Regulation of Tissue Factor and Coagulation Activity;

Translation Studies with Focus on Platelet-Monocyte Aggregates and Patients with Acute Coronary Syndrome

CHRISTINA CHRISTERSSON
Dissertation presented at Uppsala University to be publicly examined in Enghoffsalen, Ingång 50 bv, Uppsala Universitetets Sjukhus, Uppsala, Saturday, May 17, 2008 at 10:00 for the degree of Doctor of Philosophy (Faculty of Medicine). The examination will be conducted in Swedish.

Abstract

Myocardial infarction (MI) is often caused by a disruption of an atherosclerotic plaque with activation of coagulation, platelets and inflammation. The aims were; to investigate whether the oral direct thrombin inhibitor, ximelagatran affected markers for coagulation, platelet and inflammation in a patient cohort with recent MI and if the coagulation markers could identify patients with increased risk of new ischemic events; to evaluate some of the mechanisms involved in formation of platelet-monocyte aggregates (PMAs).

In a biomarker substudy patients with recent MI were randomized to 24-60 mg of ximelagatran or placebo for six months. There was a persistent dose-independent reduction of coagulation markers (F1+2, D-dimer) by ximelagatran treatment. 60 % reduced their D-dimer levels after one week and that group had less ischemic events during treatment. There was an early increase of the platelet activation marker and ximelagatran in higher doses attenuated these increased levels. Both in vivo and in vitro the direct thrombin inhibitor diminished procoagulant activity and tissue factor (TF) presenting microparticles. In contrast, the inflammatory markers increased after six months of ximelagatran treatment. The PMA-levels were elevated for long-term after MI. In vitro thrombin inhibition diminished formation of PMAs. Formation of PMAs in stimulated whole blood was P-selectin dependent and induced TF expression through phosphorylation of the Src-family member Lyn in monocytes.

Addition of an oral direct thrombin inhibitor reduces coagulation and platelet activation markers for long-term after a MI together with reduced procoagulant activity which may contribute to the clinical benefit of the drug. Early reduction of D-dimer levels seems to be suitable to identify patients with reduced risk of new ischemic events independent of antithrombotic treatment. Circulating PMAs persist after a MI connecting coagulation to inflammation. Within these aggregates P-selectin induces TF, the main initiator of coagulation, partly through phosphorylation of Lyn.

Keywords: Myocardial infarction, Coagulation, Platelet-monocyte aggregates, Tissue factor, Thrombin inhibition

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ISSN 1651-6206
ISBN 978-91-554-7177-4
urn:nbn:se:uu:diva-8669 (http://urn.kb.se/resolve?urn=urn:nbn:se:uu:diva-8669)
To my family and to science
This thesis is based on the following papers


III. Christersson C, Oldgren J, Wallentin L, Siegbahn A. Treatment with an oral direct thrombin inhibitor for long-term after a myocardial infarction reduces platelet activity but increases markers for inflammation. *Manuscript*.

IV. Christersson C, Johnell M, Siegbahn A. Thrombin but not ADP induced formation of platelet-monocyte aggregates and tissue factor expression are reduced by direct thrombin inhibitors. *Manuscript*.

V. Christersson C, Johnell M, Siegbahn A. Tissue factor and IL8 production by P-selectin-dependent platelet-monocyte aggregates in whole blood involves phosphorylation of Lyn and is inhibited by IL10. *Journal of Thrombosis and Haemostasis* 2008. In press

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Patients and control populations .......................................................... 34
Whole blood collection and activation .................................................. 34
Purification and activation of human monocytes ................................. 35
Differentiation and stimulation of U-937 .............................................. 35
Flow cytometry analysis of cell markers .............................................. 35
Flourogenic analysis of thrombin generation ...................................... 37
Soluble IL-8 and MCP-1 ..................................................................... 37
mRNA preparation and quantification ................................................. 37
Phosphorylation of Lyn ....................................................................... 38
Statistical analysis ................................................................................ 38

Results and Discussion ......................................................................... 39
Coagulation activity after myocardial infarction ................................. 39
Baseline characteristics of patients ...................................................... 39
Thrombin generation and fibrin turnover at aspirin treatment (paper I) ............................................................................................................. 40
The additional effect of an oral direct thrombin inhibitor ................. 40
Reduced coagulation activity and risk of new ischemic events (paper II) ............................................................................................................. 42
Bleeding events, coagulation activity and APTT ................................. 44
Platelet and inflammatory activity in patients with recent myocardial infarction (paper III) ................................................................. 46
Studies of formation of platelet- leukocyte aggregates and cross-talk therein ............................................................................................................. 49
Formed platelet-monocyte aggregates in patients with recent myocardial infarction (paper IV) ................................................................. 49
The influence of thrombin inhibitors on formation of platelet-leukocyte aggregates and tissue factor production in vitro ............................................. 50
Microparticles and early tissue factor expression upon stimulation of whole blood ......................................................................................... 52
The importance of P-selectin for formation of platelet-monocyte aggregates and the cross-talk therein (paper V) ............................................. 53
TRAP-induced production of tissue factor and IL-8 is dependent of P-selectin ............................................................................................................. 54
The importance of Src-family kinases with focus on Lyn for P-selectin/TRAP-induced cellular signalling in whole blood ......................... 55
The anti-inflammatory cytokine IL-10 diminishes tissue factor and IL-8 production through reduced Lyn phosphorylation ............................. 57

General Discussion .............................................................................. 59
Conclusions ........................................................................................... 61
Sammanfattning på svenska ................................................................. 63
Acknowledgements.................................................................................................67
References..................................................................................................................69
Abbreviations

ADP  Adenosine diphosphate
APC  Activated protein C
APTT  Activated partial thromboplastin time
AT  Antithrombin
CD  Cluster of designation
CD40L  CD40 ligand
COX  Cyclooxygenase
CRP  C reactive protein
DTT  Dithiothreitol
EDTA  Ethylene-diamine-tetraacetic acid
ELISA  Enzyme-linked immunosorbent assay
EPCR  Endothelial cell protein C receptor
ESTEEM  Efficacy and Safety of the oral direct Thrombin inhibitor ximelagatran in patients with recent Myocardial damage
F1+2  Prothrombin fragment 1 and 2
FITC  Fluorescein-isothiocyanate
Gp  Glycoprotein
HRP  Horseradish peroxidase
IL  Interleukin
INR  International normalized ratio
ITAM  Immunoreceptor tyrosine-based activation motif
LDL  Low density lipoprotein
LMW  Low molecular weight heparin
LPS  Lipopolysaccharide
MAPK  Mitogen-activated protein kinase
MCP-1  Monocyte chemotactic protein-1
MFI  Mean channel fluorescence intensity
MI  Myocardial infarction
MP  Microparticle
mRNA  Messenger ribonucleic acid
NFκB  Nuclear factor κB
NSAID  Non steroid anti-inflammatory drugs
PAF  Platelet activating factor
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>PAR</td>
<td>Protease-activated receptor</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCI</td>
<td>Percutaneous coronary intervention</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
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<tr>
<td>PDGF</td>
<td>Platelet-derived growth factor</td>
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<tr>
<td>PE</td>
<td>Phycoerythrin</td>
</tr>
<tr>
<td>PGA</td>
<td>Platelet-granulocyte aggregate</td>
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<tr>
<td>PLA</td>
<td>Platelet-lymphocyte aggregate</td>
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<td>PMA</td>
<td>Platelet-monocyte aggregate</td>
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<td>PPP</td>
<td>Platelet poor plasma</td>
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<tr>
<td>PSGL-1</td>
<td>P-selectin glycoprotein-1</td>
</tr>
<tr>
<td>RANTES</td>
<td>Regulated on activation, normal T cell expressed and secreted</td>
</tr>
<tr>
<td>RH</td>
<td>Recombinant human</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SFK</td>
<td>Src-family kinase</td>
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<td>TAFI</td>
<td>Thrombin activatable fibrinolysis inhibitor</td>
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<td>TF</td>
<td>Tissue factor</td>
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<tr>
<td>TFPI</td>
<td>Tissue factor pathway inhibitor</td>
</tr>
<tr>
<td>THRIVE</td>
<td>THRombin Inhibitor in Venous thromboEmbolism</td>
</tr>
<tr>
<td>TNFα</td>
<td>Tumor necrosis factor alpha</td>
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<tr>
<td>TRAP</td>
<td>Thrombin receptor activator peptide</td>
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<tr>
<td>TXA₂</td>
<td>Thromboxane A₂</td>
</tr>
<tr>
<td>TXB₂</td>
<td>Thromboxane B₂</td>
</tr>
<tr>
<td>VASP</td>
<td>Vasodilator-stimulated phosphoprotein</td>
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Introduction

Myocardial infarction (MI), due to rupture of an atherosclerotic plaque with thrombus formation, is the leading cause of death worldwide and cardiovascular deaths is projected to increase in the coming decades [1, 2]. Within the last 10 years, acute treatment with antithrombotic drugs and percutaneous coronary intervention (PCI) followed by secondary prevention have reduced the risk for death in-hospital and early after discharge. However, the one-year mortality, for the growing group of patients above 70 years, is still 20%. Several new oral antithrombotic drugs targeting platelets or the “coagulation cascade” are emerging with promising results. Concerns are being raised regarding combinations of these drugs and possible unexpected side-effects. The knowledge of the effect of drugs, which interfere with biological substances with multitude functions, given as long-term treatment is limited. Development of “clinical tools” for monitoring the antithrombotic effect and side-effects of these drugs has just started.
Background

Atherosclerosis-Artherothrombosis

Atherosclerosis is a systemic inflammatory disease involving medium and large arteries. Under normal conditions the endothelium regulates the vascular tone, and controls platelet aggregation and fibrin production through the release of prostacyclin, tissue factor pathway inhibitor (TFPI) and plasminogen activator inhibitor, respectively [3]. Among others hypercholesterolemia with elevated light dense lipoprotein (LDL) levels, smoking with production of free radicals and diabetes mellitus are risk factors which change the properties of the endothelium. During the progression of atherosclerosis endothelial cells expose adhesion molecules and release cytokines leading to increased permeability, accumulation of lipids through adherence of LDL and migration of monocytes into the subendothelium. The activated monocytes transformed to macrophages produce tissue factor (TF), the main initiator of coagulation in vivo, and cytokines such as monocyte chemotactic protein (MCP) -1 and interleukin (IL)-8 resulting in an enhanced inflammatory process [4]. In addition to the inflammatory process both the amount of lipids within the fibrous cap and the location of the plaque are important in determining the instability of the plaque. Upon disruption of an atherosclerotic plaque the exposure of TF forming complexes with VIIa initiates the coagulation process and aggregation of activated platelets. This process promotes a burst of thrombin and fibrin generation resulting in thrombus formation and occlusion of the coronary artery [5].

Platelets and their receptors

During the last 30 years the knowledge of platelets as important cells, not only for clot formation, but also for the inflammatory response has emerge. Platelets are anucleated and formed upon fragmentation of megakaryocytes. Through a two-step process initiated by a change of the cytoskeleton in megakaryocytes with formation of tubular structures filled with granules and organelles pro-platelets are formed. These cells are then divided into platelets. The lifecycle of platelets are 7-10 days.
Table 1. Some of the important substances located within platelets. Modified from ref [6, 7].

<table>
<thead>
<tr>
<th><strong>ALPHA GRANULE</strong></th>
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<tr>
<td><strong>Localized within α granulae</strong></td>
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<tr>
<td>Adhesive glycoprotein;</td>
<td>Von Willebrand factor</td>
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<td></td>
<td>Fibronectin</td>
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<td>Haemostatic factors;</td>
<td>Fibrinogen</td>
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<tr>
<td></td>
<td>Factor V, VII, XI, XIII</td>
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<td></td>
<td>Protein S</td>
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<td></td>
<td>Plasminogen</td>
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<tr>
<td>Protease inhibitors;</td>
<td>Tissue factor pathway inhibitor (TFPI)</td>
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<td></td>
<td>Plasmin activator inhibitor (PAI)-1</td>
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<td></td>
<td>α2- antiplasmin</td>
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<td>Cellular motigenes;</td>
<td>Platelet derived growth factor (PDGF)</td>
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<td></td>
<td>Transforming growth factor β (TGF β)</td>
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<td>Vascular endothelial growth factor (VEGF)</td>
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<td>Epidemic growth factor (EGF)</td>
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<td></td>
<td>Endothelial cell growth factor (ECGF)</td>
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<td></td>
<td>Interleukin 1 β (IL1 β)</td>
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<td>Proteoglycans specific for platelets;</td>
<td>Platelet activating factor 4 (PAF4)</td>
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<tr>
<td></td>
<td>β-tromboglobulin (βTG)</td>
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<tr>
<td><strong>Localized with the α granulae membrane</strong></td>
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<tr>
<td>Antigens and receptors;</td>
<td>P-selectin</td>
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<tr>
<td></td>
<td>GpIIb/IIIa</td>
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<td></td>
<td>GpIb-IX</td>
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<tr>
<th><strong>DENSE GRANULE</strong></th>
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<tr>
<td><strong>Pro-aggregation factors:</strong></td>
<td>Adenine nucleotides; ATP, ADP</td>
</tr>
<tr>
<td></td>
<td>Guanine nucleotides; GTP, GDP</td>
</tr>
<tr>
<td></td>
<td>Serotonin (5 HT), Histamine, Calcium</td>
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<th><strong>CYTOPLASMA</strong></th>
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<td>CD40 ligand</td>
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<tr>
<td>Tissue factor</td>
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Two thirds of the platelets are circulating in the blood stream and one third is stored in the spleen. Under resting conditions platelets express several receptors on their surfaces such as Gp Ib-V-IX for von Willebrand factor, GpIa and VI for collagen, P2Y2 and P2Y1 for adenosine 5-diphosphate (ADP), protease-activated receptor (PAR)-1 and -4 for thrombin, and GpIIb and GpIIIa separately for fibrinogen. Within the granules or the cytosol platelets store several chemotactic factors, coagulation factors, growth factors and proteins important for cell interactions [6, 8].

