40% vs. 7%, $p=0.008$).

# DISCUSSION

## 1. Thrombin and myocardial damage after CABG

The present study (**I**) showed that thrombin generation during reperfusion after CABG associated with postoperative myocardial damage and postoperative pulmonary vascular resistance. This finding is novel. Even though we demonstrated an association and not a causal relationship between thrombin and myocardial damage, previous experimental studies have established that thrombin is a possible mediator of myocardial ischemia-reperfusion injury. Thrombin has been shown to increase cell death of cultured cardiomyocytes subjected to simulated ischemia-reperfusion dose-dependently (Mirabet *et al.* 2005) and other experimental studies have shown that either direct or indirect inhibition of thrombin in myocardial ischemia-reperfusion injury is beneficial (Erlich *et al.* 2000, Jormalainen *et al.* 2004, Jormalainen *et al.* 2007, Snow *et al.* 1991). Previous clinical studies also support the notion that the observed thrombin burst may be harmful after CABG. Dixon and coworkers (Dixon *et al.* 2005) demonstrated that a marker of thrombin generation (F1+2) correlated with measurements of organ dysfunction after CABG, including left ventricular stroke work index, $Pa0_2$/ fraction of inspired oxygen ratio, and serum creatinine. Interestingly, other investigators showed that levels of F1+2 and a marker of fibrin turn over (D-dimer) after off-pump CABG also associated with early postoperative cognitive decline (Lo *et al.* 2005).

## 2. Impact of different kinetics of thrombin and APC

In agreement with previous studies (Chandler and Velan 2003, Knudsen *et al.* 1996, Petäjä *et al.* 1999), we demonstrated a burst in thrombin generation during early reperfusion (**I**). This massively upregulated systemic generation of thrombin is explained only partly by local tissue factor expression in the reperfused post-ischemic myocardium, as transcoronary gradients of thrombin generation during reperfusion are modest (Kalweit *et al.* 2005, Petäjä *et al.* 1999). A possible contribution of the pulmonary and splanchnic circulations to reperfusion-induced thrombin generation is unknown. Also, release of prothrombotic microparticles into the circulation from the myocardium during reperfusion might explain the observed systemic burst in thrombin generation but remains to be investigated.

APC is activated on the endothelial surface by thrombin, which is bound to thrombomodulin (Gomez *et al.* 2005). Therefore our finding of clearly delayed protein C activation in relation to thrombin generation during CABG was unexpected, but the underlying mechanism is beyond the scope of our study (**II**). However, in the setting of CPB and cardiac surgery our results do not support the hypothesis that low levels of thrombin generate increased levels of APC (Griffin 1995). In fact, throughout the study a dominance of thrombin generation over protein C activation was indicated by the APC/ F1+2 ratio, which was below the preoperative level. It can be speculated that the dynamic association of APC levels with postoperative hemodynamics might reflect an insufficient and delayed APC response to an underlying thrombin challenge. Overall, our results (**I** and **II**) suggest that hypercoagulation after CABG, especially during reperfusion, may be clinically important.

## 3. Regulation of thrombin during CPB

Since efficient control of thrombin during CPB and especially during reperfusion after CABG would probably be of benefit we undertook studies that addressed the regulation of thrombin during CABG (**III** and **IV**). Several thrombophilic factors associate with increased basal thrombin generation (Bauer *et al.* 1988, Mannucci *et al.* 1992, Regnault *et al.* 2004, Zöller *et al.* 1996) and therefore we hypothesized that thrombophilic factors might enhance CPB-related activation of coagulation. Our study (**III**) showed that a preoperative thrombophilic state did not associate with perioperative generation of thrombin or its procoagulant activity suggesting that other mechanisms that enhance thrombin generation during CPB overwhelm any possible effect of thrombophilia on the activation of coagulation during CPB.

Because reperfusion during CABG and the initiation of CPB cause bursts of thrombin generation rather than a continuous progressive increase in thrombin levels (Chandler and Velan 2003) accurate knowledge of the extent of anticoagulation during the various phases of surgery could be of importance. Therefore, we measured heparin effects during various relevant time points during CPB and found that the lowest heparin levels during CPB associated with higher subsequent F1+2 levels after heparin reversal (**IV**). This suggests that more accurate monitoring of heparin anticoagulation might aid in controlling thrombin during CPB. However, point-of-care methods to achieve this goal are lacking. Indeed, we showed that there is poor agreement even between laboratory-based methods of anticoagulation monitoring in the challenging setting of CPB. We also demonstrated that patients with high heparin levels during CPB received fewer transfusions than other patients did (**IV**). This potentially practically important finding is in agreement with a previous randomized study by Despotis and coworkers (Despotis *et al*. 1995).