Activation of platelets by collagen, ADP or thrombin will transform the platelets leading to changes in the lipid core with an increased amount of phosphatedylserine providing a negative charged surface for the coagulation cascade. Moreover, this Ca$^{2+}$-dependent change of lipid content promotes formation of microparticles (MP) [9]. Upon degranulation of platelets, the granules migrate and fuse with the platelet surface, releasing coagulation factors, fibrinogen, ADP and calcium. There will be a shift of GpIIb/IIIa and the intergrins α2β1, increasing the affinity to fibrinogen. Thrombin bound to the PAR-receptors is the most potent platelet activator and increased amount...
of P-selectin and CD40 ligand (CD40L) will be exposed at the platelet surface.

Platelets interaction with endothelium

Platelets interaction with endothelium is initially a loose adhesion: “rolling” of platelets through P-selectin expressed by endothelial cells interacting with GpIb-V-IX complexes on platelets, where GpIb is the most important receptor for the interaction [10]. P-selectin glycoprotein-1 (PSGL-1) on platelets, as a counterreceptor to P-selectin, becomes more important for rolling at high shear. Upon plaque disruption, exposed von Willebrand factor and collagen stabilize the interaction with activated platelets, generating a firm adhesion of platelets to the endothelium through the intergrins. This interaction together with exposure of CD40L increases adhesion molecules on endothelial cells, promoting recruitment of lymphocytes and monocytes.

Platelet-platelet aggregates

Circulating fibrinogen can not adhere to the GpIIb and GpIIIa receptors on resting platelets. The shift of GpIIb/IIIa upon activation increases the affinity for fibrinogen and tethering of platelets, through fibrinogen binding to GpIIb/IIIa, is initiated. This process is strongly dependent on Ca\(^{2+}\) concentrations and, after an initial loose interaction, becomes irreversible within seconds and form substrates for the thrombus.

Platelet-monocyte aggregates

The activated platelets will form aggregates with leukocytes initially through platelet P-selectin binding to its counter receptor PSGL-1 on leukocytes. This association leads to upregulation of CD11b/CD18 (Mac-1) on leukocytes and a firm adhesion between the cells is formed through interaction with GpIIb/IIIa bridged by fibrinogen and CD40L binding to CD40 on leukocytes [11]. The formation of aggregates is suggested as a more efficient marker for platelet activation than P-selectin expressed on the platelet surface. The degranulated platelets rapidly lose the surface P-selectin. The aggregates formed are dynamic in vivo and platelets favor monocytes before neutrophiles [12, 13]. Increased levels of platelet-monocyte aggregates (PMAs) are found in patients with coronary artery disease both in acute MI and stable angina [14, 15]. These PMAs form rapidly and are therefore an early marker for acute MI with the highest levels measured four hours after start of chest pain [16].
Figure 1. Platelets interaction with endothelium starts with “rolling” (A). The activated platelets with GPIIb/IIIa form aggregates with other platelets (B). The injured endothelium with exposed von Willebrand factor (vWF) and collagen further strengthen the platelet-endothelium formation and recruitment of leukocytes (C). Platelets with P-selectin and CD40L at their surfaces will also form aggregates with leukocytes (D).

P-selectin interactions with PSGL-1 and cellular signalling

P-selectin, is a transmembrane protein localized in platelets’ α-granule and also in Weibel-Palade bodies in endothelial cells. Upon activation of platelets, P-selectin is incorporated into the platelet membrane through exocytose [17]. The major counter receptor to P-selectin is PSGL-1 a transmembrane protein expressed on all leukocytes. Two P-selectin proteins bind to one PSGL-1 dimer with high affinity [18, 19]. Besides mediating adhesion of leukocytes to endothelial cells, the interaction between P-selectin and PSGL-1 also induces cellular signal pathways. P-selectin induces production of TF both in purified monocytes and in whole blood [20, 21]. P-selectin also initi-
ates production MCP-1 and IL-8 in neutrophiles and monocytes. Although, P-selectin ought to be accompanied with other platelet activating agents, such as platelet activating factor (PAF) or regulated on activation, normal T cell expressed and secreted (RANTES), to induce production of these cytokines [22-24]. The cellular pathways induced by P-selectin bound to PSGL-1 are not fully understood.

Figure 2. Three hypothetic ways of P-selectin-PSGL-1 induced cellular signalling in human monocytes

PSGL-1 induces tyrosine phosphorylation leading to activation of mitogen-activated protein kinase (MAPK) in neutrophiles, and induction of Mac-1 generating, a firm adhesion of neutrophiles to platelets [25, 26]. Moreover, it was recently described that Src-protein tyrosine kinases were involved in P-selectin induced upregulation of Mac-1 [27].

Src-family kinases (SFK), proteins located within the cells, are important in intracellular signal transduction at inflammatory response. SFK consist of nine members, with Hck, Fgr and Lyn the most predominant in monocytes [28]. Upon phosphorylation, SFK can both be positive and negative regulators of cellular signalling. The immunoreceptor tyrosine-based activation motif (ITAM) is a protein used by classical immunoreceptors and located in the membrane of all myeloid cells. Receptor ligation to ITAM leads to SFK-mediated tyrosine phosphorylation and activation of spleen tyrosine kinase (Syk). Recent data has described a PSGL-1 induced phosphorylation of Syk [29, 30]. Thus, taken together P-selectin expressed on activated platelets...
binds to PSGL-1 on leukocytes and initiates phosphorylation of Syk and SFK through an interaction with ITAM. This signal might activate MAPK directly or through other intracellular proteins. Although, the phosphorylated SFK could also acts through a pathway independent of MAPK.

**CD40 ligand**

CD40L is a transmembrane protein identified in T lymphocytes and mast-cells. Within platelets, it seems like CD40L is located within the cytoplasm and not in the α-granule membrane [7]. It is rapidly expressed on platelet surface upon activation and takes part in the formation of platelet-leukocyte aggregates [31]. CD40L interacts with CD40 expressed by B lymphocytes, monocytes and endothelial cells. Moreover, CD40L has recently been found to bind to Mac-1 independently of CD40 [32]. CD40L induces TF production in endothelial cells and in purified monocytes, and TF correlates to CD40L in atherosclerotic plaque [33]. Interruption of the CD40L-CD40 induced signalling reduces atherosclerotic plaque formation [34]. However, inhibition of CD40L in whole blood only generates a small and varying effect on TF expression on monocytes [21].

**IL-8 and MCP-1**

Both IL-8 and MCP-1 are chemokines able to induce cellular locomotion. They are important for the inflammatory response in atherosclerosis, coordinating leukocytes to endothelium [35]. IL-8, released by endothelial cells and monocytes upon activation with tumor necrotic factor α (TNFα) and IL-1β, is a potent mediator for neutrophile recruitment and chemotactic for lymphocytes. MCP-1 induced among others by IL-6 and IL-8 effectively recruits monocytes and neutrophiles. Both IL-8 and MCP-1 are therefore involved in the progression of atherosclerosis and also in the myocyte damage during MI [36].

**Tissue factor**

Tissue factor, a 45 kDa glycoprotein with an extracellular domain, a transmembrane part and a short intracellular domain, is located in a variety of cells. In addition to its role as the main initiator of coagulation, TF also participates in inflammatory processes, tumor biology and angiogenesis. TF forms complexes with the clotting factor VIIa, starting the coagulation process. The complex also initiates cellular signalling leading to production of growth factors and cytokines among others IL-8. The TF/VIIa complex in-
interacts with PAR-2 receptors. In the coagulation process the factor Xa formed binds to the TF/VIIa complex and these complexes induce cellular signalling through interaction with both PAR-1 and PAR-2 [37]. Platelet-derived growth factor (PDGF) is involved in chemotaxis and TF/VIIa complex increases cell migration induced by PDGF [38]. The cytoplasmatic domain of TF together with PAR-2 phosphorylation or PAR-2 phosphorylation alone is both important cellular signalling pathways leading to cell migration [39, 40]. It has recently been described that TF/VIIa induces phosphorylation of the SFK members Src and Yes. Moreover, TF/VIIa has for the first time been found to transactivate the PDGFβ-receptor [41]. These mechanisms are of importance for angiogenesis, cell migration, cancer and the progression of the atherosclerotic process [42, 43].

High concentrations of TF have been found in brain, heart, lung and placenta, all of which are sensitive for injury with a demand of rapid haemostatic response. Within the normal vessel wall, TF is predominantly localized in the fibroblasts in the adventitia. However, there is a variety within the vessel tree, whereas the carotid and femoral arteries contain the highest and lowest TF activity, respectively [44, 45]. In contrast to the normal vessel, increased concentration of TF protein is found in the atherosclerotic plaque, predominantly localized within macrophage foam cells and in the extracellular matrix.

Nor endothelial cells neither monocytes express TF under resting condition, but cytokines such as TNFα, IL-1β and also CD40L induce TF production in endothelial cells [46-48]. Besides endotoxin (lipopolysacharide, LPS), the most investigated inducer of TF in monocytes, CD40L, C-reactive protein (CRP), angiotensin II, oxidized LDL PDGF-BB, MCP-1 and P-selectin stimulate production of TF in purified monocytes, and in whole blood [20, 21, 49-52]. Platelets and neutrophiles have also been suggested to produce TF. It has not been described that megacaryocytes produce TF, and thus the TF within platelets may originate from other cells and be incorporated upon stimulation [53, 54]. Neutrophiles have phagocyte properties and this could be an explanation for the TF measured within these cells [55, 56]. So the question remains whether others blood cells, besides monocytes, are able to produce TF.

There is a reproducible interindividual variation in TF production in monocytes upon LPS stimulation [52, 57]. When the TF gene contains the 5466 AG genotype monocytes respond with pronounced TF activity to LPS. In acute coronary syndrome this genotype predicts risk of cardiovascular death [58].

**Blood-borne tissue factor**

Added to the traditional way, with TF as an extravascular protein exposed only at major injury, evidence for blood-borne TF has emerged during the
The main source of blood-borne TF is contributed to microparticles (MP), defined as membrane vesicles rich in phosphatidylserine with TF on their surfaces. This concept with both the initiator of coagulation and the negative charged surface is suitable for propagation of coagulation within the blood stream [60]. The cell types that generate MP are not well-defined. Monocytes contribute to TF-bearing MP found in the atherosclerotic plaque [61]. Platelet-derived MP has also been described as the major source of blood-borne TF, where TF originates from platelets or is transferred from leukocytes in a P-selectin/PSGL-1-dependent way [62, 63]. Increased levels of procoagulant MP has been recorded in several diseases, including acute coronary syndrome. Further investigation is needed to understand the biological mechanisms involved in MP formation, clinical relevance of MP determination, and the standardization of methods [64, 65].

The coagulation process

Except for factor VIII, all coagulation factors are synthesized in the liver and the production of factor II, VII, IX and X is vitamin K-dependent. Under resting conditions, small amounts of factor VIIa without proteolytic properties are circulating in the blood. Accumulating data have clearly estimated that the coagulation process is a cell-regulated reaction divided into three steps. Upon exposure of TF, for example from a disrupted atherosclerotic plaque, TF binds to factor VII to form TF/VIIa complexes. These complexes further activate factor IX and X, favoring factor X, and activated factor Xa promotes generation of IXa. When bound to membrane, factor Xa will, together with factor Va, produce small amounts of thrombin in the initiation phase of coagulation [66, 67]. In the amplification phase, the small amounts of thrombin produced activate the platelets through the Gplb-V-IX and this interaction enhances the effect of thrombin on PAR-1 and PAR-4. Thrombin also cleaves factor V released from platelets, activates factor XI and cleaves factor VIII from von Willebrand factor. The activated platelets increase the amount of phosphatidylserine on their surfaces, generating a surface suitable for the coagulation process. In the propagation phase, the initial created factor IXa form complexes with VIIIa and the complex enhances factor Xa, which, together with Va located on the platelet surface, creates a burst of thrombin. Thrombin cleaves fibrinogen to fibrin and a thrombus is formed.

The coagulation process is regulated by inhibitors to balance the procoagulant response to exposed TF. Tissue factor pathway inhibitor (TFPI) released from endothelial cells neutralizes TF/VIIa/Xa complexes in the initial phase of coagulation. Antithrombin (AT) circulates in higher concentrations than TFPI and has potential to neutralize all factors not forming complexes, including thrombin. The third inhibitor to the coagulation process is a dynamic system. The thrombin created binds to thrombomodulin and endothel-
The cell-based coagulation process is divided into three steps: initiation, amplification, and propagation.

**Thrombin - the central coagulation factor**

Thrombin is a serine protease formed upon cleavage of prothrombin. It consists of one active site, two exosites and a Na\(^+\)-binding site, all important for the interactions with its substrates [71, 72]. Due to high/low affinity, these interactions are concentration-dependent and also regulated through the requirement of cofactors for some substrates. Thrombin has a low affinity to fibrinogen but, due to high concentrations of fibrinogen, thrombin cleaves it to fibrin [73]. Together with the thrombin cleavage of factor V and VIII these three interactions could continue without the need of cofactors. The fibrin-bound thrombin can dissociate and interact with factor XIII or the PAR receptors on platelets for further stabilization of the clot. However, these two interactions rely on supply of fibrin as cofactor.

For participation in the anticoagulant process, thrombin needs cofactors such as thrombomodulin and heparin sulfate, both with high affinity for thrombin, leading to dissociation of thrombin from the fibrin within the clot,
initiating the anticoagulant process [74]. The formed APC downregulates production of thrombin through cleavage of factor Va and VIIIa. Besides the procoagulant and anticoagulant properties of thrombin, the protein also participates in inflammatory processes through binding to PAR-1. New data show that PAR-1 induces several cytokines important in sepsis [75]. The anti-inflammatory effect of thrombin proceeds through APC. Together with EPCR, APC activates PAR-1 on endothelial cells, leading to reduced adhesion of leukocytes and production of cytokines [76-78]. Moreover, thrombin-activated fibrinolysis inhibitor (TAFI) is activated by thrombin, and TAFI has also been found to reduce inflammatory response in mouse models [79].

*Figure 4.* A schematic view of some of the properties of thrombin, as an actor in coagulation-, anticoagulation-, inflammatory- and anti-inflammatory-processes in blood.
Biomarkers of the coagulation process, the platelet and the inflammatory activity in clinical settings and experimental models

Prothrombin fragment 1+2 and D-dimer

When prothrombin is cleaved to thrombin, there is a release of prothrombin fragment 1+2 (F1+2). The formed thrombin cleaves fibrinogen to fibrin, which forms a stable clot together with factor XIIIa and the platelet-platelet aggregates. The fibrinolytic system with plasmin reduces the thrombus formed by cleavage of cross-linked fibrin, leading to D-dimer production. D-dimer can therefore be used as a measurement of both thrombin generation and fibrin turnover.

F1+2 is stable over time and can be used for calculation of the thrombin generation and analyzed by immunoassays. The concentrations of F1+2 is influenced by exercise [80]. Increased levels have been found in patients long-term after MI and high levels of F1+2 during acute MI predict risk of death and reinfarction at one month. However, for long-term, both high and low concentrations of F1+2 are associated with adverse events [81-84].

As a measurement for fibrin turnover, D-dimer is, as expected significantly related to F1+2 both in men and women. Moreover, D-dimer concentration is higher in women than in men and increases with age both in a healthy population and in patients with coronary artery disease. D-dimer levels are related to the burden of atherosclerosis and predict risk of future MI in healthy men [85, 86]. D-dimer is also used in risk stratification of acute pulmonary artery disease and deep venous thrombus [87]. In contrast, due to low specificity, no benefit has been found upon addition of D-dimer in early risk stratification of patients with suspected acute coronary syndrome [88]. However, high D-dimer levels during acute MI and also two months after an acute event predict the risk of mortality and recurrent ischemic events for long-term [89, 90]. The concentration of D-dimer is related to inflammatory markers such as IL-6 and CRP both in patients with or without previous MI, although it is a better predictor of coronary risk than the inflammatory markers [91, 92].

Soluble P-selectin

P-selectin is present both in endothelial cells and platelets and the origin of soluble P-selectin is debated. Most studies suggest that the soluble form predominantly originates from platelets and it is related to platelet count and reflects platelet activity [93, 94]. Increased levels of sP-selectin have been described in patients with coronary artery disease, both in stable angina and in MI, and also in peripheral arterial occlusive disease and hypertension [95-97]. Moreover, in healthy women and in patients with hypertension, sP-
selectin, analyzed in citrate plasma independently predicts the risk of future cardiovascular events [98, 99]. In contrast to these results, sP-selectin evaluated in serum could not predict future cardiovascular events [100].

Soluble tissue factor
TF expressed by macrophages within the disrupted plaque and by circulating MP is essential for initiation of thrombus formation in an acute MI. The source of the soluble form of TF originates from truncated TF, enzymatically cleaved from extravascular cells, TF-bearing MP derived from monocytes or platelets, and also from alternative spliced human TF [101]. The procoagulant properties of these last forms of sTF and the optimal method for evaluation of procoagulant activity of sTF are currently debated. Increased levels of sTF have been reported both in patients with stable angina and patients with acute MI [102, 103]. There are conflicting data presented regarding sTF levels in patients with recent MI and the risk of new events. In a large trial in patients with recent acute coronary syndrome, the sTF levels were not related to risk of death or new MI [58]. In contrast, other studies have found that in patients with recent MI high sTF predicted risk of death and reinfarction for long-term [104, 105].

IL-18
IL-18, a member of IL-1β family, is expressed in a variety of cells, and the main source in hematopoietic cells is macrophages. IL-18 is activated through cleavage by the enzyme caspase-1. Besides promoting T lymphocyte differentiation, IL-18 also induces production of IL-6, IL-8 and adhesion molecules [107]. Accumulation of IL-18 has been described in atherosclerotic plaques and, in animal studies injection with IL-18 further enhanced plaque progression [108, 109]. Furthermore, the concentration of IL-18 is a strong predictor for cardiovascular death in patients with coronary artery disease and also in healthy men without influence of age and traditional risk factors [110, 111].

C-reactive protein
CRP is primarily synthesized by hepatocytes as a response to IL-6. It is an acute phase inflammatory protein that increases in, among others, inflammatory diseases and infections. When CRP is determined with a high sensitive CRP (hsCRP) assay for detection of low levels, CRP predicts future risk of cardiovascular events in healthy men and women [112, 113]. In acute coro-
nary syndrome, CRP increases in relation to the amount of damaged myocytes. High CRP levels predict risk of recurrent ischemic events for long-term and, together with Troponin I, it identifies the group of patients with the highest cardiovascular risk [114-116].

Medical treatment for long-term after myocardial infarction

Antiplatelet drugs

There are three kinds of antiplatelet drugs, targeting different receptors/functions in platelets, which have been found effective in preventing new ischemic events in patients with coronary artery disease.

Aspirin

Cyclooxygenases 1 (COX-1) is involved in the metabolism of arachidonic acid to thromboxane A₂ (TXA₂), a metabolite that promotes platelet aggregation and blood vessel constriction. TXA₂ has a short half-life and is rapidly metabolized to thromboxane B₂ (TXB₂). Aspirin irreversibly inhibits COX-1, thereby reducing platelet aggregation and vasoconstriction. Aspirin was introduced 20 years ago as long-term treatment after MI and effectively reduces the risk of death and new MI by 25% [117, 118]. Today, the efficacy of aspirin also includes patients with stable angina, ischemic stroke and peripheral arterial occlusive disease. The high doses of aspirin used in the first studies have been replaced with lower doses. Recommended doses for long-term treatment today are 75-100 mg. In addition to reducing platelet aggregation, aspirin also reduces TNFα and MCP-1 in patients with stable coronary artery disease, and treatment with higher doses, i.e., 300 mg decreased the concentrations of IL-6 and CRP [119, 120]. The anti-inflammatory effect of aspirin depends among others functions on reduced monocyte adhesion together with reduced activation of nuclear factor κβ (NFκβ) in endothelial cells [121].

Clopidogrel

ADP activates the platelets through binding to the P2Y₁ and P2Y₁₂ receptors leading to an increase of calcium, induction of the TXA₂ pathway and a shift of GpIIb/IIIa receptors and platelet aggregation. Ticlopidine was the first available oral ADP-inhibitor, but due to its side-effects, which include bone marrow depression, it has been replaced by clopidogrel. Initially, clopidogrel was used together with aspirin at PCI. However, today the indication for long-term treatment with clopidogrel in combination with aspirin includes both ST- and non ST-elevation MI [122, 123]. In experimental studies and also in vivo, clopidogrel reduces the P-selectin expression on platelets and
the formation of platelet-leukocyte aggregates [124]. Moreover, besides its anti-inflammatory properties similar to aspirin, clopidogrel also reduces CD40L and sTF in stable coronary artery disease [7, 125]. However, clopidogrel has no effect on the markers for thrombin generation and fibrin turnover [126].

GpIIb/IIIa inhibitors
The change of GpIIb/IIIa upon platelet activation increases the affinity to fibrinogen, leading to formation of platelet-platelet aggregates. Intravenous drugs with inhibitory effect on the GpIIb/IIIa receptors have been found to be effective in reducing platelet activity and new ischemic events in acute coronary syndrome, mainly in patient undergoing PCI. However, the addition of oral GpIIb/IIIa inhibitors to aspirin showed no beneficial effects in large phase III trials for long-term treatment [127, 128]. The oral treatment with GpIIb/IIIa inhibitors even increased mortality, which led to premature termination of one trial and no oral GpIIb/IIIa inhibitor is available for clinical use today.

Resistance to antiplatelet drugs
The increased risk of new ischemic event and cardiovascular death after MI, despite the dual antiplatelet therapy, has initiated a debate of resistance to aspirin and clopidogrel. In this debate, several different methods have been used to evaluate resistance to aspirin/clopidogrel, without any defined “gold standard”. However, the recommendation today is to use more specific methods to evaluate the effect. The TXA2 pathway, should be determined by serum TXB2, and the ADP pathway, through measurement of ADP-induced phosphorylation of a protein vasodilator-stimulated phosphoprotein (VASP), in platelets [129]. Aspirin resistance is extremely rare, with an incidence of only 1-2% [130]. For clopidogrel, there is a wide variability of response. When the effect was evaluated with more specific methods as many as 30% of the patients were defined as poor responders [131]. Recent data have described that the variation in response to clopidogrel is due to absorption of the drug rather than heterogeneity of the P2Y12 receptor [132]. There is no recommendation for determination of resistance to antiplatelet drugs in clinical practice today.

Oral anticoagulant treatment
Warfarin
Warfarin interferes with vitamin K and changes the N-terminal region of the vitamin K-dependent coagulation factor II, VII, IX and X leading to reduced coagulation activity. Several clinical trials have shown that addition of warfarin treatment with an INR >2.0 to aspirin reduces the risk of reinfarction
and death, and it can therefore be recommended for long-term treatment [133-135]. The frequency of bleedings increases with the combination of antiplatelet and anticoagulant drugs but it is still low in clinical trials. There is a large interindividual variation in dose and a small therapeutic window between efficacy and risk of bleedings. Warfarin treatment thus requires frequent controls of INR. The addition of warfarin for long-term treatment after MI in clinical practice is therefore restricted to selected patients. Warfarin treatment effectively reduces the thrombin generation and fibrin turnover measured as F1+2 and D-dimer for long-term. In contrast, sTF is increased by warfarin as a result of reduced consumption [136].

**Factor Xa inhibitors**

Factor Xa inhibitors are pentasaccharides, a heparin derivate, which selectively inhibits Xa through binding to antithrombin without specific inhibition of thrombin. When administrated subcutaneously during acute coronary syndrome the Xa inhibitor, fondaparinux, was as effective as low molecular weight (LMW) heparin in reducing ischemic events but with a reduced frequency of major bleedings [137]. Several new oral Xa inhibitors for long-term treatment are under clinical development and results of these trials will be presented in the coming years.