## 4. Methodological aspects

Accurate quantification of thrombin generation became possible with the introduction of a radioimmunoassay for F1+2 (Teitel *et al.* 1982). We used a subsequently developed commercially available enzyme-linked immunoassay for F1+2 to measure thrombin generation (Pelzer *et al.* 1991)(**I-IV**). Because of the multiple interactions of thrombin, we also used commercially available immunoturbidimetric analyses to measure markers of the procoagulant activity of thrombin (SFC) and a marker of fibrin turnover (D-dimer), which is a surrogate marker of both thrombin and plasmin function (Mustonen *et al.* 1998). Importantly, we also utilized an enzyme-capture assay, which is not widely available, for the measurement of free circulating APC (Gruber and Griffin 1992)(**II**). We were therefore able to diversely quantify thrombin and its interactions.

PiCT is a recently introduced coagulation test that uses the entire prothrombinase complex to trigger coagulation (Calatzis et al. 2000). It is a final-stage coagulation test that has better reproducibility and linearity than coagulation tests that activate the coagulation cascade at an early stage (Fenyvesi et al. 2002). It has also been suggested to be less sensitive to coagulation factor levels, which are altered during CPB (Fenyvesi et al. 2002). Because PiCT is sensitive to heparin and has potential advantages in monitoring heparin levels during CPB, we compared its performance in this setting with two chromogenic anti-Xa assays, the present standard of laboratory-based monitoring of heparin treatment (**IV**). There was poor agreement between PiCT, ACT, and anti-Xa. Possible reasons for the poor agreement between these assays are

discussed in detail in study **IV** and can be partly explained by limitations of each assay in the challenging setting of CPB. However, the correlation between PiCT and one chromogenic assay (anti-Xa A) was at least as good as the correlation between the two chromogenic assays and much better than the correlation between ACT and any of the other assays. Therefore, our results suggest that PiCT could be an alternative to the chromogenic anti-Xa assays.

PiCT is sensitive to both the anti-IIa and anti-Xa activities of heparin (Calatzis et al. 2000) while the chromogenic anti-Xa assays are sensitive only to the anti-Xa activity of heparin. It was therefore of interest also to specifically test whether there are differences between assays measuring either the anti-Xa activity or both the anti-Xa activity and the anti-factor IIa activity of heparin in detecting thrombin-controlling effects of heparin or the effects of heparin on blood loss or transfusion requirements. However, our study could not show that either PiCT or anti-Xa measurements were superior in this respect (**IV**).

## 5. Study limitations

There are some limitations to the present study. The study setting was observational and therefore conclusions about causal relationships can not be made. The study does not reveal possible mechanisms by which thrombin could mediate myocardial damage or by which APC associates with hemodynamic performance. The outcome measures of the study were limited to measurements of laboratory parameters and hemodynamic performance. There was no angiographic evaluation of graft patency or long-term follow-up of patients. However, postoperative elevation of the cardiac biomarkers, Ck-Mbm and TnT, after CABG has been shown to associate with both early and late mortality (Costa *et al.* 2001, Januzzi *et al.* 2002, Kathiresan *et al.* 2004, Klatte *et al.* 2001). Therefore, cardiac biomarker levels can be considered highly relevant endpoints.

# CONCLUSIONS

In this study we showed that the generation of thrombin and its procoagulant and anticoagulant activities associated with clinically important sequelae after CABG. Furthermore, we presented novel findings on the regulation of thrombin in patients undergoing CABG. Specifically we demonstrated that

1. Thrombin generation during reperfusion after CABG associated with postoperative myocardial damage and increased pulmonary vascular resistance.

2. Protein C activation during CPB was clearly delayed in relation to both thrombin generation and fibrin formation. APC associated dynamically with postoperative hemodynamic performance but did not associate with postoperative myocardial damage.

   Overall, these results suggest that hypercoagulation after CABG, especially during reperfusion, might be clinically important.

3. Preoperative thrombophilic variables did not associate with perioperative thrombin generation or its procoagulant activity in patients undergoing CABG. Our results do not support routine thrombophilia screening before CABG.

4. There was poor agreement between a novel coagulation assay (PiCT) and two chromogenic anti-Xa assays and between the two anti-Xa assays in monitoring heparin levels in the challenging setting of CPB. Further studies are needed to establish optimal laboratory-based methods for monitoring high heparin levels during CPB, but our results suggest that PiCT could be an alternative to the chromogenic anti-Xa assays. However, heparin levels evaluated both with the chromogenic anti-Xa assays and with PiCT during CABG correlated positively with the thrombin-controlling effects of heparin and inversely with transfusion requirements.

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