**Direct thrombin inhibitors**

The first available oral direct thrombin inhibitor, ximelagatran binds directly and reversible to the active site of circulating and fibrin-bound thrombin [138]. It is a prodrug, rapidly absorbed and then transformed to the active compound melagatran, and it is mainly excreted through the kidneys. Compared to warfarin, melagatran has a wider therapeutic window and there is no need for monitoring [139, 140]. Ximelagatran was investigated in a program including clinical trials both as prophylactic antithrombotic treatment in orthopedic surgery and in atrial fibrillation. In these trials, ximelagatran was as effective as LMW heparin and warfarin respectively [141, 142]. In the THRIVE trials, ximelagatran was compared to warfarin for treating venous thromboembolism, and reduced the risk of recurrence of thromboembolic events for long-term [143, 144]. The problem with ximelagatran was the association with increased lever enzymes during treatment. Due to the risk of hepatotoxicity the compound was withdrawn from the market in February 2006.

In the ESTEEM trail, ximelagatran was investigated in atherothrombosis. This was a dose finding phase II study for safety and efficacy of ximelagatran for long-term treatment in combination with aspirin in patients with recent MI. 1883 patients where randomized within 14 days after an acute MI to placebo or one of four doses of ximelagatran twice daily in combination with aspirin for 6 months [145]. Upon addition of ximelagatran to aspirin there was a 24% relative reduction in death, MI and severe recurrent ische-
mia without difference between the ximelagatran doses. There was no increased risk of major bleeds in the ximelagatran groups, but the total bleeding risk increased with the dose of ximelagatran. Today, new oral direct thrombin inhibitors are under progress in clinical trials. Dabigatran etexilate has recently been approved for prophylactic treatment in orthopedic surgery and is currently investigated in atrial fibrillation and in coronary artery disease.

The risk of long-term treatment with thrombin inhibitors has been debated. Concerns have been raised due to the mechanism of thrombin not only serving as an actor in the coagulation process but also its involvement in several inflammatory pathways. The effects of long-term treatment with an oral thrombin inhibitor, regarding thrombin generation, fibrin turnover, platelet activity and inflammation markers, have not been investigated. We know from acute myocardial infarction trials that an intravenous direct thrombin inhibitor was comparable to unfractionated heparin regarding clinical effect and the effect on coagulation markers, but after cessation there was an increase in events and a rebound of coagulation activity [146, 147].

Anti-inflammatory drugs
The atherosclerotic process with atherothrombosis involves several inflammatory mechanisms and both statins and ACE-inhibitors, recommended drugs for long-term treatment after a MI, have anti-inflammatory properties. In experimental atherosclerotic mouse models statins and ACE-inhibitors both inhibit MCP-1 and TNFα, leading to reduced monocyte recruitment to endothelium [148, 149]. Moreover, both drugs reduce TF expression in monocytes interacting with endothelial cells [150].

Traditional anti-inflammatory drugs such as prednisolone, non-steroid anti-inflammatory drugs (NSAID) and COX-2 inhibitors are not related to reduced inflammatory activity in atherosclerosis. In contrast, several studies have found a dose related increased risk of MI upon treatment with these drugs [151, 152].

The anti-inflammatory cytokine IL-10 inhibits monocyte/macrophage activation, leading to reduced production of pro-inflammatory cytokines and reduction of atherosclerotic lesions [153, 154]. IL-10 also decreases TF production induced by LPS in whole blood [57]. Treatment with IL-10 has not been investigated in coronary artery disease. However, in patients with inflammatory bowel diseases, IL-10 effectively prevents inflammation in selected patients [155].
Aims

Atherosclerosis is an inflammatory disease in large and medium arteries with manifestations in several organs. When affecting the coronary arteries, the disease gives symptoms in form of angina pectoris. In an acute occlusion of a coronary artery related to an atherosclerotic plaque, a MI, there is pronounced platelet and coagulation activity. The coagulation activity is important for risk of new ischemic events in patients with MI. Long-term treatment with anticoagulant drugs such as warfarin interferes with normal lifestyle and requires regular monitoring. New drugs with potential effect on both coagulation and inflammation are under progression and the goal is to reduce the risk of recurrence of ischemic events with a low risk of side-effects. There is also a need to identify markers that reflect coagulation activity in order to monitor the new drugs and to identify patients at high risk.

The aims of Paper I-III were to evaluate whether the first available oral direct thrombin inhibitor, ximelagatran, affected coagulation markers and APTT for long-term in patients with recent MI. We further planned to investigate whether the levels of coagulation markers and change in these were related to risk of new ischemic events. Thrombin is a potent platelet agonist and it is also involved in inflammatory and anti-inflammatory processes. Therefore, evaluation of markers reflecting platelet and inflammation activity was planned in the patient cohort with recent MI.

The cross-talk between platelets and leukocytes connects inflammation and coagulation and with new techniques this interaction could be investigated in more depth. From earlier results, we know that patients with MI have elevated levels of circulating platelet-leukocyte aggregates and that these aggregates express TF. The aim in Paper IV and V was to investigate these aggregates for long-term after MI and to determine whether the oral direct thrombin inhibitor could affect blood-borne TF. The aim was also to perform in-depth studies regarding:

- the importance of adhesion proteins i.e., P-selectrin and CD40 ligand, in the formation of platelet-monocyte aggregates and the induction of TF and pro-inflammatory cytokines.
- further evaluation of the induced P-selectin-PSGL-1 cellular signalling pathways regulating the expression of TF and pro-inflammatory cytokines.
- the role of IL-10 in the formation of platelet-monocyte aggregates and regulation of TF and pro-inflammatory cytokines.
Methods

Clinical studies (paper I-III)

Patient population
In the ESTEEM trial high risk patients were enrolled within 14 days of a MI.

Table 1. *Inclusion and exclusion criteria in the ESTEEM trial*

<table>
<thead>
<tr>
<th>INCLUSION CRITERIA:</th>
</tr>
</thead>
<tbody>
<tr>
<td>• Raised biochemical markers (troponin I, troponin T, creatine kinase-MB above upper limit or creatine kinase above upper limit when cardiac specific markers were unavailable)</td>
</tr>
<tr>
<td>• New ischemic electrocardiogram changes (ST-segment elevation, ST-segment depression of &gt;0.05 mV or acute T-wave inversion &gt;0.1 mV, or both)</td>
</tr>
</tbody>
</table>

Additional risk factors:
• Age ≥ 65 years
• Diabetes mellitus
• Previous myocardial infarction
• Known multivessel coronary disease
• Previous ischemic stroke
• Peripheral arterial occlusive disease
• Symptomatic congestive heart failure or left ventricular ejection fraction of < 40%
• New left bundle branch block
• ST-segment depression of 0.1 mV or greater associated with index event
• History of hypertension

<table>
<thead>
<tr>
<th>EXCLUSION CRITERIA:</th>
</tr>
</thead>
<tbody>
<tr>
<td>• Performed percutaneous coronary intervention within the last 4 months or planned with 60 days</td>
</tr>
<tr>
<td>• Conditions related to increased risk of bleeding</td>
</tr>
<tr>
<td>• Known liver diseases or high concentrations of liver enzymes (more than twice the upper limit of normal)</td>
</tr>
</tbody>
</table>

The 1883 patients were randomized in a 1:1:1:1:2 fashion to one of four doses of ximelagatran (24, 36, 48 or 60 mg) or placebo twice daily in combination with aspirin 160 mg for 6 months. 518 of the patients randomized in Sweden, Norway and Denmark took part in a biomarker substudy.
Definition of ischemic events and bleedings

New ischemic events in form of death, myocardial infarction, severe recurrent ischemia, and ischemic stroke were all pre-specified endpoints in the ESTEEM study. New myocardial infarction was diagnosed by at least two of three criteria; raised biochemical markers of myocardial damage defined as creatine phosphokinase-MB twice the upper limit of normal; symptoms suggestive of myocardial infarction; or development of significant Q wave. Severe recurrent ischemia was defined as severe, prolonged or repetitive anginal pain at rest despite optimal medical treatment and at least one of the following: new ischemic electrocardiographic changes (ST-elevation ≥ 0.05 mV, ST-depression ≥ 0.05 mV, or T wave inversion in at least two adjacent leads); an increase in creatinine phosphokinase-MB not fulfilling the criteria of myocardial infarction, or raised concentration of troponin I or T; or admission leading to unplanned coronary angiography or intervention. Stroke was diagnosed as abrupt onset of focal neurological deficit persisting more than 24 hours. All stokes were assessed by CT or MRI scan.

Bleeding was classified as major or minor; the former defined as one or more of the following criteria: fatal bleeding; clinical overt bleeding associated with a fall in haemoglobin of at least 20 g/L, or leading to transfusion of two or more units of whole blood or erythrocytes, intracranial, intraspinal, intraocular, pericardial, or atraumatic intra-auricular bleeding. A major
bleeding was defined as an endpoint in the study leading to cessation of study treatment.

Blood sampling and laboratory methods

Venous blood was drawn using a 21/22 gauge needle into vacutainer tubes containing citrate or ethylene-diamine-tetraacetic acid (EDTA). The blood was centrifuged within 30 minutes in 2000 g for 20 minutes and stored at -20°C or -70°C until analysis. The time-points used in the study were: at randomization (before start of study treatment); and at 1 week - before intake of morning dose (trough) and after 4 hours (peak); 8 weeks - 2-3 hours post dose; 26 weeks - trough and peak; and 2 weeks after cessation of the drug - following 6 months treatment or premature discontinuation (follow up). The plasma concentration of melagatran was measured at 1 and 26 weeks trough and peak. The APTT was determined at randomization and at the same time-points as the plasma concentration of melagatran using a Medical Laboratory Automation (MLA) Electra 1400C analyser (Beckman Coulter, Fullerton, CA) with a reagent from Dade-Behring. F1+2 and D-dimer were determined at all time-points in the study. sP-selectin and IL-18 were analysed at randomization and at 1 and 26 weeks trough. sTF and hsCRP were measured in the 60 mg ximelagatran group and in the placebo group at the same time-points as sP-selectin and IL-18. Immunoassays: Enzygnost® (Dade-Behring) and TintElize®D-dimer (Biopool®) were used for F1+2 and D-dimer respectively. sP-selectin, IL-18, sTF and hsCRP were analyzed with immunoassays from R&D Systems, MBL, American Diagnostics and ABBOT, respectively.

There were 297, 269, 493, 492, 490 and 487 samples available for analysis of the concentration of melagatran, APTT, F1+2, D-dimer, sP-selectin and IL-18 respectively at start. At 26 weeks of treatment, there were samples from 61% of the patients and follow-up samples from 75% of the patients.

Statistical analysis

For demographic variables proportion or mean values are given. The plasma concentration, APTT and all markers for coagulation, platelet and inflammation activity are presented as medians (25th-75th percentiles). Within each group the changes from randomization (in per cent) were presented as medians. The parameters were not normally distributed; therefore non parametric tests were used. Wilcoxon signed-ranks test for within-group comparison and Kruskal-Wallis and Mann-Whitney U-test (Wilcoxon two-sample test) for between-group comparisons. Spearman’s rank correlation coefficient was used to relate plasma concentration of melagatran to APTT and the change of the different markers. Pearson’s correlation coefficient was used to calcu-
late the correlation between the change of markers for platelet activation and inflammation.

Clinical endpoints were described in frequencies and compared between the different strata of patients based on median levels of coagulation markers at randomization. The change of the coagulation markers after 1 week was dichotomized as increased or unchanged/decreased. The change of APTT within the ximelagatran groups was divided according to APTT ratio; levels less than two times the level at randomization compared to levels greater or equal to two times the level at randomization. Kaplan-Meier estimates with log-rank test were used to illustrate cumulative frequencies over time. Differences were evaluated with \( \chi^2 \) test or Fisher’s exact test when appropriate. Cox regression with models including interaction term treatment *F1+2 at randomization, treatment *D-dimer at randomization and also the interaction between treatment and change of coagulations markers after 1 week were calculated. P-value <0.05 was considered significant.

Experimental studies (paper IV and V)

Patients and control populations

Twenty patients in the ESTEEM trial randomized at Uppsala University Hospital participated in a platelet activity substudy. Twelve healthy age matched men and women without risk factors were used as a control population.

Blood was drawn with no stasis into vacutainer tubes containing citrate (3.8%) and preparation of the blood was performed within 30 minutes. In the patient population, samples were collected at randomization, after 1 week and after 6 months. In the control population samples were collected at one time-point.

Whole blood collection and activation

In paper IV, blood was collected from healthy volunteers as described above. Preparation of the blood was performed within 30 minutes. PephablocFG®, a peptide with high affinity to fibrin, were added to the whole blood before stimulation to avoid clot formation and platelet aggregation [156]. To investigate the properties of direct thrombin inhibitors, we used two direct thrombin inhibitors: melagatran, the active compound of ximelagatran, and dabigatran etexilate in concentrations evaluated in clinical phase II studies [157]. Whole blood with or without the direct thrombin inhibitors was stimulated with 5nM thrombin or 20 μM ADP and incubated at 37°C for up to 1 hour, gently rotated after 30 minutes.
In paper V, whole blood was collected as described above and, in these experiments, we stimulated the blood with thrombin receptor activator peptide (TRAP) 20 μM for up to two hours in 37°C. Recombinant human (rh)IL-10 5 ng/mL, 5 μg/mL neutralizing antibodies to P-selectin (9E1, R&D systems) or CD40L (clone TRAP1, Beckman Coulter), the inhibitors to SFK; SU6656 2 μM or PP2 1 μM (Calbiochem®, Merck KGaA) were added 30 minutes before stimulation in some experiments.

Purification and activation of human monocytes

In paper V, we isolated mononuclear cells from heparinised blood. Platelets were removed by centrifugation and the platelet poor plasma (PPP) was brought back to the leukocytes and erythrocytes. The blood was incubated with RosetteSep®, human monocyte enrichment cocktail (StemCell Technologies) for 20 minutes. Followed by dilution with phosphate buffered saline (PBS), and subjected to density gradient centrifugation using Ficoll-Paque (GE Healthcare) [158]. The final cell suspension contained ≥ 90% monocytes. The monocytes were diluted in cell medium, RPMI1640, and incubated with or without 150 or 250 ng/mL rhP-selectin for up to 2 hours.

Differentiation and stimulation of U-937

The human monoblastic cell line U-937 was maintained and differentiated by exposure to 0.1 μM of vitamin D3 for 72 hours. The differentiation resulted in growth-arrested, CD14 positive cells with inducible TF expression [159]. The vitamin D3-differentiated U-937 was incubated with or without rhP-selectin for up to 2 hours.

Flow cytometry analysis of cell markers

Flow cytometry is a technique where antigen on cell surfaces is detected by fluorochrome-conjugated antibodies. The laser beam within the instrument irradiates each cell passing through the file. The laser light is then scattered and detectors in the flow cytometer are placed to detect light along the forward axis (forward scatter) and at a 90° angle (side scatter) from the incoming laser beam. Forward scatter reflects the size of the cells and side scatter internal granules and membranes. Platelets and the different leukocyte populations are distinguished by forward and side scatter. Four fluorochromes with different wavelength can be used for detection of antigens on cell surfaces in the same sample. In paper IV and V, we have used the fluorochromes fluorescein isothiocyanate (FITC) and phycoerythrin (PE). The signals from the detectors are presented in histogram, and the percentage of positive stained cells in the population is also shown. Moreover, the intensity
of staining on each cell, mean fluorescence intensity (MFI), is presented and used as a measurement for the amount of antigen on each cell. The instrument provides a sensitive technique where small sample volumes can be used for analysis. A disadvantage with the method is the need of fresh samples.

Figure 6. Flow cytometric analysis of platelet-monocyte aggregates. Leukocytes positive for CD45 are gated by A. The CD45-positive cells are discriminated by their side scatter properties and monocytes are gated by B. CD42a is a platelet marker (GpIX) and monocytes are analyzed on the basis of their positivity for CD42a.

Whole blood was labelled with fluorescence-conjugated antibodies for detection of surface expression of CD14, CD42a, CD62P, CD154 and CD142 to define single free platelets, platelets-monocyte aggregates (PMAs), platelet-granulocyte aggregates (PGAs) and platelet-lymphocyte aggregates (PLAs) and their expression of P-selectin and CD40L. Annexin V determined the amount of MP in the population with a size $< 1\mu\text{M}$. The surface expression of TF was evaluated in the platelet, leukocyte and microparticle populations. In paper V, we also used flow cytometry for detection of intracellular phosphorylated Lyn within monocytes after permiabilization and staining with anti-Lyn (pY396) antibody and FITC-conjugated secondary antibody.
Flurogenic analysis of thrombin generation

In paper IV, PPP was prepared from citrated whole blood in the patient population or from thrombin and ADP stimulated whole blood. Thrombin generation assay was performed using the Calibrated Automated Thrombogram (Thromboscope) measured in a 96-well plate fluorometer [160]. PPP was mixed with standardized reagent to a final concentration of 4 μM phospholipids to determine the activity of endogenous TF. The fluorometric measurements were performed after automated addition of fluorescent substrate and calcium chloride. Thrombin generation was followed for 60 minutes. The lag time reflected the initiation phase of the coagulation process where the endogenous levels of TF determine the time to start of thrombin generation.

![Figure 7](image)

*Figure 7.* This is a schematic representation of thrombin generation. The time-to-start of thrombin generation (lag time) is 10 minutes in a patient without antithrombotic treatment (placebo). After treatment with a thrombin inhibitor the lag time is prolonged to 40 minutes.

Soluble IL-8 and MCP-1

In paper V, PPP was prepared through centrifugation of unstimulated or TRAP stimulated whole blood. Soluble IL-8 and MCP-1 were determined with Quantikine® immunoassays (R&D Systems).

mRNA preparation and quantification

In papers IV and V, the total mRNA from 1-2 ml of stimulated whole blood was prepared by using QIamp®RNA Blood Mini Kits (QIAGEN). In paper V, the total RNA from 0.5 x 10^6 purified monocytes and 1.0 x 10^6 vit D₃ differentiated U-937 was prepared using Trizol® with Phase Lock Gel
Heavy. Reverse transcription and real time polymerase chain reaction (PCR) were carried out with the TaqMan real-time PCR assay by an ABI PRISM™ 7000 Sequence Detection System (Applied Biosystems). TF was analysed and related to the endogenous control β2-microglobulin [161]. IL-8 and MCP-1 were carried out using predesigned “assays on demand” (Applied Biosystems).

Phosphorylation of Lyn
In TRAP and rhP-selectin stimulated whole blood, leukocytes were permeabilized with 90% MetOH and thereafter incubated with anti-Lyn (Y396) antibody to detect these phosphorylated proteins located within the cells. After labelling anti-Lyn antibodies with a FITC-conjugated secondary antibody, flow cytometry was used to determine the amount of phosphorylated Lyn(Y396) within the monocytes.

Vitamin D₃-differentiated U-937 was stimulated with rhP-selectin for up to 10 minutes. Cells were lysed in SDS-PAGE sample buffer with 40 mM DTT, homogenized, boiled, and run on a 10% SDS-PAGE gel and electro-transferred to a nitrocellulose membrane for immunoblotting. Phosphorylated Lyn was detected by incubation of the membrane with anti-Lyn antibody followed by incubation with a HRP-linked anti-rabbit IgG. The bands were detected by enhanced chemiluminiscence (ECL, GE Healthcare). For loading control, unphosphorylated Lyn protein was performed after stripping of the membrane. The membranes were exposed to autoradiographic films and the relative extent of phosphorylated protein was quantified densitometrically.

Statistical analysis
In paper IV formation of PMAs and the procoagulant activity within the patient and control population were not normally distributed and therefore median values (25th-75th percentile) were used for description. Wilcoxon signed-ranks test and Mann-Whitney U-tests, as appropriate, were used for within- and between-groups comparisons.

Results from the experiments in whole blood environment, purified monocytes and vitamin D₃-differentiated U-937 were described by mean or mean±SEM. Student’s t-test for dependent samples were performed to determine statistical significance between different data sets. P<0.05 was considered statistically significant.
Results and Discussion

Coagulation activity after myocardial infarction

Baseline characteristics of patients

Table 2. Baseline characteristics and medical treatment in the ESTEEM biomarker substudy

<table>
<thead>
<tr>
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<th><strong>XIMELAGATRAN GROUP</strong> N=339</th>
<th><strong>PLACEBO GROUP</strong> N=179</th>
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<tr>
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</tr>
<tr>
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<tr>
<td>Female (%)</td>
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<td><strong>Diagnosis:</strong></td>
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<td>ST-elevation MI (%)</td>
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<tr>
<td>Non ST-elevation MI (%)</td>
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<td><strong>Additional risk factors:</strong></td>
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<td>Previous MI (%)</td>
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<td>Symptomatic heart failure or LVEF* &lt;0.4 (%)</td>
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<td>Hyperlipidemia (%)</td>
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<td>49</td>
</tr>
<tr>
<td><strong>In-hospital treatment:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thrombolysis (%)</td>
<td>38</td>
<td>34</td>
</tr>
<tr>
<td>Heparin or LMW heparin (%)</td>
<td>84</td>
<td>80</td>
</tr>
<tr>
<td><strong>Medical treatment at randomization:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aspirin (%)</td>
<td>99</td>
<td>100</td>
</tr>
<tr>
<td>Beta blockers (%)</td>
<td>93</td>
<td>94</td>
</tr>
<tr>
<td>Statins (%)</td>
<td>65</td>
<td>57</td>
</tr>
<tr>
<td>ACE† inhibitors or AT• II blocking agents (%)</td>
<td>54</td>
<td>54</td>
</tr>
<tr>
<td>Nitrates (%)</td>
<td>75</td>
<td>81</td>
</tr>
</tbody>
</table>

*Left ventricular ejection fraction; †Angiotensin converting enzyme; •Angiotensin
There were no significant differences between the placebo group and the combined ximelagatran group regarding risk factors, medical treatment at the acute event or medication for secondary prevention.

Thrombin generation and fibrin turnover at aspirin treatment (paper I)

179 patients with recent MI were randomized to placebo treatment for 6 months. Within the placebo group the median F1+2 levels was 0.94 (0.74-1.19) nmol/L at randomization with an increase to 1.02 nmol/L after 1 week when continuing with aspirin as antiplatelet treatment (p=0.006). The median D-dimer levels at randomization was 127 (66-243) ng/mL, and we also found an increase of the D-dimer levels to 146 ng/mL after 1 week (p<0.001). In previous studies in acute MI, initial treatment with unfractionated heparin or LMW heparin reduces F1+2, but after 1-2 days a gradual increase was found despite ongoing treatment [84, 147]. In the present study the patients were enrolled 6 days (mean) after the acute event when antithrombotic treatment with heparin or LMW heparin had reduced most of the initial enhancement of coagulation activity. Therefore, both F1+2 and D-dimer levels were in the upper range of normal at randomization. The elevation of thrombin generation and fibrin turnover found after one week, which persisted during the study period, indicates a reactivation phenomenon after cessation of the acute antithrombotic treatment when continuing with only aspirin.

The additional effect of an oral direct thrombin inhibitor

There were no significant difference between the levels of F1+2 and D-dimer when comparing the four doses of ximelagatran during the study period. Moreover, there were no differences in F1+2 and D-dimer levels when comparing trough and peak values. We could therefore pool all results from the four dose groups of ximelagatran to a combined ximelagatran group. The F1+2 levels at randomization within the combined ximelagatran group was 0.94 (0.78-1.31) nmol/L with a significant reduction to 0.80 nmol/L found after 1 week (p<0.001). These reduced levels remained stable during the study period. After cessation of ximelagatran, the F1+2 returned to the initial level seen at randomization (Figure 8).
Figure 8. The relative change from randomization in F1+2 for the placebo (•) and the ximelagatran groups (•24 mg, □36 mg, ○48 mg and ○60 mg).

Similar results but even more pronounced was found for the change of D-dimer levels. In the combined ximelagatran group, the D-dimer levels at randomization was 135 (74-263) ng/mL, not significant different from the placebo group. After 1 week the D-dimer levels had decreased to 97 ng/mL (p<0.001) with further decreases at 8 and 26 weeks. At follow-up the D-dimer concentration had increased but was still reduced compared to randomization (p=0.003) (Figure 9).

The change of F1+2 and D-dimer levels compared to randomization were not related to the plasma concentration of melagatran. The effect of melagatran on both circulating thrombin and thrombin bound to fibrin might explain the proportional larger reduction of D-dimer, which reflects the fibrin turnover, while F1+2 is a more specific marker for thrombin generation. Ximelagatran consistently reduced both F1+2 and D-dimer without any signs of reactivation. The non-dose-dependent response to ximelagatran, with similar effect on thrombin generation and fibrin turnover at 24 mg as at 60 mg, might explain the similarity in clinical outcome in the ESTEEM trial [145]. After the acute MI under more stable conditions small amount of thrombin is generated during the initiating phase of the coagulation process and, under such circumstances, low concentrations of melagatran is enough to interfere with the formed thrombin. Upon plaque disruption, a burst of thrombin is
generated and under such conditions there is a need for higher concentrations of melagatran [162].

![Figure 9](image)

**Figure 9.** The relative change from randomization of D-dimer for the placebo (•) and the ximelagatran groups (•24 mg, □36 mg, ◊48 mg and ◊60 mg).

Reduced coagulation activity and risk of new ischemic events (paper II)

In the ESTEEM trial, addition of ximelagatran to aspirin reduced the risk of new ischemic event from 16.3% to 12.7% [145]. In the biomarker substudy cohort, there were 64 ischemic events in form of death (n=3), myocardial infarction (n=32), severe recurrent ischemia (n=25) or ischemic stroke (n=4) during study treatment.

Elevated levels of F1+2 or D-dimer at the acute MI or 2 months afterwards have been attributed an elevated risk, both short and long-term, of new cardiac events [89, 90, 163]. Treatment with ximelagatran significantly reduced the risk of new ischemic events in patients with above-median F1+2 and/or D-dimer levels at randomization (p=0.03 and p=0.009 respectively). In contrast, there appeared to be little beneficial effect of the direct thrombin inhibitor in patients with lower levels of F1+2 and D-dimer.

After 1 week the levels of F1+2 were reduced in 63% of the patients in the total substudy cohort. In the combined ximelagatran group, 76% had
decreased F1+2 compared to 39% in the placebo group (p<0.001). Patients with reduction versus no reduction of F1+2 tended to have a lower frequency, 10% versus 14%, of ischemic events during the period of randomized treatment (Figure 10).

60% of the total population had reduced D-dimer levels after 1 week. D-dimer levels were reduced in 72% of ximelagatran treated patients compared to 40% in the placebo group (p<0.001). In the group with decreased D-dimer levels, there were significantly less ischemic events during treatment (9%) compared to the group with unchanged or increased levels (16%) (Figure11). A reduction in D-dimer levels, regardless whether the reduction occurred spontaneously or by ximelagatran treatment, predicts improved clinical outcome and a similar trend was found for reduced F1+2 levels. Increased D-dimer levels has been identified as a risk marker for future MI among healthy men, for predicting cardiovascular events in other clinical settings and for reflecting the severity of atherothrombotic disease [85, 86, 164, 165]. The benefit of early reduced D-dimer levels that we found further supports the concept of D-dimer as a useful marker to estimate the risk of new thrombotic events in atherothrombotic disease.

Figure 10. The cumulative incidence of death, MI, severe recurrent ischemia and ischemic stroke during treatment in relation to reduction (solid line) or no reduction (dotted line) of F1+2 after 1 week
Figure 11. The cumulative incidence of death, MI, severe recurrent ischemia and ischemic stroke during treatment in relation to reduction (solid line) or no reduction (dotted line) of D-dimer after 1 week.

Bleeding events, coagulation activity and APTT

Major and minor bleedings occurred in 31% of the patients in the ximelagatran group compared to 16% in the placebo group (p<0.001). The frequency of major bleedings was 2% during the study, with no difference between the ximelagatran and placebo groups.

The frequency of major and minor bleedings during the treatment was not related to the levels of F1+2 or D-dimer at randomization or the change of levels after 1 week. Neither was there any significant difference of the frequency of bleeding events when comparing the patients with reduced F1+2 or D-dimer levels with the patients with increased levels of these markers.
Figure 12. The cumulative frequency of major and minor bleeding events in relation to the change of APTT value after 1 week. Placebo group (solid line), ximelagatran group with APTT < 2x the level at randomization (dotted line) and ximelagatran group with APTT ≥ 2x the level at randomization (broken line). P-value comparing the two ximelagatran groups.

Activated partial thromboplastin time, APTT, is an analysis that evaluates the activity of coagulation factors in vitro. APTT is used for dose-guiding of treatment with unfractionated heparin. Most of the available anticoagulant drugs given in acute MI have therefore been evaluated with APTT. For the ximelagatran groups, there was a significant difference between the APTT levels both at 1 week and 26 weeks comparing the four dose groups (p<0.001) and the APTT concentrations at peak were 1.3 fold higher than trough in all four dose groups (p<0.001). The linear dose-response relation between the plasma concentration of melagatran and the APTT values (r=0.70-0.76, p<0.001) was in accordance with previous results [166]. After 1 week, 98% of ximelagatran treated patients showed increased APTT levels, with 24% demonstrating a level of APTT twice or more times higher than the level at randomization. The optimal therapeutic APTT range during treatment with direct thrombin inhibitors is poorly defined. Elevation of APTT indicates a risk of bleeding even at levels below the therapeutic range, and higher APTT levels during treatment were related to increased risk of new ischemic events during treatment with inogatran, an intravenous direct
thrombin inhibitor [167, 168]. In the ximelagatran group, the degree of APTT elevation after 1 week was significantly related to bleeding events during treatment (Figure 12). In contrast the frequency of ischemic events in patients receiving ximelagatran did not differ in the group with an APTT after 1 week <2 x level at randomization (10%) compared to the group with APTT after 1 week ≥ 2 x level at randomization (8%) (p=0.8). APTT does not seem to be a suitable marker for clinical efficacy of ximelagatran and it does not reflect ximelagatran effects on thrombin generation and fibrin turnover.

Platelet and inflammatory activity in patients with recent myocardial infarction (paper III)

In the biomarker substudy population in ESTEEM, soluble P-selectin significantly increased after 1 week and persisted elevated during the study both in the placebo group and in all ximelagatran groups (p<0.001). The combined ximelagatran group had lower levels of sP-selectin after 1 week compared to the placebo group. The two highest ximelagatran dose groups (48 and 60 mg) showed significantly less increase in sP-selectin levels compared to the placebo group and the lower-dose ximelagatran groups (Figure 13). The change of sP-selectin correlated to the change of F1+2 (r=0.23, p<0.001) and D-dimer (r=0.39, p<0.001) after 1 week in the ximelagatran group. Aspirin has no effect on markers for platelet activity in patients with stable angina and, for the period immediately following a MI, the platelets are even more activated than in a stable cohort [119, 120]. Little is described of the effect of direct thrombin inhibitors on platelet activity for long-term. It has recently been reported that the oral direct thrombin inhibitor dabigatran even increased TXB2 in urine [169]. The soluble form of P-selectin originates mostly from platelets and might be a better marker for platelet activity than thromboxane in urine. P-selectin is also important for formation of platelet-leukocyte aggregates, linking coagulation to inflammation. Evaluation of sP-selectin may therefore also reflect the inflammatory activity. The effect of ximelagatran on platelet activity was more modest and also dose-dependent compared to its effect on the coagulation markers.

Soluble TF reflects both TF without procoagulant activity represented by the cleaved extracellular domain of TF, and TF presenting microparticles, and alternative spliced TF with procoagulant properties [101]. In the placebo group sTF correlated to both D-dimer (r=0.52, p<0.001) and sP-selectin (r=0.27, p=0.03) levels for up to 6 months after the MI. After 1 week on study treatment reduced levels of sTF was found in the 60 mg ximelagatran group (p=0.008). However, after 6 months the sTF had returned to the levels found at randomization. At aspirin treatment sTF is related to coagulation
and platelet activity. In contrast an oral direct thrombin inhibitor interferes with the balance. P-selectin is important for formation of microparticles, and the initial reduction of sTF in the ximelagatran group may therefore reflect its effect on platelet activity [63, 170]. In this small cohort the increase found after six months needs further investigation although one explanation could be that sTF also are related to the inflammatory activity.

![Figure 13](image-url)

Figure 13. The relative change from randomization of soluble P-selectin for the placebo group, the 24+36 mg ximelagatran groups and the 48+60 mg ximelagatran groups, after 1 week and 6 months.

CRP is an acute phase inflammatory protein, which is an established marker of acute MI, and initial high CRP levels decrease over time [171]. We found similar results in this population with a CRP reduction of 65% and 85% after 1 week and 6 months compared to randomization in the placebo group (p<0.001). The CRP levels or change in levels in the ximelagatran group after 1 week were not significant different compared to the placebo group. However, after 6 months of ximelagatran treatment, the low CRP levels was different compared to the levels in the placebo group (p=0.002).

IL-18 is correlated with the severity of atherosclerosis and predict death in MI [172]. Little is known of IL-18 for long-term after MI. In the patient population with recent MI, the concentration of IL-18 was 240 (176-342) pg/mL. These levels persisted stable for up to 6 months after the acute event in the placebo group.
The available amount of thrombin is reduced upon treatment with an oral direct thrombin inhibitor. Therefore, through interaction with thrombomodulin and the endothelial EPCR and PAR-1 receptors, the anti-inflammatory properties of thrombin may be diminished, and the amount of APC produced is limited [77, 78]. In this paper, we found that after 6 months of ximelagatran treatment, IL-18 levels increased in all four dose groups compared to randomization (p=0.01-0.003). There was a significant increase of the IL-18 levels in the ximelagatran-treated patients compared to the placebo group (Figure 14). IL-18 promotes T lymphocyte differentiation and also induces production of IL-6 and IL-8 [107]. The change of IL-18 and CRP was correlated at both 1 week and 6 months in the ximelagatran group. These results indicate that there is a change in balance between inflammation and anti-inflammation by addition of an oral direct thrombin inhibitor. The effect on inflammatory markers through blockage of thrombin progress more slowly than the effect on coagulation markers.

Figure 14. The relative change from randomization of IL-18 in the placebo group, the 24+36 mg ximelagatran groups and the 48+60 mg ximelagatran groups, after 1 week and 6 months.
Studies of formation of platelet-leukocyte aggregates and cross-talk therein

Formed platelet-monocyte aggregates in patients with recent myocardial infarction (paper IV)

In whole blood from a healthy control population, 3.6% (2.1-5.5%) of the monocytes form aggregates with platelets under resting conditions. In a small cohort of patients with recent MI, 89.2% (65.2-98.1%) of the monocytes formed aggregates with platelets early after the MI and these levels persisted elevated up to 6 months after the acute event. The amount of platelets adhered per monocyte, analyzed as the MFI, was also significantly increased. (Figure 15).

PMAs have been described as an early marker in acute MI and correlate to inflammatory markers and the severity of atherosclerosis [16, 173, 174]. In this study, we describe a persisted elevation of PMAs for up to 6 months after an acute MI, indicating ongoing inflammation. In this small cohort of patients, we could not find any difference in PMAs when comparing ximelagatran and placebo treatment. However, as previously described, the sP-selectin levels were reduced by addition of ximelagatran, and P-selectin is important for formation of platelet-leukocyte aggregates.

Figure 15. The amount of platelet-monocyte aggregates (A) and the mean fluorescence intensity (MFI) of CD42a on each monocyte (B) in patients with recent MI. ***p ≤ 0.001 and *p < 0.05.
The influence of thrombin inhibitors on formation of platelet-leukocyte aggregates and tissue factor production in vitro

We further investigated whether two direct thrombin inhibitors, i.e., melagatran and dabigatran, could modulate the formation of platelet-leukocyte aggregates upon thrombin and ADP stimulation in vitro. In whole blood from healthy volunteers, thrombin and ADP induced formation of platelet-leukocyte aggregates with the most pronounced effect obtained by thrombin, with an increase of PMAs (p=0.002), platelet-granulocyte aggregates (PGAs) (p=0.001) and platelet-lymphocyte aggregates (PLAs) (p=0.008). The mean fluorescence intensity for P-selectin was increased 2.4±0.2 and 3.7±0.5 times in the formed PMAs and PGAs, respectively.

Melagatran has previously been described to reduce P-selectin expression in a dose-dependent manner on stimulated platelets [175]. We could confirm these results both with melagatran and dabigatran, and they also reduced the thrombin-induced CD40L on single platelets (p=0.001). The thrombin inhibitors significantly diminished the amount of platelets tethered to each monocyte and granulocyte and the P-selectin expression within these aggregates upon thrombin stimulation. The most pronounced effect was obtained on PGA formation (Figure 16). The direct thrombin inhibitors also reduced TF mRNA levels recorded in whole blood after 1 hour of thrombin stimulation (p=0.002 and p=0.04 for melagatran and dabigatran, respectively). Formation of PMAs with adhesion of platelets to monocytes induces a cellular signal important for induction of TF production. Activated platelets induce an inflammatory response in granulocytes leading to increased levels of inflammatory markers in MI [21, 176, 177]. The effect of the direct thrombin inhibitors on PMA and PGA formation and the signalling events induced by cellular cross-talk therein may be beneficial during long-term treatment in patients with increased risk of new atherothrombosis.

In one of the performed studies with prolonged ximelagatran treatment, concerns have raised of increased risk of MI [178]. In that trial, ximelagatran was given without a combined antiplatelet therapy after deep venous thrombosis. In our in vitro model, when ADP was used as platelet agonist, pre-incubation with direct thrombin inhibitors did not affect the amount of PMAs formed, and in fact achieved increased formation of PGAs. Moreover, the ADP induced TFmRNA production was not affected by the direct thrombin inhibitors. ADP induced formation of PMAs describes another mechanism where ADP activates platelets through the P2Y12 receptor. Under these circumstances, the expressed P-selectin involved in PMA formation and TF production could not be affected by the direct thrombin inhibitors. Clopidogrel has been found to reduce PMA formation in patients with peripheral arterial occlusive disease [179, 180]. The lack of effect of the direct thrombin inhibitors in this study may indicate a need for both antiplatelet
and anticoagulant therapy that targets both ADP and thrombin-induced activation.

Figure 16. The effect of direct thrombin inhibitors on formation of platelet-monocyte and platelet-granulocyte aggregates. Formation of platelet-monocyte and platelet-granulocyte aggregates are described as mean fluorescence intensity (MFI) of CD42a in the different leukocyte populations in mean±SEM. *** p ≤ 0.001 , **p ≤ 0.005 and *p < 0.05.
Microparticles and early tissue factor expression upon stimulation of whole blood

MP, platelets and monocytes have all been described as the source of blood-borne TF and are of importance in a situation with acute activation through a ruptured atherosclerotic plaque [21, 59, 181]. In unstimulated whole blood from healthy volunteers, we found that 2.2±0.2% of the free platelets expressed TF. There were also low concentrations of circulating MP with TF on their surfaces. Thrombin stimulation increased the amount platelets with TF expression two-fold compared to unstimulated control (p=0.004). The amount of TF presenting MP was also increased by thrombin. ADP stimulation had no effect with respect to the platelets TF expression, but significantly increased the amount of MP (p=0.001).

Both melagatran and dabigatran reduced the amount of TF presenting MP by 42±12% and 36±12%, respectively, in thrombin stimulation. Similar results were found in ADP-stimulated whole blood with a decrease of 21±7% and 39±6% upon pre-incubation with melagatran and dabigatran, respectively. The amount of TF presenting platelets was also decreased by the thrombin inhibitors upon thrombin activation. The result was confirmed as a reduced procoagulant TF activity with prolonged lag time upon pre-incubation with thrombin inhibitors before stimulation (p=0.02). However, the TF within the formed PMAs was not affected by thrombin inhibition.

To evaluate whether the direct thrombin inhibitor ximelagatran had any effect on the procoagulant activity in a patient population post MI, we analyzed the time-to-start of thrombin generation with the Calibrated Automated Thrombogram 1 week after start of study medication. Patients treated with 60 mg ximelagatran twice daily together with aspirin were compared with patients receiving only aspirin. We found a significant increase of the time-to-start of thrombin generation in the ximelagatran treated patients (<0.001) (Figure 17). The result in paper III, with reduced sTF in the ximelagatran treated group was in accordance with the prolongation of time-to-start of thrombin generation.

The effect of the direct thrombin inhibitors on the blood-borne TF, with reduced amount of MP and reduced TF expression on single platelets, contributes to a delay in the initiation phase of the coagulation process. Together with the effect on the TF production for long-term, this effect on the circulating procoagulant TF might be of importance when discussing the clinical effect of oral direct thrombin inhibitors.
Figure 17. Tissue factor activity in patients with recent myocardial infarction. The time to thrombin generation (lag time) was analyzed in plasma from ESTEEM trial patients treated with aspirin or ximelagatran 60 mg and aspirin for 1 week. *** p ≤ 0.001.

The importance of P-selectin for formation of platelet-monocyte aggregates and the cross-talk therein (paper V)

In paper V, we focused on the P-selectin-induced cellular signalling upon formation of PMAs. We used thrombin receptor activator peptide (TRAP), a PAR-1 agonist, as stimulating agent. In the experimental environment, TRAP has advantages to thrombin, due to the multitude properties of thrombin. Several adhesion molecules are important for formation and stability of PMAs and we therefore initially investigated the role of both P-selectin and CD40L in this context. In whole blood, the TRAP-induced formation of PMAs was significantly and sustainably reduced by 80±2% by a neutralizing P-selectin antibody (p=0.001) (Figure 18). In contrast, the neutralizing CD40L antibody had no effect on the amount of PMAs formed in whole blood.
CD40L induces TF production in monocytes and endothelial cells and inhibition of CD40L results in a varying effect on surface expression of TF in purified monocytes [21, 33]. In the whole blood environment, however, the formation of PMAs was not dependent of CD40L, while P-selectin was of major importance.

**TRAP-induced production of tissue factor and IL-8 is dependent of P-selectin**

TRAP-induced TF mRNA expression in formed PMAs with an increase of 5.4±1.4 times the levels in unstimulated control blood after 1 hour of stimulation (p=0.03). These increased TF mRNA levels was in accordance with the increase of the amounts of PMAs expressing TF at their surface after 2 hours of TRAP stimulation, 18.4±3.3% compared to 9.3±3.0% in unstimulated whole blood (p=0.001). One hour of TRAP stimulation induced the maximum levels of IL-8 mRNA, with an increase of 2.2±0.2 times compared to unstimulated control (p=0.008). The plasma levels of soluble IL-8 was also elevated after 4 hours in stimulated whole blood (p=0.02).

Neutralizing P-selectin antibody reduced the TF and IL-8 mRNA levels by 41±12% (p=0.04) and 61±11% (p=0.01), respectively, in whole blood.
upon TRAP stimulation. In highly purified human monocytes, P-selectin stimulation for 1 hour increased both TF (p=0.03) and IL-8 (p=0.02) mRNA.

The maximum MCP-1 mRNA levels was found after 2 hours of TRAP stimulation together with increased soluble MCP-1 in plasma (p=0.02). The neutralizing P-selectin antibody had no effect on mRNA for MCP-1 and P-selectin stimulation did not significantly increase the MCP-1 production in purified human monocytes.

The importance of Src-family kinases with focus on Lyn for P-selectin/TRAP-induced cellular signalling in whole blood

SFK are important for intracellular signal transduction and may act as both positive and negative regulators. Whole blood was pre-incubated with two SFK-inhibitors: SU6656 with effect on the SFK family members Src, Fyn, Yes and Lyn, or PP2 with effect on Src, Fyn, Lck and Hck before stimulation with TRAP. Addition of SU6656 reduced TF mRNA levels to 72±8% and IL-8 mRNA to 76±3% of the levels in stimulated whole blood without SU6656 (Figure 19).

![Figure 19. TF (A), IL-8 (B) and MCP-1 (C) mRNA levels in whole blood pre-treated with SU6656 or PP2 before stimulation with TRAP for 60 minutes. Data are expressed relative to mRNA levels in TRAP-stimulated whole blood. **p ≤ 0.005 and *p<0.05.](image)

No inhibitory effect of SU6656 was recorded on the MCP-1 mRNA levels. SU6656 did not affect the amount of formed PMAs. In contrast, PP2 had neither effect on the production of TF nor IL-8 or MCP-1.

The predominant SFK members in monocytes are Hck, Fyn and Lyn, and based on the results obtained by the SFK-inhibitors we further investigated the involvement of Lyn. TRAP stimulation of whole blood increased the amount of monocytes with phosphorylated Lyn. SU6656 and neutralizing P-selectin antibody reduced these levels. We confirmed the involvement of P-
selectin for Lyn phosphorylation in monocytes in whole blood stimulated with P-selectin (Figure 20).

The SFK is easily activated upon the purification procedure and we therefore decided to verify the obtained results with vitamin D₃-differentiated U-937 cells. Stimulation with P-selectin increased phosphorylation of Lyn in these cells compared to unstimulated cells (p<0.001).

Figure 20. The amount of monocytes with phosphorylation of Lyn(Y396) in TRAP- or P-selectin-stimulated whole blood and the effect of pre-incubation with SU6656, neutralizing anti-P-selectin antibody or recombinant human IL-10. Data are expressed relative to the monocytes with phosphorylation of Lyn(Y396) in TRAP- or P-selectin-stimulated blood respectively ***p ≤ 0.001 and *p<0.05.

In experimental models with purified monocytes, activated platelets increase expression of TF and the pro-inflammatory cytokines, although the P-selectin-PSGL-1 induced cellular signalling pathway leading to upregulation of TF and these cytokines is less well known [20-23]. In mouse models and
mononuclear cells a P-selectin-β-integrin interaction with platelet-leukocytes induces a phosphorylation process that is inhibited by PP2, which also reduced the number of formed platelet-leukocyte aggregates [27, 182]. We found no effect of PP2 in stimulated whole blood regarding TF and IL-8 production. In contrast, the SFK inhibitor SU6656 with effect on Yes and Lyn reduced both TF and IL-8 without affecting the amount of formed PMAs. We show for the first time that P-selectin-PSGL-1 pathway increases phosphorylation of Lyn in monocytes. ITAM has recently been described to be involved in PSGL-1 activation of non-regulatory tyrosine kinases, Syk [29]. The activation of ITAM by PSGL-1 may involve Src-mediated ITAM phosphorylation, leading to Syk activation and further downstream signals.

The anti-inflammatory cytokine IL-10 diminishes tissue factor and IL-8 production through reduced Lyn phosphorylation

Upon stimulation with LPS, IL-10 interferes with several cellular signalling pathways in myeloid cells, but the effect initiated by IL-10 on P-selectin induced pathways has not been explored [183, 184].

Pre-incubation of whole blood and highly purified human monocytes with IL-10 before stimulation with TRAP or P-selectin significantly reduced TF mRNA (p=0.002 and p=0.03 respectively) and IL-8 mRNA (p=0.009 and p=0.001 respectively) (Figure 21).

Figure 21. TF (A), IL-8 (B) and MCP-1 (C) mRNA levels in whole blood pre-incubated with IL-10 (dark grey) before stimulation with TRAP. Data are expressed relative to mRNA levels in TRAP-stimulated whole blood (grey) in the same experiment. **p ≤ 0.005 and *p<0.05.

No effect of IL-10 was found regarding the amount of formed PMAs, indicating that the inhibitory effect of IL-10 involves P-selectin-PSGL-1-mediated cellular signalling in monocytes. In contrast to the inhibitory effect
on TF and IL-8, MCP-1 mRNA was increased by IL-10 in TRAP-stimulated whole blood.

The induced Lyn phosphorylation in monocytes in TRAP-stimulated whole blood and in P-selectin-stimulated highly purified monocytes was reduced by IL-10 (Figure 20). This result was confirmed in P-selectin-stimulated vitamin D$_3$-differentiated U-937 cells (p=0.002). The reduced phosphorylation of Lyn in P-selectin-stimulated human monocytes in whole blood could explain part of the inhibitory effect of IL-10 on P-selectin-induced TF and IL-8 production.
General Discussion

The present studies, have investigated the change of markers for coagulation-, platelet-, and inflammatory activity for long-term in patients with recent MI, and the effect of the first available oral direct thrombin inhibitor on these markers. To further understand the mechanisms involved in formation of platelet-monocyte aggregates that were found in the patient cohort with recent MI we have applied an in vitro model in whole blood. The focus in the experimental situation has been to understand part of the cellular signalling induced by formation of platelet-monocyte aggregates leading to production of tissue factor and pro-inflammatory cytokines, which all contribute to the interplay between coagulation and inflammation in the in vivo situation.

The main result from Paper I is that an oral direct thrombin inhibitor persistently, without signs of reactivation, reduces markers for thrombin generation and fibrin turnover. The most pronounced effect was found on D-dimer levels. An other important message in Paper I is that the effect of the direct thrombin inhibitor is dose-independent. Today when we treat the patients with several antiplatelet drugs for secondary prevention, there is an increased risk of bleeding. Therefore, the knowledge that low doses of a direct thrombin inhibitor is as effective as higher doses regarding its antithrombotic properties is important for the opportunity of safe and effective treatment of patients for long-term.

The most important result in Paper II is that an early change of D-dimer can predict the risk of future thromboembolic events. In clinical practice, we have few tools to evaluate the efficacy of the antithrombotic treatment given to the patient for long-term. Monitoring of D-dimer early after a MI could add information of the patient’s risk of future ischemic events and guide the intensity of treatment needed.

In Paper III patients with recent MI are shown to increase sP-selectin as a marker for platelet activity for long-term after the acute event. The direct thrombin inhibitor could diminish these increased levels but higher doses were needed and the efficacy was more moderate compared to the results found for markers of thrombin generation and fibrin turnover. The IL-18 levels as a marker for inflammation are stable after MI. The finding that long-term treatment with the direct thrombin inhibitor increases the inflammatory markers needs to be evaluated in new, longer clinical trials.
Patients with recent MI have increased levels of circulating platelet-monocyte aggregates for long-term, which is described for the first time in Paper IV. In vitro these aggregates can be modulated by direct thrombin inhibitors, leading to reduced tissue factor production, and this interaction with the cell-cross talk and cellular signalling can be of importance for the described clinical beneficial effect of the drug. Another result of interest is that microparticles together with the procoagulant activity, were reduced by direct thrombin inhibitors. How important is the effect on the blood-borne TF in secondary prevention after MI? The direct thrombin inhibitors lack of effect on ADP-induced activation is another result that generates new questions.

In Paper V, it is for the first time described that P-selectin-PSGL-1-induced cellular signalling generating tissue factor and IL-8 production involves phosphorylation of the Src-family member Lyn in monocytes. The knowledge of the signalling pathway involved in formation of platelet-monocyte aggregates has just atarted. To delineate and identify actors in the P-selectin-PSGL-1 pathway is an exploration of new possible targets for intervention. Another result of interest in Paper V is that IL-10 treatment interferes with the P-selectin-induced TF and IL-8 production through reduced Lyn phosphorylation. Atherosclerosis is an inflammatory disease but specific anti-inflammatory treatment has not given promising results in clinical trials. New anti-inflammatory drugs may arise in the future with development of more specific drugs targeting a specific biological mechanism of interest.
Based on this work, the following conclusions can be made:

- The oral direct thrombin inhibitor, ximelagatran, effectively reduced markers for thrombin generation and fibrin turnover with no variation between doses of 24-60 mg b.i.d and no signs of reactivation during treatment.

- Reduction of initially high coagulation activity in patients with recent myocardial infarction, preferably measured by D-dimer levels, identifies patients with decreased risk of new ischemic events regardless whether the D-dimer reduction occurs spontaneously or is induced by pharmacological means.

- The frequency of bleedings was not related to the markers for thrombin generation or fibrin turnover or changes in these markers.

- There was a linear dose-response relation between plasma concentration of melagatran and APTT levels. No correlation was found between APTT levels and markers for thrombin generation and fibrin turnover. The increase of APTT occurred almost exclusively in the ximelagatran group. The degree of elevation was related to bleeding events during treatment.

- Soluble P-selectin, a marker for platelet activity, increases after a myocardial infarction. An oral direct thrombin inhibitor at higher doses diminished the elevation.

- The inflammatory marker IL-18 is stable for long-term in serial samples from patients with recent myocardial infarction. The direct thrombin inhibitor, ximelagatran, increases these levels when given for long-term.

- Patients with recent myocardial infarction show elevated levels of platelet-monocyte aggregates for long-term.

- Thrombin but not ADP-induced formation of platelet-monocyte aggregates and production of tissue factor is reduced by direct thrombin inhibitors.

- Direct thrombin inhibitors reduce the formation of tissue factor expressing microparticles and the procoagulant activity in vitro and in patients with recent myocardial infarction.

- P-selectin bound to P-selectin glycoprotein-1 is important for formation of platelet-monocyte aggregates. P-selectin induces production...
of tissue factor and IL-8 partly through a cellular signalling pathway involving phosphorylation of the Src-family member Lyn.

- In a whole blood environment, IL-10 reduces the P-selectin-induced production of tissue factor and IL-8 partly through decreased phosphorylation of Lyn.
Sammanfattning på svenska

Akut hjärtinfarkt är den vanligaste dödsorsaken i världen. Den akuta behandlingen av hjärtinfarkt omfattar dels farmakologisk behandling med läkemedel som påverkar koagulationssystemet och dels en bedömning av kranskärlens utseende. Den akuta behandlingen har dramatiskt minskat risken för död och ny hjärtinfarkt i akut skedet. Den uppföljande, s.k. sekundärpreventiva, behandlingen omfattar livsstilförändringar och läkemedelsrekommendationer utifrån en bedömning av patientens riskfaktorer. Trots god sekundärpreventiv behandling kvarstår en 10-20% risk att återinsjukna i ny hjärtinfarkt.


Aktiverade trombocyter förändrar sin cellyta så att de hämmande proteinerna inte kommer att koagulationsfaktorerna. En trombocyt inneåller en rad ämnen såsom koagulationsfaktorer, ADP och kalcium som frisätts vid stimulering med exempelvis trombin eller ADP. Trombocyter som aktiveras kommer att förmånga fibrinogenreceptorn så att benägenhet att binda fibrinogen ökar. Uttrycket av CD40 ligand (CD40L) och P-selectin på trombocytyn kan öka och de kan binda till CD40 respektive P-selectin glycoprotein ligand-1 (PSGL-1) på vita blodkroppar och komplex bildas mellan trombocyter och vita blodkroppar.

Vid en hjärtinfarkt har man en ökad koagulationsaktivitet som kan mätas i blodet som förhöjda nivåer av protrombin fragment 1+2 (F1+2) och D-dimer. När protrombin klyvs till trombin frisätts F1+2 och D-dimer är ett mått på fibrinomsättningen eftersom det frigörs då fibrinet bryts ned. Studier har visat att höga värden av F1+2 och D-dimer vid det akuta hjärtinfarktsjuknandet är kopplade till en ökad risk för död och ny hjärtinfarkt. I blodet
cirkulerar också förhöjda koncentrationer av inflammationsmarkörer såsom MCP-1 och IL-8, två cytokiner som aktiverar monocyter. Koagulation och inflammation är nära sammankopplade vid arteriosklerossjukdom bl.a genom cirkulerande trombocyt-monocytkomplex.

Till patienter som haft en hjärtinfarkt rekommenderas idag behandling med acetylsalicylsyra (ASA) och clopidogrel, trombocythämmande läkemedel, som minskar risken att insjukna i ny tromboembolisk händelse.

Målet för denna avhandling har dels varit att studera eventuella effekter av den första tillgängliga orala direkt trombinhämman, ximelagatran, på koagulations-, trombocyt- och inflammationsmarkörer i en patientpopulation med nyligen genomgången hjärtinfarkt. Vidare att utvärdera om en tidig förändring av koagulationsmarkörer kan påverka risken att på nytt insjukna i en tromboembolisk händelse. Vidare så planerades fördjupade studier av mekanismer som påverkar bildningen av trombocyt-monocyt komplext och delar av de cellulära signalvägar som aktiveras och leder till produktion av TF och pro-inflammatoriska cytokiner.

ESTEEM studien var en säkerhets och dosguidande studie där fyra doser av den oral direkta trombinhämman ximelagatran gavs i kombination med ASA och jämfördes med enbart ASA behandling (placebo). I studien fanns man att med tillägg av ximelagatran så minskade risken för att återinsjukna i en ny tromboembolisk händelse. I en biomarkörstudie i ESTEEM omfattade 518 patienter togs blodprover vid randomisering (c:a 6 dagar efter den akuta infarkten), och efter 1, 8 och 26 veckors behandling. Behandling med studieläkemedlet avslutades efter 26 veckor och ytterligare ett blodprov togs efter 2 veckor.

Hos de patienter som behandlades med ASA som antitrombotisk behandling steg F1+2 och D-dimer nivåerna efter en vecka och dessa förhöjda nivåer kvarstod sedan under hela studietiden. Ximelagatran minskade effektivt både F1+2 och D-dimer redan efter en vecka och de reducerade nivåerna bibehölls så länge behandlingen pågick. Effekten på F1+2 och D-dimer var oberoende av vilken dos av ximelagatran som patienterna behandlades med.

Under studietiden förekom 64 nya tromboemboliska händelser (död, ny hjärtinfarkt, allvarlig kärlkramp eller ischemisk stroke). De grupper av patienter som hade höga nivåer av F1+2 och/eller D-dimer dvs. över medianvärdet vid randomisering, hade nytta av ximelagatran behandling för att minska återinsjuknandet i ny händelse. I gruppen med lägre F1+2 och/eller D-dimer så förändrades inte frekvensen av händelser av tillägg med ximelagatran. 63% av patienterna minskade sin F1+2 koncentration och 60% reducerade D-dimer nivåerna efter en vecka i hela studiepopulationen. I gruppen med sänkt F1+2 förekom 10% nya tromboemboliska händelser jämfört med 14% i gruppen med oförändrat eller ökat F1+2 värde (p=0.3). Den grupp av patienter som sänkte sitt D-dimer värde efter en vecka hade färre tromboemboliska händelser jämfört med gruppen med oförändrat eller ökat D-dimer vär-
Information om vad som händer med trombocytmarkörer såsom lösligt P-selectin efter en genomgången hjärtinfarkt är begränsad. Utvärdering av lösligt P-selectin i biomarkörstudien visade att i alla patientgrupper, de fyra ximelagatrangrupporna och placebogruppen så ökade nivåerna efter en vecka och dessa nivåer kvarstod sedan under 6 månader. Behandling med ximelagatran i högre dos (48 och 60 mg) gav en lägre ökning jämfört med ximelagatran (24 och 36 mg) och också jämfört med placebo gruppen. Effekten av ximelagatran på trombocytmarkören var mindre jämfört med effekten på koagulationsmarkörernamn och den var också beroende av en högre dos.

IL-18 är en cytokin som finns i höga koncentrationer i arteriosklerotiska plack och koncentrationen av IL-18 mätt i blodet är relaterad till graden av arteriosklerotisk sjukdom. I biomarkörsutiden visade seriella mätningar av IL-18 på oförändrade nivåer upp till ett halvår efter hjärtinfarkten i placebo gruppen. Detta till skillnad från C-reaktivt protein (CRP) som liksom i ett flertal tidigare studier sjönk efter den akuta händelsen. Trombin har förutom att det är centralt i koagulationprocessen också inflammatoriska och antiinflammatoriska egenskaper. Efter sex månaders ximelagatranbehandling noterades en ökning IL-18 koncentrationerna i samtliga ximelagatrangrupper. Mekanismerna bakom detta fynd är oklart och här behövs ytterligare studier utföras.


I blodet finns det cirkulerande mikropartiklar som kommer både från trombocyter och från monocyter. Vissa av dessa mikropartiklar ändras TF och kan snabbt initiera trombildning vid exempelvis en skada. I helblodet minskade de direkta trombinhämmarna antalet mikropartiklar både vid trombin och vid ADPaktivering. I patientpopulationen studerades tiden till trombinbildning i plasma. Om man tillsätter standardiserade mängder av koagulationsfaktorer så är tiden beroende av mängden TF i plasman. I ximelagatran grupperna var tiden till trombinbildning förlängd jämfört med placebo gruppen talande för en mindre mängd cirkulerade TF.

IL-10 är ett cytokin som har antiinflammatoriska egenskaper och som används vid behandling av vissa utvalda grupper med inflammatorisk tarmsjukdom. Effekten av IL-10 vid P-selectin stimulerad TF produktion är inte känt men om andra stimuli som exempelvis endotoxin används har IL-10 en hämmande effekt på TF produktionen. IL-10 minskade både TF och IL-8 mRNA, i helblod vid TRAPstimulering och vid P-selectinstimulering av rena human monocyter. En del av den effekt som IL-10 uppvisade berodde på en minskad fosforylering av Src-medlemmen Lyn.

Sammanfattningsvis så har direkta trombinhämmare en icke dosberoende reducerade effekt på den ökade koagulationsaktiviteten som uppmätts hos patienter efter en nyligen genomgången hjärtinfarkt. För att påverka trombocytyaktiviteten behövs dock högre doser av läkemedlet. Långtidsbehandling med orala direkta trombinhämmare ökar vissa inflammationsmarkörer där mekanismen inte är känt och behöver studeras ytterligare.

I would like to express my sincerely gratitude to all of those, who in one way or another have contributed to the fulfilment of this thesis, with special mention to:

**Agneta Siegbahn**, my supervisor, for given me the opportunity to learn so much about biological science, for guiding me with her knowledge in the “thromboembolic field”, for fruitful discussions and distinct comments when I have been lost in the coagulation factors or signalling pathways, for encouragement and for just being a fantastic mentor.

**Lars Wallentin**, my co-supervisor, for given me the opportunity to take part in the cardiology research group and participate in clinical trails, for generously sharing his great knowledge in clinical research and for sharing his enthusiasm of the importance of atherothrombosis.

**Jonas Oldgren**, my co-supervisor, for sharing all his knowledge about coagulation and thrombin inhibitors with me, for being encouraging, and for all the hours he has spent reading drafts and offering enthusiasm and fruitful comments.

**Matilda Johnell**, my colleague at the coagulation laboratory, for sharing and teaching me about the experimental work, for skilful guidance in the laboratory, for all the collaboration in the experimental manuscripts and for her friendship.

**Anders Bylock**, co-author in papers I and II, for productive collaboration.

**Gunnar Frostfeldt, Bertil Lindahl, Bo Lagerqvist, Gerhard Wikström, Stefan James, Kai Eggers, Erik Björklund, Claes Held, Bertil Andrén, Christof Varenhorst, Ziad Hijazi, Emil Hagström, Axel Åkerblom, Johan Sundström, Birgitta Jönelid**, my colleagues and members of the research group on ischemic heart diseases, for encouragement and fruitful discussions.

**Birgitta Fahlström, Helena Vretman, Anders Mälarstig, Mikael Åberg, and Teet Velling** my colleagues in the coagulation research.
group, for teaching me coagulation basic research and for many interesting discussions.

**Gerd Ålsjö**, research nurse, for collaboration in the ESTEEM trial in recruiting and taking care of “our” patients, and for all her knowledge about clinical trials.

**Sylvia Olofsson**, bio-statistician for all her patience in discussing statistical issues and for her work with the statistics in the “clinical papers”.

**Nina** and **Lisa**, colleagues and friends, for support and relaxed lunches with laughs and gossip during all this time.

**Albert, Malin, Johanna and Oskar**, my family for everything!

This study was supported by grants from the Swedish Heart and Lung Foundation, Uppsala County Association against Heart and Lung Diseases and Erik, Karin and Gösta Selander Foundation. The ESTEEM study was supported by AstraZeneca AB, Mölndal, Sweden.
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83


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